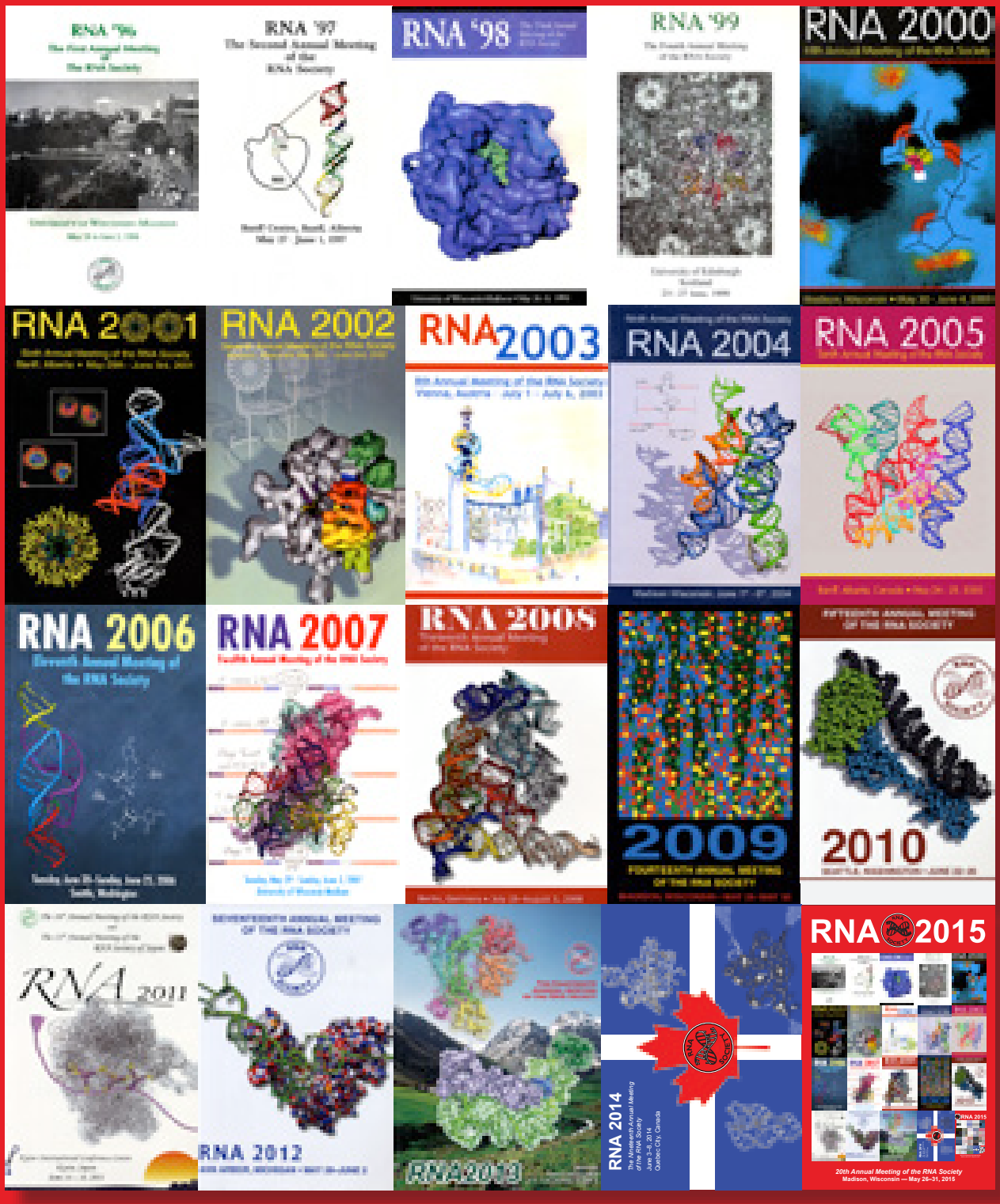
RNA 2015



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Madison, Wisconsin — May 26–31, 2015

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RNA 2015

The Twentieth Annual Meeting of the RNA Society

PROGRAM & ABSTRACTS

May 26–31, 2015
University of Wisconsin – Madison

David Brow, *University of Wisconsin – Madison*
Matt Hentze, *European Molecular Biology Laboratory*
Amy Pasquinelli, *University of California, San Diego*
Anna Pyle, *Yale University*

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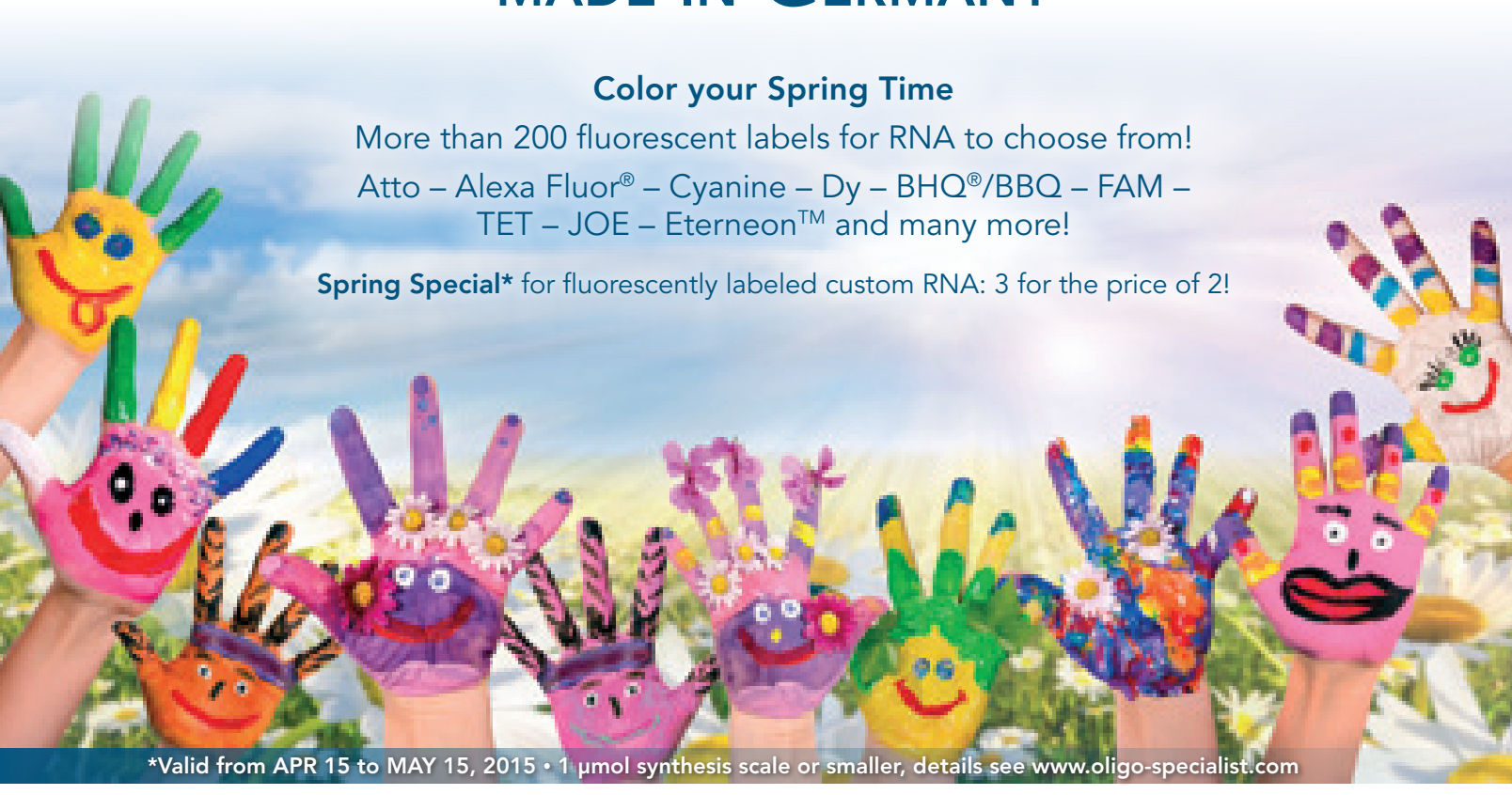

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GENERAL INFORMATION

Throughout the Program listing, the numbers next to the titles refer to corresponding oral or poster abstract numbers in the Abstract section of this book. These abstracts should not be cited in bibliographies. Material contained herein should be treated as personal communication, and should be cited only with the consent of the author.



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USE OF SOCIAL MEDIA: The official hash tag of the 20th Annual Meeting of the RNA Society is #RNA2015. The organizers encourage attendees to tweet about the amazing science they experience at the meeting, so that those who could not come to Madison can join in from afar. However, please respect these few simple rules when using the #RNA2015 hash tag or talking about the meeting on Twitter and other social media:

- Be polite and respectful of others in all of your messages.
- Do not transmit photographs of slides or posters under any circumstances.
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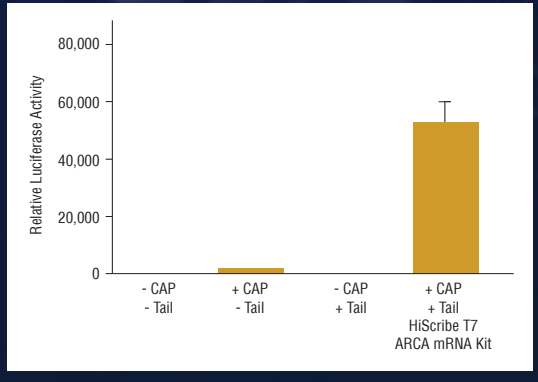

Luciferase expression in U2OS cells. Purified Cypridina luciferase RNA produced as indicated was co-transfected into U2OS cells with purified Gaussia luciferase mRNA. mRNAs produced using the HiScribe T7 ARCA mRNA Kit (with Tailing) are 5´- capped and have 3´- poly(A) tails. After 16 hours incubation at 37 °C, cell culture supernatants from each well were assayed for CLuc and GLuc activity. Luminescence values were recorded and used to calculate relative luciferase activity.

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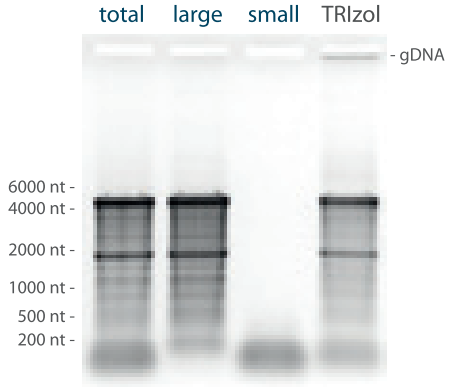

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The 21st Annual Meeting of the RNA Society will be held in Kyoto, Japan from June 28-July 2, 2016, at the Kyoto International Conference Center.

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1997	James Dahlberg	Univ of Wisconsin-Madison
1998	Harry Noller	Univ of California-Santa Cruz
1999	Iain Mattaj	EMBL
2000	John Abelson	California Institute of Technology
2001	Christine Guthrie	Univ of California-San Francisco
2002	Marvin Wickens	Univ of Wisconsin-Madison
2003	Anita Hopper	Ohio State Univ
2004	Olke Uhlenbeck	Northwestern Univ
2005	Eric Westhof	IBMC-CNRS
2006	Lynne Maquat	Univ of Rochester Sch of Med & Dentistry
2007	Brenda Bass	Univ of Utah
2008	David Tollervey	Univ of Edinburgh-Inst of Cell & Molec Biol
2009	Reinhard Lührmann	Max Planck Inst for Biopyhsical Chem
2010	Roy Parker	Univ of Arizona-HHMI
2011	Manuel Ares	Univ of California-Santa Cruz
2012	Douglas Black	UCLA-HHMI
2013	Rachel Green	Johns Hopkins Univ Sch of Med; HHMI
2014	Adrian Krainer	Cold Spring Harbor Laboratory

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Elsebet Lund
Peter Moore
Norm Pace

1997 (Banff)
Dave Brow
Nancy Martin
Tom Steitz
Jon Warner

1998 (Madison)
Dave Brow
Ray Gestland
Angela Krämer
Anna Pyle

1999 (Edinburgh)
Jennifer Doudna
Matt Hentze
Angela Krämer
David Tollervey

2000 (Madison)
Jim Dahlberg
Alan Weiner
Eric Westhof
Sarah Woodson

2001 (Banff)
Benoit Chabot
Matt Hentze
Jamie Williamson
Sandy Wolin

2002 (Madison)
Juli Feigon
Betsy Goodwin
Paula Grabowski
David Tollervey

2003 (Vienna)
Andrea Barta
Lynne Maquat
Reneé Schroeder
Scott Strobel
Juan Valcárcel

2004 (Madison)
Phil Anderson
Marty Fedor
Richard Jackson
Angus Lamond

2005 (Banff)
David Lilley
Tim Nilsen
Marina Rodnina
Sandy Wolin

2006 (Seattle)
Andrea Barta
Adrian Ferré-D'Amaré
Elisa Izaurralde
Alan Weiner

2007 (Madison)
Sam Butcher
Maria Carmo-Fonseca
Rachel Green
Erik Sontheimer

2008 (Berlin)
Elena Conti
Volker Erdmann
Witek Filopowicz
Reinhard Lührmann
Joan Steitz
Juan Valcárcel

2009 (Madison)
Benoit Chabot
Andrew Feig
Fátima Gebauer
Narry Kim

2010 (Seattle)
Doug Black
Juli Feigon
Elisa Izaurralde
Tim Nilsen

2011 (Kyoto)
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Lynne Maquat
Yoshi Nakamura
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Jamie Williamson

2012 (Ann Arbor)
Rachel Green
Melissa Moore
Gerhart Wagner
Nils Walter

2013 (Davos)
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Witold Filipowicz
Adrian Krainer
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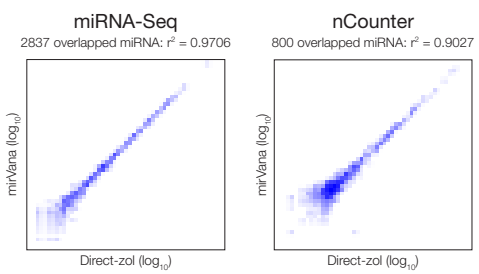
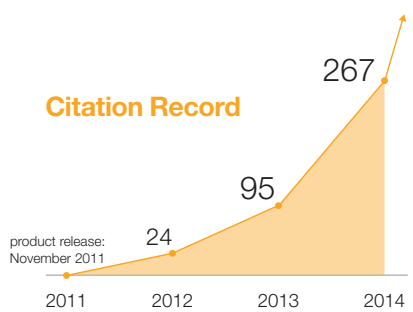


The data show RNA purified from TRIzol® samples using the Direct-zol™ RNA MiniPrep compared to an unbiased method (mirVana™, Ambion). Micro-RNA analysis was performed using miRNA-Seq (MiSeq®, Illumina) and a direct hybridization assay (nCounter®, Nanostring).

Highly cited



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Invitation to Membership

The RNA Society was established in 1993 to facilitate sharing and dissemination of experimental results and emerging concepts in RNA research. The Society is an interdisciplinary, cohesive intellectual home for those interested in all aspects of RNA Science. We welcome new members from all disciplines and we look forward to sharing the new perspectives they bring to the Society.

Our members work in numerous areas of RNA science including but not limited to:

RNAi and miRNA
Ribosomes and Translation Regulation
Splicing Regulation and Alternative Splicing
RNA Turnover and Surveillance
Integration of Nuclear Gene Expression Processes
RNA Regulation in Neurons and Specialized Cells
RNA Structure and Folding
RNA and Disease: Therapeutic Strategies
Viral RNA Mechanisms
Methods in RNA and RNP Research
Noncoding RNA
Splicing Mechanisms
3′End Formation and Riboregulation of Development
RNA Transport and Localization
RNP Biosynthesis and Function
RNP Structure and RNA-Protein Interactions
RNA Catalysis
Heterochromatin Silencing
Telomerases
Bioinformatics

Our members receive:

- Subscription to the Society journal, *RNA* (IF 6.051) with
  - 50% discount on page charges
  - 50% discount on first color figure charge (a savings of $225)
  - Those members who wish to have their articles completely open access immediately upon publication can do so at a reduced cost of $1500 (a $500 savings from non-member fee)
- Reduced registration fees for the annual meeting of the Society (a savings of $175)
- The RNA Society Newsletter, a forum for disseminating information to members and discussing issues affecting the Society and RNA Science
- Numerous opportunities for junior scientists to become involved in the Society
- The Directory of Members, available online
- Free job postings on the Society website
- Opportunities to request Travel Fellowships and Meeting Support for RNA-related meetings you are organizing

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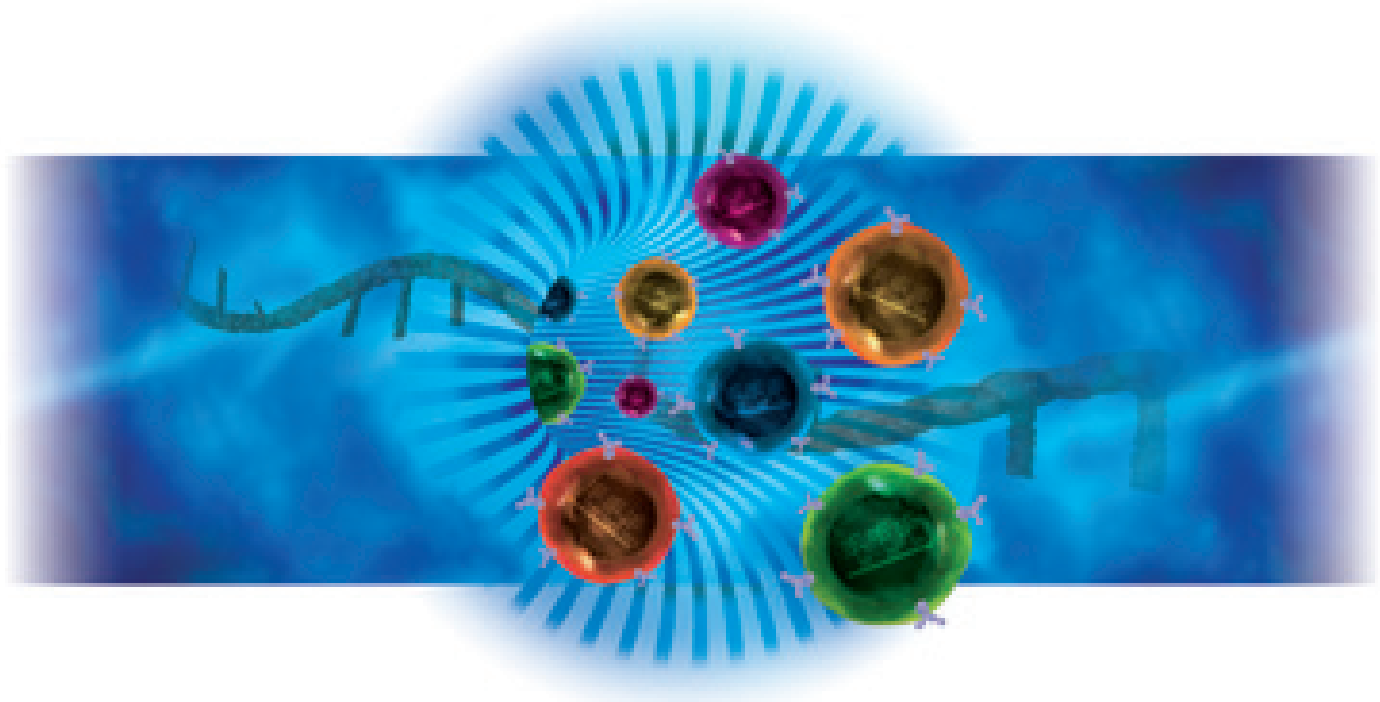

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PROGRAM–RNA 2015

The Twentieth Annual Meeting of the RNA Society
Madison, Wisconsin
May 26–31, 2015

(See room locations floor plan at the back of the book.)

Tuesday May 26

1:00 – 7:30 pm	Registration	Main Lounge
5:00 – 7:15 pm	Opening reception/light dinner	Tripp Commons/Tripp Deck
7:30 – 7:45 pm	Welcome and meeting overview	Shannon Hall
7:45 – 10:00 pm	**Keynote talks** *Harry Noller, UC Santa Cruz* *Narry Kim, Seoul National University* *Elena Conti, Max-Planck-Institute of Biochemistry*	Shannon Hall

Wednesday May 27

7:30 am – 8:30 pm	Poster installation	Gordon Dining and Event Center
8:00 am – 6:30 pm	Registration continues	Annex
9:00 am – 12:30 pm	**Plenary session 1: Ribozymes, riboswitches & RNA structure (1-12)** *Chair: Rob Batey, University of Colorado, Boulder*	Shannon Hall
12:30 – 2:00 pm	Lunch	Inn Wisconsin/Main Lounge
12:30 – 2:00 pm	Meetings Committee lunch/meeting	Class of ’24 Reception Room
2:00 – 3:30 pm	**Concurrent session 2A: Ribosome assembly and function (13-19)** *Chair: Gloria Culver, University of Rochester*	Shannon Hall
	Concurrent session 2B: RNA transport and localization (20-25, 616) *Chair: Samie Jaffrey, Weill Cornell Medical College*	Great Hall
4:00 – 5:30 pm	**Workshops**	
	W1: RNA and disease *Chair: Chonghui Cheng, Northwestern University* (Abstracts 629, 448, 424, 420, 539, 527, 254, 600, 426)	Shannon Hall
	W2: Deciphering the mRNP code *Chairs: Niels Gehring, University of Cologne and Utz Fischer, Universität Würzburg* (Abstracts 249, 393, 328, 606, 563, 404, 113, 464, 357)	Great Hall
	W3: Careers in RNA science *Chair: Allison Didychuk, University of Wisconsin – Madison*	Play Circle Theater

Note: Numbers in parenthesis in session listings correspond to abstract numbers.

W4: RNA structure prediction Humanities 1111
Chair: Tamar Schlick, New York University
(Abstracts 210, 207, 202, 197, 209, 198, 200, 195, 204)

W5: RNA synthetic biology Humanities 1121
Chair: Hans-Joachim Wieden, University of Lethbridge
(Abstracts 594, 344, 258, 470, 337, 566, 407, 288)

6:30 – 8:00 pm **Science and Society Dinner** Varsity Hall/Union South
Speaker: Jon Lorsch, NIH National Institute for General Medical Sciences

8:30 – 11:00 pm **Poster session 1 and beer hall (even numbers)** Gordon Dining and Event Center
Sponsored by Promega

Abstracts	Topic
(103 – 123)	Emerging & High-throughput Techniques
(124 – 131)	Interconnections between Gene Expression Processes
(132 – 136)	Mechanisms of RNA Interference
(137 – 182)	Non-coding and Regulatory RNAs
(183 – 190)	Ribosome Biogenesis
(191 – 192)	RNA and Epigenetics
(193 – 211)	RNA Bioinformatics
(212 – 230)	RNA Catalysis and Riboswitches
(231 – 238)	RNA Chemistry
(239 – 261)	RNA Editing and Modification
(262 – 267)	RNA in Neurobiology
(268 – 297)	RNA Processing
(298 – 331)	RNA Structure and Folding
(332 – 340)	RNA Synthetic Biology
(341 – 342)	RNA Systems Biology
(343 – 356)	RNA Transport and Localization
(357 – 381)	RNA Turnover
(382 – 419)	RNA-protein Interactions
(420 – 450)	RNAs in Disease
(451 – 465)	RNP Structure, Function and Biosynthesis
(466 – 482)	Small RNAs
(483 – 506)	Splicing Mechanisms
(507 – 539)	Splicing Regulation
(540 – 552)	Therapeutic RNAs
(553 – 558)	Transcriptional Regulation by RNA
(559 – 582, 636)	Translation Mechanisms
(583 – 612)	Translational Regulation
(613 – 626)	tRNA, snRNA, snoRNA, rRNA
(627 – 635)	Viral RNAs

Thursday May 28

8:00 am – 6:00 pm	Registration continues	Annex
9:00 am – 12:30 pm	**Plenary session 3: Pre-mRNA splicing (26-37)** *Chair: Charles Query, Albert Einstein College of Medicine*	Shannon Hall
12:30 – 2:00 pm	Mentor-Mentee lunch *Coordinator: Beth Tran, Purdue University*	Inn Wisconsin/Main Lounge
12:30 – 2:00 pm	Conference lunch (anyone not attending M/M lunch)	Gordon Dining and Event Center
2:00 – 3:30 pm	**Concurrent session 4A: Alternative splicing (38-44)** *Chair: Jernej Ule, UCL Institute of Neurology*	Shannon Hall
	Concurrent session 4B: **Interconnections between RNA processes (45-51)** *Chair: Melissa Moore, University of Massachusetts Medical Center/HHMI*	Great Hall
4:00 – 5:30 pm	**History of RNA research panel discussion** *Moderator: Marv Wickens, University of Wisconsin – Madison* *Jean Beggs, University of Edinburgh* *Jim Dahlberg, University of Wisconsin – Madison* *Christine Guthrie, UC San Francisco* *Reinhard Lührmann, Max Planck Institute for Biophysical Chemistry* *Iain Mattaj, EMBL* *Harry Noller, UC Santa Cruz* *Joan Steitz, Yale University/HHMI* *Olke Uhlenbeck, Northwestern University*	Shannon Hall
5:30 – 6:30 pm	Junior Scientist Social *Coordinated by the Junior Scientist Committee*	Tripp Deck
6:00 – 8:00 pm	Picnic dinner *Free evening (posters and beer hall open 8 – 10:30 pm)*	Lakeshore dorms Gordon Dining and Event Center

Friday May 29

8:00 am – 6:00 pm	Registration continues	Annex
9:00 am – 12:30 pm	**Plenary session 5: Short non-coding RNAs (52-64)** *Chair: Erik Sontheimer, UMass Medical School*	Shannon Hall
12.30 – 2:00 pm	Lunch	Inn Wisconsin/Main Lounge
2:00 – 3:30 pm	**Concurrent session 6A: Long non-coding RNAs (65-70)** *Chair: Howard Chang, Stanford University School of Medicine*	Shannon Hall
	Concurrent session 6B: RNA processing/3′ end formation (71-76, 453) *Chair: Elmar Wahle, Martin Luther University Halle-Wittenberg*	Great Hall
	Concurrent session 6C: RNA modification and editing (77-82) *Chair: Jane Jackman, Ohio State University*	Play Circle Theater

4:00 – 5:30 pm **Workshops**

W6: Emerging techniques Shannon Hall
Chair: Marv Wickens, University of Wisconsin – Madison
(Abstracts 117, 111, 174, 138, 104, 270, 120, 121)

W7: Interface of theory and experiments in functional RNAs Great Hall
Chairs: Darrin York, Rutgers University & Phil Bevilacqua, Pennsylvania State University
(Abstracts 220, 303, 232, 217, 213, 223, 221, 306, 216, 238)

W8: RNA therapeutics Play Circle Theater
Chair: Brett Monia, Isis Pharmaceuticals
(Abstracts 550, 547, 543, 540, 549)

W9: La and related proteins Humanities 1111
Chair: Rich Maraia, NIH NICHD
(Abstracts 416, 454, 386, 372, 376, 451, 442, 588)

W10: Splicing structure and mechanism Humanities 1121
Chair: Stephen Rader, University of Northern British Columbia
(Abstracts 489, 465, 460, 493, 504, 485, 523, 528, 498)

6:00 – 7:30 pm Dinner Inn Wisconsin/Main Lounge

6:00 – 7:30 pm Board of Directors dinner/meeting Class of ’24 Reception Room

8:00 – 10:30 pm **Poster session 2 and beer hall (odd numbers)** Gordon Dining and Event Center

Abstracts	**Topic**
(103 – 123)	Emerging & High-throughput Techniques
(124 – 131)	Interconnections between Gene Expression Processes
(132 – 136)	Mechanisms of RNA Interference
(137 – 182)	Non-coding and Regulatory RNAs
(183 – 190)	Ribosome Biogenesis
(191 – 192)	RNA and Epigenetics
(193 – 211)	RNA Bioinformatics
(212 – 230)	RNA Catalysis and Riboswitches
(231 – 238)	RNA Chemistry
(239 – 261)	RNA Editing and Modification
(262 – 267)	RNA in Neurobiology
(268 – 297)	RNA Processing
(298 – 331)	RNA Structure and Folding
(332 – 340)	RNA Synthetic Biology
(341 – 342)	RNA Systems Biology
(343 – 356)	RNA Transport and Localization
(357 – 381)	RNA Turnover
(382 – 419)	RNA-protein Interactions
(420 – 450)	RNAs in Disease
(451 – 465)	RNP Structure, Function and Biosynthesis
(466 – 482)	Small RNAs
(483 – 506)	Splicing Mechanisms
(507 – 539)	Splicing Regulation

	(540 – 552)	Therapeutic RNAs
	(553 – 558)	Transcriptional Regulation by RNA
	(559 – 582, 636)	Translation Mechanisms
	(583 – 612)	Translational Regulation
	(613 – 626)	tRNA, snRNA, snoRNA, rRNA
	(627 – 635)	Viral RNAs
10:30 – 11:00 pm	Remove posters	

Saturday May 30

8:00 am – 6:00 pm	Registration continues	Annex
7:30 – 9:00 am	Remove posters	Gordon Dining and Event Center
9:00 – 12:30 pm	**Plenary session 7: mRNA turnover and translational control (83-94)** *Chair: Ambro van Hoof, University of Texas Health Science Center*	Shannon Hall
12:30 – 2:00 pm	Lunch	Inn Wisconsin/Main Lounge
2:00 – 4:30 pm	**Plenary session 8: RNP biogenesis, structure and function (95-102)** *Chair: Reinhard Lührmann, Max Planck Institute for Biophysical Chemistry*	Shannon Hall
5:00 – 6:00 pm	Awards ceremony	Shannon Hall
6:30 – 7:30 pm	Reception	Varsity Hall Lobby & Terrace/Union South
7:30 – 9:00 pm	Dinner	Varsity Hall/Union South
9:00 – 11:30 pm	Dance and “Gomeroke” to music by the Gomers	The Sett/Union South

Sunday May 31

Conference concludes

5:00 – 11:00 am Shuttle bus runs from Ogg Hall to Dane County Airport

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RNA 2015 AWARDS

The RNA Society Lifetime Achievement Award

The RNA Society Lifetime Achievement Award acknowledges the impact of an outstanding RNA scientist on the general scientific community. Each year, beginning in 2003, the Board of Directors has selected the recipient who receives a lifetime membership in the RNA Society in recognition of their outstanding contributions. The award is presented at the Annual RNA Meeting, where the recipient gives a special address to the RNA Society. Previous winners include Joan Steitz (2003), Harry Noller (2004), John Abelson (2005), Christine Guthrie (2006), Walter Keller (2007), Norm Pace (2008), Thomas Cech (2009), Fritz Eckstein (2010), Witold Filipowicz (2011), Olke Uhlenbeck (2012), Phillip Sharp (2013) and Reinhard Lührmann (2014).

*Congratulations to **Anita Hopper** who is the winner of the 2015 RNA Society Lifetime Achievement Award.*

The RNA Society Service Award

The RNA Society Service Award is given in appreciation of outstanding service to the RNA community. The overall mission of the RNA Society is to facilitate sharing and dissemination of experimental results and emerging concepts in RNA research. Each year, the Board of Directors identifies the recipient of this award who has made exemplary contributions to these goals. Previous winners include Tim Nilsen (2003), Chris Greer (2004), Jean Beggs (2005), Olke Uhlenbeck (2006), Marvin Wickens (2007), Eric Westhof (2008), Anita Hopper (2009), Lynne Maquat (2010), Evelyn Jabri (2011), Brenda Peculis (2012) and Ann Marie Micenmacher (2014).

*Congratulations to **David Lilley** who is the winner of the 2015 RNA Society Service Award.*

The RNA Society/Scaringe Award

The RNA Society/Scaringe Young Scientist Award was established to recognize the achievement of young scientists engaged in RNA research and to encourage them to pursue a career in the field of RNA. In 2004 and 2005, the RNA Society/Scaringe Award was made to the student author(s) of the best paper, as selected by the editors, published during the previous year in RNA. The winners of the 2004 and 2005 awards were Stefano Marzi and Ramesh Pillai, respectively. In 2006, this award was revamped and opened to all junior scientists (graduate students or postdoctoral fellows) from all regions of the world who have made a significant contribution to the broad area of RNA. The award is no longer restricted to authors who have published in the RNA journal. The award includes a cash prize and support for travel and registration costs for the awardee(s) to attend the annual RNA Society meeting. Previous graduate student winners include: Jeff Barrick (2006), Malte Beringer (2007), Qi Zhang (2008), Jeremey Wilusz (2009), John Calarco (2010), Jasmine Perez (2011), Chenguang Gong (2012), Tatjana Trcek Pulisic (2012), Wenwen Fang (2013) and David Weinberg (2014). Previous postdoctoral fellow winners include Megan Talkington (2006), Zefeng Wang (2007), Alexei Aravin (2008), Shobha Vasudevan (2009), Luciano Marraffini (2010), Hani Zaher (2011), Kotaro Nakanishi (2012), Dipali Sashital (2012), Je-Hyun Yoon (2013) and Jinwei Zhang (2014).

SCARINGE
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*Congratulations to graduate students **Samuel Sternberg** and **Katherine Warner**, and postdoctoral fellows **Olga Anczukow-Camarda** and **Schraga Schwartz**, who are the winners of the 2015 RNA Society/Scaringe Award.*

The Lexogen Poster Prizes

Lexogen is pleased to recognize junior scientists with three poster prizes to be awarded at RNA 2015. The prizes are for 'outstanding RNA research using next generation sequencing' and each includes a $250 cash award.

The New England BioLabs Poster Prizes

New England BioLabs is pleased to recognize junior scientists with three poster prizes to be awarded at RNA 2015. The prizes are for 'general excellence in RNA research', and each consists of a $200 cash prize.

The RNA Society Poster Prizes

The RNA Society is pleased to provide an additional six poster prizes at RNA 2015. Each consists of a $200 cash award, and will be awarded in the following categories.

- innovation in RNA research
- outstanding interdisciplinary RNA research
- outstanding RNA structural biology research
- outstanding RNA molecular biology research
- outstanding RNA research in genetics and development
- innovation in computational RNA research
- outstanding RNA biochemistry research

All graduate students and postdoctoral fellows presenting posters at the meeting are eligible for these prizes.



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RNA 2015 MEETING FELLOWSHIP DONORS

The organizers gratefully acknowledge the generous contributions to the RNA 2015 Meeting Fellowship fund from the following individuals:

David and Mary Ann Brow

James Dahlberg

Elsebet Lund

James McSwiggen

Brenda Bass

Natalia Broude

Ren-Jang Lin

Rich Maraia

Jeff Wilusz

Yi-Tao Yu

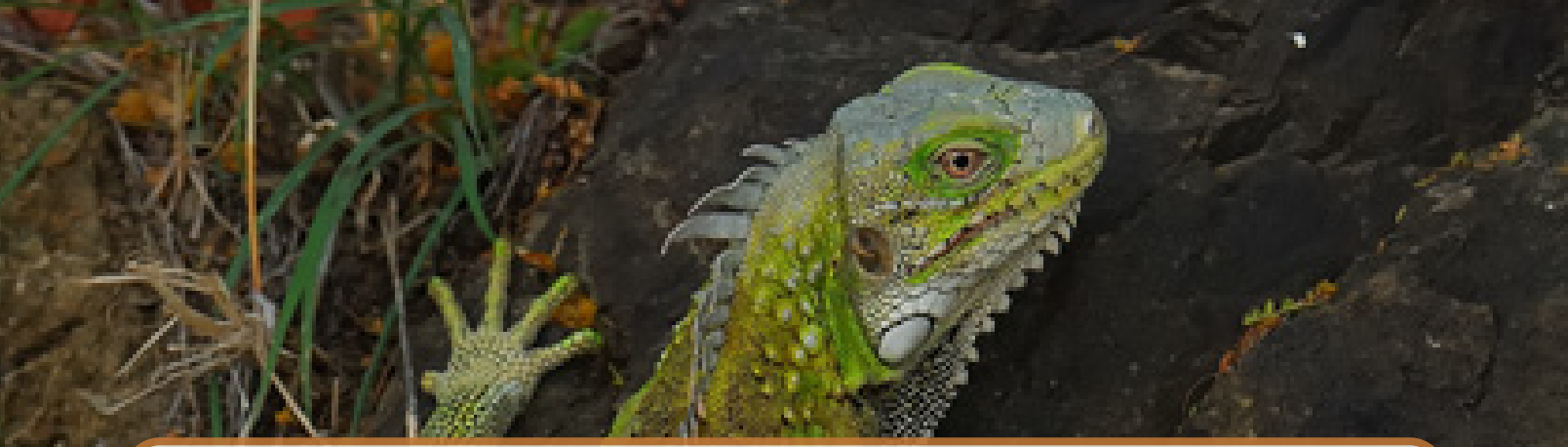
Thomas Cooper

Mark McNally

Katlin Massirer

Anonymous (3)

Personal donations from these individuals allowed us to award 10 additional full registration waivers to graduate students and postdocs attending the meeting. Thank you!



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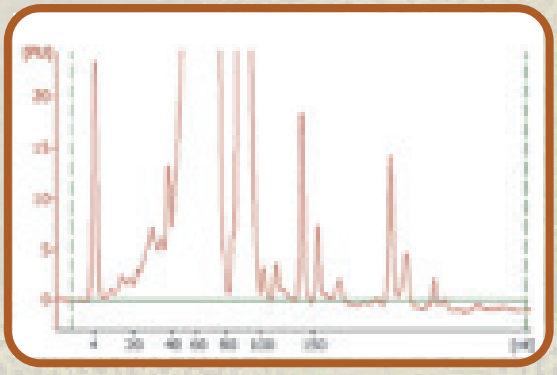

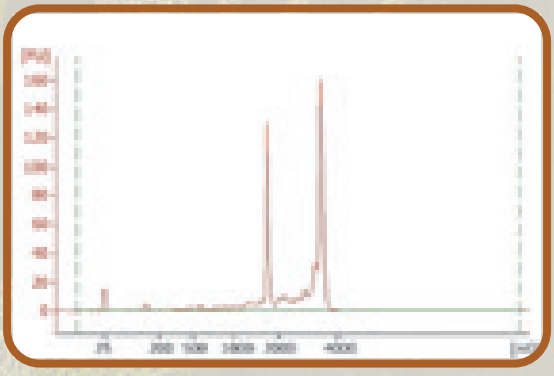

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ADDITIONAL SCHEDULED EVENTS AT RNA 2015

Tuesday, May 26

2:30 – 5:00 PM **Junior Scientists - Wisconsin State Capitol Tour**

- Open to all attendees / No additional charge, no registration required
- Meet at Ogg Hall for the short walk to the Capitol

The Junior Scientists will be starting the meeting off on the right foot by visiting the **Wisconsin State Capitol** *building for a tour! This iconic building in the center of Madison is rich with history (and marble!). If you're interested in coming on this fun guided tour, meet outside* **Ogg Hall** *on the Wisconsin-Madison campus at* **2:30 pm.** *On our way back, we will stop in at* **The Great Dane**, *a local brewpub, for some beer and socializing before heading back to the conference for the opening reception.*

Wednesday, May 27

12:30 – 2:00 PM **Meetings Committee Meeting** Class of '24 Reception Room

- Open to the Meetings Committee, the Board of Directors, meeting sponsors, and (due to space constraints) a small number of additional observers

The venues for future RNA Society meetings are reviewed and selected at this meeting. Any member of the RNA Society is welcome to attend, but due to space constraints, one should request participation in advance by sending an email to the Meetings Committee Chair, Benoit Chabot (benoit.chabot@USherbrooke.ca).

4:00 – 5:30 PM **Junior Scientists Careers in RNA Science Workshop** Play Circle Theater

- Open to all attendees / No additional charge, no registration required

This year's workshop, which is an annual fixture, will feature a panel of scientists who went into fields including industry, consulting, intellectual property, and research administration. Come to hear about their transition into the workplace and make sure to bring your questions! Featured panelists:

- Michael Bender, NIGMS (federal grants administrator)
- Brett Monia, Isis Pharmaceuticals (industry)
- Molly Nyholm, Cellscript (industry)
- Victoria Sutton, Wisconsin Alumni Research Foundation (intellectual property)
- Peter Watson, McKinsey (consulting)
- Crystal Young, Eckerd College (small liberal arts college)

6:30 – 8:00 PM **Science and Society Dinner** Varsity Hall/Union South

- Featured speaker: Jon Lorsch, NIH National Institute for General Medical Sciences
- Open to all attendees / No additional charge, but advance registration required

The Science and Society dinner offers an opportunity to dine with your fellow Society members and enjoy our featured speaker, Jon Lorsch.

Thursday, May 28

12:30 – 2:00 PM **Mentor/Mentee Lunch** Main Lounge & Inn Wisconsin

- Open to all attendees / No additional charge, registration by May 1 required

This lunch is an informal gathering that brings together 6-7 graduate students and post docs with one to two academic and industry mentors to answer student questions about careers. Topics include the pros and cons of academic vs industry careers, finding jobs, grant applications, and of course lots of interesting science. To the extent possible, mentors and mentees with common career and geographical objectives or experiences are grouped together.

4:00 – 5:30 PM **History of RNA Research Panel Discussion** Shannon Hall

- No additional charge, no registration required

A panel of distinguished RNA Society members will discuss events surrounding the founding of the RNA Society 20 years ago, and the evolution of RNA research since that time. The audience will have a chance to ask questions and join in the discussion.

5:30 – 6:30 PM **Junior Scientists Social** Tripp Deck

- Open to all graduate students and post docs / No additional charge, no registration required

The social is a casual setting to socialize with your fellow colleagues and talk some science over drinks.

6:00 – 8:00 PM **All Society Picnic Dinner** Lakeshore Dorms

- No additional charge, no registration required

Enjoy a short stroll along Lake Mendota on your way to the All Society Picnic!

Friday, May 29

6:00 – 7:30 PM **Board of Directors Meeting** Class of '24 Reception Room

- Open to the Board of Directors and (due to space constraints) a small number of additional observers

This is the business meeting of the RNA Society. Topics include an RNA journal update, results of the Meetings Committee deliberations, a report on finances and a vote on the next year's budget, and new initiatives. Any member of the RNA Society is welcome to attend, but due to space constraints one should request participation in advance by sending an email to the CEO, Jim McSwiggen (ceo@rnasociety.org).

Saturday, May 30

5:00 – 6:00 PM **Awards Ceremony** Shannon Hall

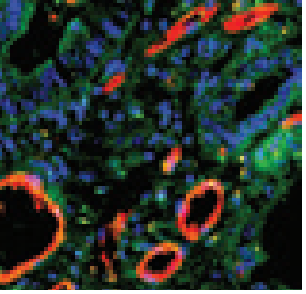
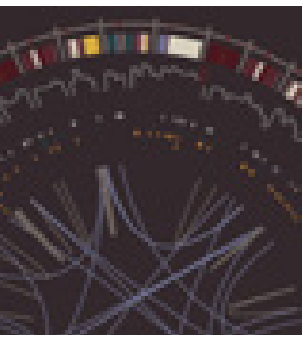
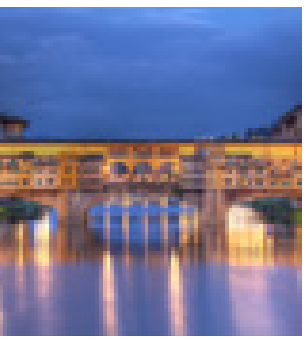
This is our opportunity to honor the people who have made significant contributions to RNA science.

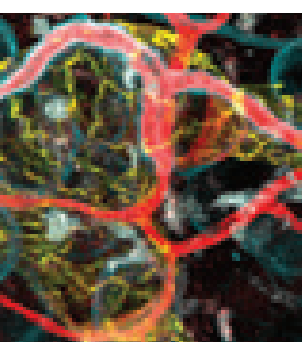
6:30 – 7:30 PM **Reception** Varsity Hall lobby and terrace/Union South

7:30 – 9:00 PM **Conference Banquet** Varsity Hall/Union South

9:00 – 11:30 PM **Dance and Gomeroke** The Sett/Union South

Provide vocals for a live back-up band, or just dance to the great music.




2015-2016 SCIENTIFIC CONFERENCES

Presenting the most significant research on cancer etiology, prevention, diagnosis, and treatment

Metabolism and Cancer
Co-Chairpersons: Ralph J. DeBerardinis, David M. Sabatini, and Almut Schulze
June 7-10, 2015 • Bellevue, WA

AACR Precision Medicine Series: Integrating Clinical Genomics and Cancer Therapy
Co-Chairpersons: Charles L. Sawyers, Elaine R. Mardis, and Arul M. Chinnaiyan
June 13-16, 2015 • Salt Lake City, UT

EACR-AACR-SIC Special Conference on Anticancer Drug Action and Drug Resistance: From Cancer Biology to the Clinic
Co-Chairpersons: Richard M. Marais, Pasi Jänne, and Riccardo Dolcetti
June 20-23, 2015 • Florence, Italy

Chromatin and Epigenetics in Cancer
Co-Chairpersons: Peter A. Jones, Sharon Y. R. Dent, and Charles W. M. Roberts
September 24-27, 2015 • Atlanta, GA

CRI-CIMT-EATI-AACR The Inaugural International Cancer Immunotherapy Conference: Translating Science into Survival
September 27-30, 2015 • New York, NY

Advances in Breast Cancer Research
Co-Chairpersons: Matthew J. Ellis, Charles M. Perou, and Jane E. Visvader
October 17-20, 2015 • Bellevue, WA

Advances in Ovarian Cancer: Exploiting Vulnerabilities
Co-Chairpersons: Kathleen R. Cho, Douglas A. Levine, and Benjamin G. Neel
October 17-20, 2015 • Orlando, FL

Fourth AACR International Conference on Frontiers in Basic Cancer Research
Chairperson: M. Celeste Simon;
Co-Chairpersons: James P. Allison, John E. Dick, Nathanael S. Gray, and Victor E. Velculescu
October 23-26, 2015 • Philadelphia, PA

Basic Science of Sarcomas
Co-Chairpersons: Robert G. Maki, Angelo Paolo Dei Tos, Jonathan A. Fletcher, Lee J. Helman, and Brian Van Tine
November 3-4, 2015 • Salt Lake City, UT

AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics
Scientific Committee Co-Chairpersons: Levi A. Garraway, Lee J. Helman, and Jean-Charles Soria
November 5-9, 2015 • Boston, MA

Pediatric Oncology
Co-Chairpersons: Scott Armstrong, Charles G. Mullighan, Kevin M. Shannon, and Kimberly Stegmaier
November 9-12, 2015 • Fort Lauderdale, FL

New Horizons in Cancer Research
Co-Chairpersons: Lewis C. Cantley and Carlos L. Arteaga
November 12-15, 2015 • Shanghai, China

Eighth AACR Conference on the Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved
Co-Chairpersons: John M. Carethers, Marcia R. Cruz-Correa, Mary Jackson Scroggins, Edith A. Perez, Beti Thompson, and Cheryl L. Willman
November 13 - 16, 2015 Atlanta, GA

Developmental Biology and Cancer
Co-Chairpersons: Hans Clevers, Stuart Orkin, and Suzanne Baker
November 30-December 3, 2015 • Boston, MA

Tumor Metastasis
Co-Chairpersons: Bruce R. Zetter, Melody A. Swartz, and Jeffrey W. Pollard
November 30-December 3, 2015 • Austin, TX

Noncoding RNAs and Cancer
Co-Chairpersons: Howard Y. Chang, Jeannie T. Lee, and Joshua Mendell
December 4-7, 2015 • Boston, MA

San Antonio Breast Cancer Symposium
Co-Directors: Carlos L. Arteaga, Virginia Kaklamani, and C. Kent Osborne
December 8-12, 2015 • San Antonio, TX

Tenth AACR-JCA Joint Conference
Conference Co-Chairpersons: Frank McCormick and Tetsuo Noda
February 16-20, 2016 • Maui, Hawaii

AACR Annual Meeting 2016
Program Committee Chairperson: Scott Armstrong
April 16-20, 2016 • New Orleans, LA

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ORAL ABSTRACT LISTING

WEDNESDAY, MAY 27, 2015: 9:00 AM – 12:30 PM

Plenary Session 1: Ribozymes, riboswitches & RNA structure, Shannon Hall

Rob Batey, Chair

Abstracts 1 – 12

1 **The catalytic mechanism of the twister ribozyme**
Timothy Wilson, Yijin Liu, Christof Domnick, Stephanie Kath-Schorr, David Lilley

2 **Crystal structure of an RNA-ligating DNA catalyst**
Almudena Ponce-Salvatierra, Claudia Höbartner, Vladimir Pena

3 **Conformational switching of the U4/U6 di-snRNA suggests a mechanism for U4/U6 unwinding during spliceosome activation**
Margaret Rodgers, Allison Didychuk, Samuel Butcher, David Brow, Aaron Hoskins

4 **Crystal structure of a RNA folding intermediate reveals a "first comes, first folds" strategy**
Chen Zhao, Marco Marcia, Kanagalaghatta R. Rajashankar, Anna Pyle

5 **Investigating Shared Molecular Recognition by the RNA and Protein Subunits of RNase P Using High-Throughput Enzymology to Measure Substrate Affinity**
Courtney N. Niland, Jing Zhao, Hsuan-Chun Lin, David R. Anderson, Eckhard Jankowsky, Michael E. Harris

6 **Using molecular simulation to model high resolution cryo-EM reconstructions**
Serdal Kirmizialtin, Justus Loerke, Elmar Behrmann, Christian Spahn, Karissa Sanbonmatsu

7 **Manganese sensing by the *yybP-ykoY* orphan riboswitch**
Ian Price, Ailong Ke

8 **Riboswitch Dynamics in Transcription Elongation Complexes by Single-Molecule FRET**
Julia Widom, Irina Artsimovitch, Nils Walter

9 **Structural features and kinetic constraints that govern ligand dependent regulatory activity of a cobalamin riboswitch**
Jacob Polaski, Erik Holmstrom, David Nesbitt, Robert Batey

10 **Activation of the Innate Immune Sensor, PKR by Three Classes of Bacterial Riboswitches**
Chelsea Hull, Philip C. Bevilacqua

11 **Validating fragment-based drug discovery for biological RNAs: Fragment-based compounds bind and remodel the TPP riboswitch specifically**
Katherine D. Warner, Ana-Maria Soto, Philip Homan, Kevin M. Weeks, Alison G. Smith, Chris Abell, Adrian R. Ferré-D'Amaré

12 **Mg^{2+}/RNA binding: insights from atomistic molecular dynamics with enhanced sampling**
Richard Cunha, Giovanni Bussi

WEDNESDAY, MAY 27, 2015: 2:00 – 3:30 PM
Concurrent Session 2A: Ribosome Assembly and Function, Shannon Hall
Gloria Culver, Chair
Abstracts 13 – 19

13 **The DEAD-box RNA helicase Ded1p prevents accumulation of aberrant ribosomes on mRNA**
Ulf-Peter Guenther, Frank Tedeschi, David Weinberg, Najwa Al-Husaini, Leah McCord, Donny Licatalosi, Jeff Coller, Eckhard Jankowsky

14 **Inhibition of ribosome production by the sequestration of the ribosome biogenesis factor GRWD1**
Maritta Küspert, Rajyalakshmi Meduri, Loren Gibson, Zhao Zhao, Raissa Schor, Mario Amend, Andreas Schlosser, Nicholas Watkins, Utz Fischer

15 **The DEAH/RHA helicase Dhr1 employs a DEAD-box like mechanism to unwind U3-pre-rRNA duplexes**
Xin Liu, Jieyi Zhu, Arlen Johnson, Carl C. Correll

16 **Structure-based insights into the eIF5A-induced protein synthesis by the eukaryotic ribosome**
Sergey Melnikov, Justine Mailliot, Byung-Sik Shin, Lukas Rigger, Sandro Neuner, Thomas Dever, Ronald Micura, Gulnara Yusupova, Marat Yusupov

17 **Initiation factor 2 stabilizes the ribosome in a semi-rotated conformation**
Clarence Ling, Dmitri Ermolenko

18 **Molecular Mechanics of Head Rotation in the Small Subunit of the Ribosome**
Srividya Mohan, John Donohue, Harry Noller

19 **Ribosome-based quality control of oxidized mRNA.**
Carrie Simms, Hani Zaher

WEDNESDAY, MAY 27, 2015: 2:00 – 3:30 PM
Concurrent Session 2B: RNA Transport and Localization, Great Hall
Samie Jaffrey, Chair
Abstracts 20 – 25, 616

20 ***Drosophila* germ granules are structured and contain homotypic mRNA clusters**
Tatjana Trcek, Markus Grosch, Andrew York, Hari Shroff, Timothée Lionnet, Ruth Lehmann

21 **Retroviral mRNA nuclear egress mediated by centrosomes**
Ginger Pocock, Jordan Becker, Paul Ahlquist, Nathan Sherer

22 **Identification of the stress granule proteome and disassembly factors**
Saumya Jain, Joshua Wheeler, Robert Walters, Roy Parker

23 **Single-molecule imaging of mRNAs in living cells during stress**
Johannes Wilbertz, James Halstead, Jeffrey Chao

24 **Common effects of ALS-associated mutations on RNA localization: a role for cytoplasmic RNA inclusions**
Kyota Yasuda, Stavroula Mili

25 **U1 snRNP is mislocalized in ALS patient fibroblasts bearing NLS mutations in FUS and is required for motor neuron outgrowth in zebrafish**
Yong Yu, Robin Reed

616 ***In vivo* biochemical analyses reveal distinct roles of β-importins and eEF1A in tRNA subcellular traffic**
Hsiao-Yun Huang, Anita K. Hopper

THURSDAY, MAY 28, 2015: 9:00 AM – 12:30 PM
Plenary Session 3: Pre-mRNA Splicing, Shannon Hall
Charles Query, Chair
Abstracts 26 – 37

26 Structure of the 800 kDa budding yeast U1 snRNP: a perspective to understand alternative splicing
Clarisse van der Feltz, Melissa Trieu, Sarah Hansen, Zhi Yang, James Lee, Aaron Hoskins, Nikolaus Grigorieff, Daniel Pomeranz Krummel

27 Structural insights into the flexible parts of U1snRNP and identification of a novel molecular link between U1snRNP and U2snRNP
Sebastien Campagne, Florian Malard, Sarah Khawaja, Frederic Allain

28 Spontaneous Toggling of the U2 snRNA between Stem IIa and IIc Conformations is Regulated by Magnesium and Cus2
U. Sandy Tretbar, Aaron Hoskins

29 Efficient annealing of spliceosomal U4 and U6 RNAs by Prp24 requires an electropositive groove, the U6 telestem, and the Lsm ring
Allison Didychuk, Eric Montemayor, David Brow, Samuel Butcher

30 Brr2 Retinitis Pigmentosa Mutations Reduce Helicase Processivity
Sarah Ledoux, Christine Guthrie

31 The architecture of the yeast spliceosomal U4/U6.U5 tri-sRNP revealed by cryo-EM
Thi Hoang Duong Nguyen, Wojciech P Galej, Xiaochen Bai, Christos J Savva, Andrew J Newman, Sjors H. W. Scheres, Kiyoshi Nagai

32 Quaternary structure of the core splicing factor Prp19
Tales Rocha de Moura, Sina Mozaffari Jovin, Jana Schmitzova, Csaba Kibedi, Mikhail Kachala, Dimitri Svergun, Reinhard Lührmann, Vladimir Pena

33 Electron-microscopic location of tagged proteins reveals the subunit architecture of the spliceosomal B complex
Norbert Rigo, Chengfu Sun, Patrizia Fabrizio, Berthold Kastner, Reinhard Lührmann

34 Small-molecule spliceosome inhibitors share a common mechanism and reveal functional roles for the core protein SF3B1 before and after splicing chemistry.
Kerstin Effenberger, Veronica Urabe, Beth Prichard, Arun Ghosh, Melissa Jurica

35 Circular RNA biogenesis can proceed through a lariat intermediate
Steven Barrett, Peter Wang, Julia Salzman

36 Spliceosomal intronogenesis
Sujin Lee, Scott Stevens

37 A hungry spliceosome reveals rapidly evolving auxiliary introns in transcribed non-protein coding regions of *Saccharomyces* genomes.
Rhonda Perriman, Lily Shiue, Elizabeth Munding, Sol Katzman, John Paul Donohue, Robert Shelansky, Manuel Ares, Jr.

THURSDAY, MAY 28, 2015: 2:00 – 3:30 PM
Concurrent Session 4A: Alternative Splicing, Shannon Hall
Jernej Ule, Chair
Abstracts 38 – 44

38 Alternative splicing regulates the expression of G9A and SUV39H2 methyltransferases, and dramatically changes SUV39H2 functions
Oriane Mauger, Roscoe Klinck, Benoit Chabot, Christian Muchardt, Eric Allemand, Eric Batsché

39 SR protein kinase phosphorylation of the branch point binding protein is required for efficient splicing of non-consensus introns
Michael C. Marvin, Jesse J. Lipp, Kevan M. Shokat, Christine Guthrie

40 Structural investigation of several SR proteins containing two RRMs reveals imbalance between the contribution of each domain
Antoine Cléry, Kyle Fowler, Ahmed Moursy, Frédéric Allain

41 Whole-transcript SHAPE-MaP reveals alternative splicing events in a 5' UTR that conserve structure
Amanda Solem, Lela Lackey, Meredith Corley, Gabriela Phillips, Ben Ziehr, Nathaniel Moorman, Alain Laederach

42 Changes in serotonin receptor 2C alternative splicing deregulate food intake
Zhaiyi Zhang, Manli Shen, Paul Gresch, Ronald Emeson, Stefan Stamm

43 Antisense Oligonucleotides that Correct Alzheimer's Disease-Associated Alternative Splicing Improve Learning and Memory in a Mouse Model
Anthony Hinrich, Francine Jodelka, Daniella Brutman, Angela Bruno, Bryan James, Grace Stutzmann, David Bennett, Steven Miller, Frank Rigo, Robert Marr, Michelle Hastings

44 A mammalian-specific alternative splicing event shapes evolutionary differences between vertebrate transcriptomes
Serge Gueroussov, Thomas Gonatopoulos-Pourtnazis, Manuel Irimia, Bushra Raj, Benjamin Blencowe

THURSDAY, MAY 28, 2015: 2:00 – 3:30 PM
Concurrent Session 4B: Interconnections Between RNA Processes, Great Hall
Melissa Moore, Chair
Abstracts 45 – 51

45 The CSR-1 RNAi pathway promotes germline transcription and defines the chromatin landscape
Germano Cecere, Sebastian Hoersch, Sean O'Keeffe, Ravi Sachidanandam, Alla Grishok

46 Primary microRNA processing is functionally coupled to RNAP II transcription in vitro
Shanye Yin, Yong Yu, Robin Reed

47 SERRATE: a key protein involved in the communication between microRNA biogenesis and splicing machineries in plants
Agata Stepien, Dawid Bielewicz, Michal Taube, Katarzyna Dorota Raczynska, Mateusz Bajczyk, Zofia Szweykowska-Kulinska, Artur Jarmolowski

48 Mutations in Integrator complex subunit INTS5 and INTS8 lead to altered gene expression and abnormal brain development.
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49 The Swi/Snf chromatin remodeling complex is a key regulator of meiotic splicing in Saccharomyces cerevisiae
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50 **Transcription elongation factor regulates polyadenylation and splicing in plasma cells**
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51 **The Ccr4-Not complex plays a key role in defining gene expression homeostasis**
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Erik Sontheimer, Chair

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52 **Determination of in vivo regulation kinetics of small non-coding RNA in bacteria**
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53 **Two distinct DNA binding modes guide dual roles of a CRISPR-Cas protein complex: a single-molecule FRET study**
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54 **Rational design of a split-Cas9 enzyme complex**
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55 **Piwi-piRNA regulates association of linker histone H1 with target transposon loci in *Drosophila***
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56 **dsRNA termini and Loquacious-PD modulate alternate reaction states of *Drosophila* Dicer-2 in an ATP- and helicase- dependent manner.**
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57 **Dissection of RISC assembly and function by single-molecule imaging**
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59 **ADAR1 is required for differentiation and neural induction by regulating microRNA processing in a catalytically independent manner**
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61 **Uridylation of hairpin-RNAs by Tailor confines the emergence of miRNAs in *Drosophila***
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62 **Sensing the end: how TUT7 controls the fate of precuror miRNAs by uridylation**
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63 **Distinct targeting by let-7 miRNA family members**
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64 **Cotranslational microRNA mediated messenger RNA destabilization**
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65 **Biosynthesis and Functions of circRNAs in Drosophila**
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66 **Natural Antisense Transcript from MALAT1 locus modulates the 3' end processing and maturation of MALAT1 lncRNA**
Xinying Zong, Shinichi Nakagawa, Susan M. Freier, Supriya G. Prasanth, Kannanganattu V. Prasanth

67 **Pervasive, novel ncRNA transcription in histone H3 lysine 36 mutants links chromatin-mediated RNA processing with organismal viability and development**
Michael P. Meers, Telmo Henriques, Karen Adelman, A. Gregory Matera

68 **Exploring the architecture of lncRNA RepA, a key player in X-chromosome inactivation**
Fei Liu, Anna Pyle

69 **Structural basis for RNA-mediated regulation of lysine specific demethylase-1**
Zigmund Luka, William Martin, Lioudmila Loukachevitch, Conrad Wagner, Nicholas Reiter

70 **Towards a therapy for Angelman syndrome by targeting a long non-coding RNA to active UBE3A**
Amanda Ward, Linyan Meng, Seung Chun, C. Frank Bennett, Arthur Beaudet, Frank Rigo

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75 **The nucleic acid-binding protein FUS/TLS plays important role in replication-dependent histone gene expression by interaction with U7 snRNP**
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78 **Profiling ribonucleotide modifications at full-transcriptome level: a step towards MS-based epitranscriptomics**
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79 **Modified Nucleosides in the tRNA Anticodon Accelerate Decoding to Maintain Protein Solubility**
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80 **5' phospho-methylation regulates fate of processed RNAs.**
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81 **A new direction: Multiple roles for 3'-5' polymerases in Dictyostelium discoideum**
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84 **Selectivity in Substrate Binding by the Exonuclease Rrp6p**
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96 Analysis of protein-RNA specificity enables targeted activation of an endogenous human transcript
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97 Molecular mechanisms of siRNA recognition by tandem dsRBD proteins in *D. melanogaster*
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235 A novel ligand that selectively targets CUG trinucleotide repeats
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255 Withdrawn

256 RNA structure is associated with essential and highly expressed genes on the distal arms of *C. elegans* autosomes.
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257 Regulated mRNA pseudouridylation in stress, development and disease
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261 Characterization of an *N6*-methyladenosine-dependent RNA structural switch
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354 Characterization of a new cis-acting signal required for the Anxa2 mRNA axonal localization by SMN
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405 RNA Mango fluorescent tag for high affinity native RNA-Protein complex purification
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446 Splicing factor hnRNP A2 activates the Ras-MAPK-ERK pathway by controlling A-Raf splicing in Hepatocellular carcinoma development
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447 Characterization of MDS-specific point mutations in serine/arginine-rich Splicing Factor SRSF2
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449 Alternative splicing shapes the phenotype of a mutation in BBS8 to cause nonsyndromic Retinitis Pigmentosa
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450 Investigating unsolved Mendelian disorders with Spliceosaurus, a software for detection and prediction of mutations that affect pre-mRNA processing
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458 Structural Analysis of a Salt Stable Spliceosome Core
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510 To what extent do Sat III RNA induction and nuclear stress bodies formation contribute to alternative splicing modulation by heat shock?
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512 Network of splice factor regulation by alternative splicing coupled with nonsense mediated mRNA decay
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513 SRSF1 Negatively Regulates Alternative Splicing of *MDM2* Under Damage
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518 Towards elucidating the role of the splicing factor DDX39B in the development of autoimmunity
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tRNA, snRNA, snoRNA, rRNA

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630 A novel combined RNA-protein interaction analysis distinguishes HIV-1 Gag protein binding sites involved in genomic RNA encapsidation from associated RNA structural change in the viral RNA leader.
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631 High-resolution structural profiling of the Chikungunya viral genome
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632 Nonsense-mediated mRNA decay (NMD) restricts replication of mammalian RNA viruses
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NOTES

NOTES

1 The catalytic mechanism of the twister ribozyme

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The twister ribozyme is a small nucleolytic ribozyme that is widely disseminated in the genomes of bacteria and eukarya. The RNA adopts a compact fold based on a double pseudoknot structure, with the active site formed by the interaction of the highly-conserved nucleosides of Loops 1 and 4. We have previously solved the crystal structure of a twister ribozyme at 2.3 Å resolution and shown that a guanine nucleobase (G45) with its Watson-Crick edge directed towards the scissile phosphate participates in catalysis. The bell-shaped dependence of cleavage rate on pH is consistent with this plus a second nucleobase of lower pK_a participating in catalysis. However initial investigations did not reveal a clear candidate for the second nucleobase and structural evidence is ambiguous as the active site is highly variable in the three extant crystal structures, with none adopting a plausibly active conformation.

We now demonstrate that the second nucleobase participating in catalysis is the highly conserved adenine (A7) immediately 3' to the scissile phosphate. Substitution of this adenine with 7-deaza-adenine results in an increase in the apparent pK_a of the cleavage reaction by 1.6 units, consistent with the difference in pK_a between adenosine and 7-deaza-adenosine, and a ten-fold increase in activity. Substitution with 1-deaza-adenine results in a fifty-fold increase in activity whereas substitution of 3-deaza-adenine yields a 10^4-fold loss of activity. We propose that catalysis is mediated through the highly acidic N3 of A7, which lies close to the 5'-oxygen leaving group in our crystal structure and is thus well placed to act as a general acid in the cleavage reaction. We discuss distinctive structural features that might serve to decrease the acidity of this nucleobase.

We show that the activity of an R_p phosphorothioate at the scissile phosphate is reduced 100-fold, whereas that of the S_p stereoisomer is unchanged, and that this effect arises from the loss of a hydrogen bond donated by the exocylic amine of G45 to the *pro*-R non-bridging oxygen. We combine these data to propose a structural model for the catalytically active ribozyme.

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2 Crystal structure of an RNA-ligating DNA catalyst

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In structural terms, the differences between functional RNA and DNA molecules are rather dramatic. RNA adopts complicated folds within various ribozymes and riboswitches. Some ribozymes, like group II introns or the ribosomal RNA, are organized by means of tertiary interactions to an overwhelming level of complexity, comparable only to the one of proteins.

In contrast, DNA generally adopts much simpler conformations. The majority of known DNA double and triple helices, four-way junctions and quadruplexes lack long-range tertiary interactions. One reason for the conformational simplicity of DNA appeared to be the absence of the 2'-OH group, resulting in greater chemical stability of DNA and rendering it suitable for its role as a long-term storage molecule of genetic information.

In recent years, synthetic DNA catalysts came under the spotlight as very intriguing constructs, since catalytic activity is expected to depend on the ability to fold into compact tertiary structures, as in the case of ribozymes or protein enzymes. Therefore, to understand how DNA molecules are organized in 3D to achieve enzymatic properties, we decided to investigate a DNA catalyst able to ligate two RNA molecules. Here we report the structure of this DNA-based RNA ligase in complex with the RNA product, which is to our knowledge the first crystal structure of a DNA molecule with catalytic properties.

The structure reveals an autonomously folding unit stabilized by a wealth of tertiary interactions, and it exhibits various features that were never observed in DNA molecules. The structure unifies a large body of biochemical data and it clearly demonstrates that DNA structures can be much more intricate and versatile than generally appreciated. Finally, the structure unveils a novel organization of the catalytic center and provides the first insight into the manner how a DNA molecule catalyzes RNA ligation.

3 Conformational switching of the U4/U6 di-snRNA suggests a mechanism for U4/U6 unwinding during spliceosome activation

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The spliceosomal small nuclear RNAs (snRNAs) undergo large-scale structural rearrangements during spliceosome assembly, activation and catalysis. For instance, the U4 and U6 snRNAs are incorporated into the spliceosome as a stably base-paired complex and are unwound in order for U6 to pair with U2 RNA and catalyze intron removal. The secondary structure of the U4/U6 di-snRNA has been proposed to contain two intermolecular helices, U4/U6 stem I and stem II. Here, using single-molecule FRET, we find reversible transitions between two U4/U6 FRET states indicative of two structures in dynamic equilibrium. Using truncations and site-specific mutations, we provide evidence for two competing structures: (1) a U4/U6 di-snRNA containing U4/U6 stems I and II and the U6 telestem and (2) a U4/U6 di-snRNA containing an additional U4/U6 helix (stem III) in place of the U6 telestem.

We propose that U4/U6 stem III and the U6 telestem are competing structures important for U4/U6 annealing and unwinding. In support of this hypothesis, we demonstrate that this structural equilibrium can be modulated by addition of DNA oligos targeting specific regions of U4 and U6 or by protein binding. Specifically, we show that binding of RNase H domain of Prp8 influences the conformation of U6 in U4/U6 potentially by binding to the central domain of U4 (Mozaffari-Jovin et al. 2012). Additionally, a cold-sensitive mutation in the U6 internal stem loop (U6-UA) can be suppressed by a mutation in U4/U6 stem III, suggesting U4/U6 stem III may form in vivo. Finally, using a novel oligo-mediated U4/U6 unwinding assay, we show that the telestem contributes to destabilization of U4/U6. We propose a model whereby U4/U6 stem III antagonizes formation of the U6 telestem, and that disruption of U4/U6 stem III accelerates oligo-induced U4/U6 unwinding by strand invasion of U4/U6 stem I. We hypothesize that a similar series of events could be occurring in the tri-snRNP as Brr2 translocates through U4/U6 stem I.

Mozaffari-Jovin S et al. 2012. The Prp8 RNase H-like domain inhibits Brr2-mediated U4/U6 snRNA unwinding by blocking Brr2 loading onto the U4 snRNA. *Genes & Development* **26**: 2422-2434.

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4 Crystal structure of a RNA folding intermediate reveals a "first comes, first folds" strategy

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Although many non-coding RNAs can form distinct three dimensional structures, our knowledge of the mechanism by which they assemble is limited. This limitation is partly due to the lack of high resolution crystal structures of RNA folding intermediates. Here we report crystal structures of an obligate group II intron folding intermediate, solved at a resolution of 3 Å. This folding intermediate is the first domain (D1) from the Oceanobacillus iheyensis (*O.i.*) group II intron, which has been shown to fold first and to serve as a scaffold, or template, for proper assembly of all other intron domains. When compared to D1 in the full-length intron ($D1_{full}$), the isolated D1 ($D1_{iso}$) adopts a native-like overall configuration, but its active-site binding cleft is closed. Comparative analysis of the backbone torsion angles reveals hinge motions that mediate the transition between the closed state as in $D1_{iso}$ and the open state as in $D1_{full}$. Furthermore, B-factor analysis shows the central 5-way junction is the critical structural motif for dictating the shape of the native-like configuration. As D1 is both the first domain and a templating intermediate for folding of group II introns, out results provide structural evidence that a "first comes, first folds" strategy is possible, and they show how this strategy can facilitate a highly precise folding pathway for a large multi-domain RNA.

5 Investigating Shared Molecular Recognition by the RNA and Protein Subunits of RNase P Using High-Throughput Enzymology to Measure Substrate Affinity

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The specificity of ribonucleoproteins is essential to their functions in biology, and often that function requires the ability to recognize and process many different substrates. Ribonuclease P, RNase P, is a multi-substrate ribonucleoprotein enzyme that removes the 5' leader from all pre-tRNAs despite their significant variation in sequence and structure. Previous work demonstrates that both the protein and RNA subunits of RNase P contact the 5' leader of pre-tRNA, however, specificity of the enzyme for different leader sequences is poorly understood. Using a new technique termed High-Throughput Sequencing Kinetics, HTS-Kin, we are able to comprehensively determine the rate constants for processing of all possible 5' leader sequences in the RNase P binding site. The resulting affinity distribution provides a complete description of enzyme specificity and reveals the full context dependence of mutations on processing rate. The E. coli RNase P holoenzyme was used to process pre-tRNA substrate pools randomized in their 5' leader sequences at the protein binding site (N(-3) to (-8) or sequences including both the protein and RNA subunit binding sites (N(-1) to N(-6)). Both reactions show the same sequence preference in the protein binding site and substrates common to both reactions provide the same rate constant in both experiments. Interestingly, traditional position weight matrix models that consider the contribution of nucleotides to binding affinity as independent do not adequately describe the data. However, including pairwise interaction terms between positions provides a better match between theory and experiment. Importantly, these data sets reveal a strong influence of P RNA subunit contacts on the effect of sequence variation in the protein subunit on the processing rate constant. Analysis of individual sequence variants is being used to confirm and investigate the mechanistic basis for this strong energetic coupling between RNA-protein and RNA-RNA interactions. This information will reveal how RNase P achieves specificity and provide deeper insight into molecular recognition by multi-substrate ribonucleoprotein enzymes.

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6 Using molecular simulation to model high resolution cryo-EM reconstructions

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An explosion of new data from high-resolution cryo-EM studies has produced a large number of data sets for many species of ribosomes in various functional states over the past few years. While many methods exist to produce structural models for lower resolution cryo-EM reconstructions, high resolution reconstructions are often modeled using crystallographic techniques and extensive manual intervention. Here, we present an automated fitting technique for high resolution cryo-EM data sets that produces all-atom models highly consistent with the EM density. Using a molecular dynamics approach, atomic positions are optimized with a potential that includes the cross-correlation coefficient between the structural model and the cryo-EM electron density, as well as a biasing potential preserving the stereochemistry and secondary structure of the biomolecule. Specifically, we use a hybrid structure-based/ab initio molecular dynamics potential.

7 Manganese sensing by the *yybP-ykoY* orphan riboswitch

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Riboswitches are structured elements that usually occur in 5'-untranslated regions (UTRs) of bacterial mRNAs. They regulate expression, generally at the level of translation or transcription, by changing conformation in response to the binding of cellular factors, such as metabolites or signalling molecules. Many putative riboswitches have been identified bionformatically but have no known ligands. Here we show that one such "orphan", the widespread yybP-ykoY RNA motif, is a Mn2+-responsive riboswitch class. Additionally, we present the crystal structure of a transcriptional yybP-ykoy riboswitch and describe its mechanism of discriminating Mn2+ from other metals by both geometry and ligand hardness. Additionally, we find that the structure of the yybP-ykoY riboswitch in the absence of Mn2+ and the structure of a binding site mutant have disrupted Mn2+ binding regions. We propose a mechanism for this riboswitch's conformation dynamics.

Using the information gleaned from these structures, we are designing fluorescent RNA sensors specific for Mn2+. The characterization of this riboswitch as Mn2+-responsive also suggests possible roles for its many uncharacterized regulated genes in manganese homeostasis or oxidative stress.

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8 Riboswitch Dynamics in Transcription Elongation Complexes by Single-Molecule FRET

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The $preQ_1$ riboswitch is a regulatory element that is found in the 5' untranslated regions of genes related to queuosine biosynthesis in numerous species of bacteria. In *Bacillus subtilis,* it operates by a transcriptional mechanism in which the binding of its ligand, the queuosine precursor $preQ_1$, causes a conformational change that favors the formation of a terminator hairpin. Previous studies have used single-molecule Förster resonance energy transfer (smFRET) to investigate the folding and conformational dynamics of the isolated riboswitch aptamer. I will present work that builds on this by incorporating the riboswitch into active transcription elongation complexes. By using smFRET to study the sampling of different conformational states by the riboswitch, I will compare the properties of the isolated aptamer to its properties when it is incorporated into a transcription bubble. I will show that both the end states and the rates at which they interconvert depend significantly on how close the aptamer is positioned to the transcription bubble, and that these properties are further altered by RNA polymerase (RNAP) binding and elongation. I will discuss the importance of co-transcriptional folding to riboswitch function, and present methods for monitoring it at the single-molecule level. Finally, I will present transcriptional pause and termination assays in which I investigate possible roles of RNAP pausing in riboswitch function. By combining biochemical and single-molecule assays in this way, I hope to obtain a complete picture of the contribution of conformational dynamics to the function of the $preQ_1$ riboswitch in its biological context.

9 Structural features and kinetic constraints that govern ligand dependent regulatory activity of a cobalamin riboswitch

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Riboswitches are elements generally found within the 5' leader region of bacterial mRNAs that directly interact with cellular metabolites to regulate gene expression. Effector binding by the receptor (aptamer) domain directs conformational changes within a regulatory domain (expression platform), which informs the expression machinery. One of the most broadly distributed riboswitches is the cobalamin clan, which comprises distinct families that selectively bind either adenosylcobalamin (Cbl-I) or hydroxo-/methylcobalamin (Cbl-II). To understand the mechanistic basis for cobalamin-dependent gene regulation, we solved the crystal structures of a member from each of these families. These structures suggested a general mechanism for all cobalamin riboswitches in which effector binding promotes formation of a kissing-loop (KL) interaction between the two domains that represses translation. While the KL interaction is generally a very stable tertiary interaction, it was proposed that features of the cobalamin riboswitch preclude KL formation in the absence of ligand. Aspects of this model were recently validated in a single molecule FRET study of a hydroxocobalamin riboswitch that monitored KL formation at varying concentrations of magnesium and ligand.

To further understand the mechanistic basis of gene regulation, we employed a combination of cell-based, biochemical, and biophysical techniques to determine sequence and structural features within the expression platform required for efficient regulatory function. First, we found that despite the ability of the aptamer and expression platform to associate in *trans* in vitro, in the cellular context the length of a single stranded linker between the two domains has a significant influence on regulatory function. Beyond an interdomain linker length of approximately 25 nucleotides, cobalamin binding cannot repress translation, suggestive of a kinetically controlled riboswitch. These data are corroborated by smFRET measurements that monitored the rate of docking between the receptor and regulatory stem loop. Second, within the KL interaction, we determined the presence of bulged nucleotides, which destabilize the KL interaction, are essential for establishing the ligand-dependent structural switch. Together, these data provide the first comprehensive map of the sequence and structural elements in a riboswitch that confer regulatory activity and further establish the mechanistic link between cobalamin binding and regulatory function.

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10 Activation of the Innate Immune Sensor, PKR by Three Classes of Bacterial Riboswitches

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The innate immune system is the first line of defense against pathogens and is known to recognize non-specific molecular patterns to protect the cell in a generalized way. The RNA-activated protein kinase, PKR is a dsRNA binding protein and an essential sensor in the innate immune response. PKR's classical role is in recognizing long stretches of viral dsRNA to promote activation by autophosphorylation and phosphorylation of eIF2α. More recently, PKR has been found to be more permissive, binding functional and non-conventional RNAs, as well as proteins.[1] Recently PKR has been found to be activated by pathogens beyond viruses including certain bacteria.[2] We characterized how PKR recognizes and interacts with three classes of bacterial riboswitches. We studied the highly structured *B. subtilis trp* leader and found that this indirect riboswitch activates PKR potently both in the absence and presence of TRAP protein and cofactor L-tryptophan. Boundary studies revealed the structural features in this RNA that lead to activation of PKR, and where expected there was a dependence on the presence of a 5'-triphosphate in the RNA. Additionally, the *V. cholerae* c-diGMP direct riboswitch and the *B. anthracis glmS* riboswitch-ribozyme were studied. We found that these riboswitches also activate PKR potently both in the absence and presence of cofactors. One physiological difference that a bacterial RNA would face upon entering a human cell is a decrease in Mg^{2+} concentration from ~2-3mM Mg^{2+} to ~0.5 mM Mg^{2+}.[3] Upon lowering the Mg^{2+} concentration to human physiological conditions, all three of the bacterial riboswitches continued to activate PKR very potently. These represent the first specific bacterial RNAs that activate PKR and the first time PKR activity has been shown in the presence of functional ligand-binding RNAs. These results suggest that PKR could act as an innate immune signaling protein for pathogenic bacteria and offer a potential explanation for the absence of riboswitches in the human genome.

[1] *Curr Opin Struct Biol*, **2011**, *21*, 119-127.

[2] *J Thorac Dis,* **2012**, *4 (2)*, 114-125. & *Int J Clin Exp Med*, **2013**, *6 (1)*, 16-25.

[3] *Proc Natl Acad Sci* **2013**, *110,* E3800-3809.

11 Validating fragment-based drug discovery for biological RNAs: Fragment-based compounds bind and remodel the TPP riboswitch specifically

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Riboswitches are structured regions of mRNA that control gene expression as a function of the intracellular concentration of a variety of small molecules. Riboswitches control essential genes in many pathogenic bacteria and are attractive targets for the development of novel antibiotics. The TPP-binding riboswitch controls transcription, translation, or splicing in response to binding thiamine pyrophosphate (TPP). In previous work, ~ 20 small molecule hits were identified in a fragment-based drug discovery screen against the *E. coli thiM* TPP riboswitch. We have characterized the binding of these fragments to the RNA through a variety of methods, including X-ray crystallography, SAXS, SHAPE and ITC. These structural and biophysical analyses indicate fragment-induced partial folding of the RNA in solution and identify a novel local structural rearrangement in the ligand binding site. The structural rearrangement we observe represents a possible mechanism for adventitious ligand discrimination by the riboswitch and suggests that off-pathway conformations of RNAs can be targeted for drug development. Observation of this idiosyncratic conformation also serves as a reminder to RNA-targeting drug discovery efforts to consider and perhaps exploit serendipitous ligand-induced structural rearrangements. Guided by structural data, synthetic development of a fragment hit has yielded a compound with an improved K_d. Structural characterization of this improved compound is underway and suggests avenues for further elaboration. More generally, fragment cocrystal structures and synthetic development, in combination with previous screening efforts, demonstrate the feasibility of fragment-based drug discovery against RNA targets.

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12 Mg^{2+}/RNA binding: insights from atomistic molecular dynamics with enhanced sampling

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Mg^{2+} cations play a major role in RNA folding and structural function both by screening electrostatic interactions and by binding to specific motifs [1]. Mg^{2+} ions can interact with RNA through a water bridge or directly bonded. This kind of interaction seems to be very important and recurrently related to specific RNA tasks and/or structural motifs [2]. We perform extensive all atoms molecular dynamics (MD) simulations in explicit solvent in order to classify and understand the thermodynamics of the Mg^{2+}/RNA interaction. We use a recently parametrization for Mg that allows proper thermodynamics to be recovered [3]. Direct Mg^{2+}/RNA binding is hindered by a free-energy barrier largely related to Mg desolvation and that is not easily accessible in plain MD. We thus use metadynamics [4] to accelerate the transition and to systematically compute the affinity of Mg^{2+} with all the possible binding sites on the four base rings, sugar, and phosphate. Results are compared with an analysis of Mg binding sites from structural databases, and can provide a validation for current force fields.

13 The DEAD-box RNA helicase Ded1p prevents accumulation of aberrant ribosomes on mRNA

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The conserved and essential DEAD-box helicase Ded1p is critical for translation initiation in *Saccharomyces cerevisiae*, but it is unknown which function the enzyme performs during this process. Here, we have used RNA-seq, ribosome profiling, iCLIP, and other molecular biology approaches to examine the function of Ded1p in translation initiation on a transcriptome-wide level.

We find that mutations in Ded1p diminish translation for most mRNAs and lead to accumulation of aberrant 80S ribosomes on the mRNAs, including in 5' and 3'UTRs. We further show that Ded1p directly binds to virtually all expressed mRNAs at multiple positions. The binding sites do not show obvious sequence or structure signatures. However, Ded1p preferentially binds to regions surrounding the start codon, and to sites where aberrant 80S ribosomes accumulate when Ded1p is defective. We also detect strong, specific binding sites of Ded1p on the small ribosomal subunit on both entry and exit sites of the mRNA channel that show a remarkable match to Ded1p interaction sites on mRNA.

Collectively, our data link Ded1p to a broad range of mRNAs and to aberrant, unproductively bound ribosomes. The data suggest that Ded1p prevents accumulation of aberrant ribosomes on mRNA, most likely by acting as a chaperone. By minimizing unproductive ribosome binding to mRNA, Ded1p ensures efficient translation for a broad range of mRNAs.

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14 Inhibition of ribosome production by the sequestration of the ribosome biogenesis factor GRWD1

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Ribosome biogenesis is a highly complex and energy consuming process. In eukaryotes, this process in initiated by the transcription and processing of rRNA precursors that ultimately generates the mature 5.8S, 18S and 28S rRNAs. During the maturation process, the rRNAs associate in an ordered manner with ribosome biogenesis factors and ribosomal proteins to yield ribosomal pre-particles and eventually mature ribosomal subunits. Ribosome biogenesis spans multiple cellular compartments, requires a multitude of trans-acting factors and is tightly linked to cell growth and proliferation. Defects in ribosome biogenesis activate the tumor suppressor p53 and cause numerous diseases known as ribosomopathies. Understanding and elucidating of the mechanism of ribosome biogenesis is hence of major interest for basic and biomedical research. Here we identify the protein encoded by the chromosome 8 open reading frame 33 (C8ORF33) as a major binding partner of the stimulatory ribosome biogenesis factor GRWD1. Both proteins localize predominantly to nucleoli and metabolic labeling experiments prove their involvement in ribosome biogenesis. Interestingly, C8ORF33 is shown to be a negative regulator of ribosome biogenesis, the opposite function as its binding partner GRWD1. Biochemical experiments suggest that the inhibitory function of C8ORF33 is due to its ability to sequester GRWD1 in an inactive complex. These data uncover a novel and unusual negative regulatory loop during ribosome biogenesis, which might play crucial roles in maintaining cellular homeostasis.

15 The DEAH/RHA helicase Dhr1 employs a DEAD-box like mechanism to unwind U3-pre-rRNA duplexes

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The conserved U3 snoRNA hybridizes to multiple sites in the pre-rRNA to promote its cleavage and folding during eukaryotic ribosome biogenesis. Because the rate of spontaneous U3-pre-rRNA dissociation is too slow to support the rates of ribosome assembly observed *in vivo*, a helicase is needed to dislodge U3. Previously, we identified the DEAH/RHA helicase Dhr1 (Ecm16) as that enzyme [1]. Here, we investigate the specificity, mechanism and stimulation of duplex unwinding by Dhr1 *in vitro*. The specificity differs from most RNA helicases because no activity was observed with general substrates that contain short duplexes flanked by either a 5'- or 3'-single stranded extensions. In contrast, duplexes that mimic two of the three genetically verified U3-pre-rRNA interactions are unwound by Dhr1 in an ATP dependent manner: U3-18S and U3-ETS2 duplexes. To examine whether ATP hydrolysis is required for unwinding we assayed a motif II mutant impaired in ATP hydrolysis; unwinding was observed in the presence of ATP under pre-steady state but not steady state conditions. To test whether single stranded RNA product release requires ATP hydrolysis we examined a motif I mutant impaired in ATP binding, which was inactive under both pre-steady state and steady state conditions. The requirement of ATP hydrolysis by Dhr1 for efficient enzyme recycling but not duplex unwinding indicates that this DEAH/RHA helicase shares a common mechanism with DEAD-box helicases but differs from viral DExH helicases, which require ATP hydrolysis for processive strand displacement. To our knowledge Dhr1 is the first DEAH/RHA helicase for which the mechanistic steps associated with ATP binding and hydrolysis have been identified. The *in vitro* unwinding rate by Dhr1 is however not fast enough to satisfy the expected U3 recycling demands during rapid cellular growth. In the abstract by Zhu et al, we identify the essential protein Utp14 as a candidate that stimulates the function of Dhr1 *in vivo*. Here, we show *in vitro* that addition of Utp14 to Dhr1 increases its unwinding rate sufficiently to satisfy the expected *in vivo* demand.

1) Sardana et al (2015) *PLOS Biology* **13(2)**:e1002083.

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16 Structure-based insights into the eIF5A-induced protein synthesis by the eukaryotic ribosome

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Protein synthesis by the ribosome is assisted by numerous proteins, among which the elongation factor eIF5A outstands for its unique ability to change catalytic properties of the ribosome. By a yet unknown mechanism, eIF5A stimulates the formation of peptide bonds, and more particularly, allows the efficient synthesis of proline-rich polypeptides, which otherwise causes ribosome stalling due to the unusual structure of prolines compared to other amino acids. Previous studies have shown that the activity of eIF5A relies on its unique and essential post-translational modification - hypusine. Here, we present the crystal structure of eIF5A bound to the 80S ribosome from S. cerevisiae that reveals the conformation of hypusine in the vicinity of the active site of the ribosome. The structure suggests that hypusine promotes the peptide bond formation in an indirect way - rather than contacting the nascent peptide chain, as was suggested previously, it is displacing the CCA-end of the P-site tRNA, thus resolving the stalling caused by the unoptimal geometry of poly-prolyl peptides in the peptidyl-transferase center.

17 Initiation factor 2 stabilizes the ribosome in a semi-rotated conformation

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During the elongation phase of protein synthesis, the movement of tRNAs through the ribosome is accompanied by large-scale conformational changes, such as intersubunit rotation and the movement of a mobile domain of the large ribosomal subunit, the L1 stalk. Although the elongating ribosome likely samples a number of transient conformations, it predominantly adopts two main structural states: the non-rotated, classical state, and the rotated, hybrid state. The L1 stalk adopts open and closed conformations in non-rotated and rotated states, respectively. However, conformational rearrangements of the ribosome during the initiation phase of translation are less well understood. The bacterial initiation factors (IFs) 1, 2, and 3 mediate the binding of initiator tRNA and mRNA to the small ribosomal subunit forming the 30S initiation complex, which subsequently associates with the large subunit. Here we use single-molecule Förster resonance energy transfer (smFRET) to monitor intersubunit rotation and the inward/outward movement of the L1 stalk of the large ribosomal subunit during the subunit-joining step of translation initiation. We show that upon subunit association, the ribosome adopts a distinct conformation in which the ribosomal subunits are in a semi-rotated orientation and the L1 stalk is observed in a half-closed state. The formation of the semi-rotated intermediate requires the presence of an aminoacylated initiator fMet-tRNA^{fMet} and IF2 the in GTP-bound state. IF1 and IF3 do not contribute to the stabilization of this intermediate. GTP hydrolysis by IF2 induces the opening of the L1 stalk and the transition to non-rotated conformation of the ribosome. Our results support a model in which L1 stalk movement is allosterically coupled to intersubunit rotation and IF2 binding. Our data also suggest that IF2-mediated positioning of ribosomal subunits in the semi-rotated orientation may promote subunit association by facilitating docking of intersubunit bridges. Subunit association in eukaryotes is assisted by the ortholog of IF2, eIF5B, hence this mechanism of subunit joining is likely conserved in eukaryotes.

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18 Molecular Mechanics of Head Rotation in the Small Subunit of the Ribosome

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Protein synthesis is universally carried out by the ribosome. The crucial process of ribosomal translocation precisely controls mRNA and tRNA movement during the elongation of the nascent polypeptide chain. Translocation requires large-scale rotation of the head domain of the small (30S) subunit of the ribosome.

It has generally been accepted that the head rotates by pivoting around the 'neck' helix (h28) of 16S rRNA, its sole covalent connection to the body domain. Surprisingly, we observe that the calculated axis of rotation does not coincide with the neck. Instead, the axis of rotation lies roughly parallel to helices h28 and h36, suggesting that the neck is not solely responsible for head movement.

By applying a novel comparative structure analysis approach across 55 crystal and cryo-EM-derived ribosome structures, we show that 30S head movement universally results from flexing at two hinge points lying within conserved elements of 16S rRNA. Hinge 1 is located within the neck and moves by straightening of the kinked helix h28 at the point of contact with the mRNA. Hinge 2 lies within a 3-way helix junction that extends to the body through a second, non-covalent connection; its movement results from flexing between helices h34 and h35 in a plane orthogonal to the movement of Hinge 1. Concerted movement at these two hinges accounts for the observed magnitudes of head rotation. Importantly, our findings explain the mode of action of spectinomycin, an antibiotic that blocks translocation by binding to Hinge 2.

19 Ribosome-based quality control of oxidized mRNA.

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RNA is vulnerable to a variety of damages caused by exogenous and endogenous agents, which can affect its functional properties and ultimately pose a challenge to the cell. In particular, the presence of reactive oxygen species (ROS) or UV irradiation can lead to oxidation of the nucleobases. The oxidized base 8-oxoguanosine has been linked to aging and neurodegeneration as well as reduced protein synthesis, however the effects of mRNA damage on the speed and accuracy of the decoding process have not been determined. With this in mind, we have introduced a single 8-oxoguanosine into mRNA and examined its effect on translation using a well-defined bacterial in vitro translation system. We find that oxidative damage has detrimental effects on the decoding process. Although 8-oxoguanosine has little to no effect on the fidelity of the selection process, it reduces the rate of peptide-bond formation by more than three orders of magnitude independent of its position within the codon. Additionally, 8-oxoguanosine was found to dramatically reduce the rate of GTP hydrolysis by EF-Tu during the initial phase of tRNA selection. In contrast, the adduct has only a marginal effect on the maximal rate of release, suggesting that defects in tRNA selection are due to altered codon-anticodon geometry. This is further supported by the observation that the aminoglycoside paromomycin partially rescues the reduced rates of tRNA selection. Collectively our *in vitro* data suggests that oxidized RNA stalls the translation machinery and would be subject to no-go decay *in vivo*. In agreement with these proposals, yeast strains deficient for no-go decay factors are sensitive to 4-nitroquinoline-1-oxide and appear to accumulate 8-oxoguanosine in their mRNAs. Finally we examined the effects of 8-oxoguanosine on mRNA decay in HEK293 cells. mRNAs with a single 8-oxoguanosine in the coding region decay ~3 times faster relative to ones containing the modification in the UTR; the latter mRNAs have half lives indistinguishable from intact mRNAs. We are currently testing the role of quality control factors in mediating the decay process. In summary, our data provide compelling evidence that cells have evolved mechanisms to deal with damaged mRNA.

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20 *Drosophila* germ granules are structured and contain homotypic mRNA clusters

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Germ granules, specialized ribonucleoprotein particles, are a hallmark of all germ cells. In some species, these particles are synthesized during oogenesis and accumulate in the germ plasm in the early embryo, while in others they form during germ cell specification. In *Drosophila*, an estimated 200 mRNAs are enriched in the germ plasm, and some of these have important, often conserved roles in germ cell formation, specification, survival and migration. How mRNAs are spatially distributed within a germ granule and whether their position defines functional properties is unclear. Here, we used single-molecule FISH and structured illumination microscopy to show that mRNAs are distributed asymmetrically within the granule whereas core germ plasm proteins are distributed evenly throughout the granule. Multiple copies of single mRNAs organize into "homotypic clusters" that occupy defined positions within the center or periphery of the granule. This organization, which is maintained during embryogenesis and independent of the translational or degradation activity of mRNAs, reveals new regulatory mechanisms for germ plasm mRNAs that may be applicable to other mRNA granules.

21 Retroviral mRNA nuclear egress mediated by centrosomes

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Retroviral genomic RNAs (gRNAs) are transcribed as pre-mRNAs and either spliced in the nucleus or exported, introns intact, to the cytoplasm to serve as viral mRNAs encoding the Gag and Gag-Pol capsid proteins and substrates bound by Gag/Gag-Pol for packaging into virus particles. HIV-1 ensures gRNA nuclear export through the activity of the Rev response element (RRE), a *cis-acting* RNA structure that binds to a complex of viral Rev proteins prior to Rev's recruitment of the cellular CRM1 nuclear export receptor. Other retroviruses are CRM1-independent and instead utilize RNA structures known as constitutive transport elements (CTEs) that directly bind to components of the NXF1/NXT export machinery that already mediates the bulk of cellular mRNA nuclear export. Why HIV-1 preferentially exploits the CRM1 pathway is unknown. Here we employed a 3-color virus imaging strategy to directly monitor HIV-1's post-transcriptional regulatory stages in single living cells. YFP-labeled, RRE-dependent, surrogate HIV-1 gRNAs (RRE-gRNAs) encoding CFP-labeled Gag proteins exhibited dramatic, Rev- and CRM1-dependent pulsiform transitions from the nucleus to the cytoplasm prior to bursts of Gag synthesis and the onset of virus particle production. Rendering HIV-1 gRNAs CRM1-independent by replacing the RRE with multiple copies of the CTE from the betaretrovirus Mason-Pfizer monkey virus largely abolished pulsiform export dynamics and, remarkably, instead directed CTE-gRNAs but not RRE-gRNAs to markedly accumulate at the microtubule organizing center (MTOC). Even more strikingly, we also observed nascent CTE-gRNAs in the nucleus rapidly targeting to centrosomal MTOCs at the onset of mitosis and in concert with mitotic spindle formation. After partitioning to daughter cells, centrosome-associated CTE-gRNAs dissipated into the cytoplasm, coincident with the onset of high-level Gag-CFP expression. Pharmacological disruption of the microtubule (MT) cytoskeleton arrested CTE-gRNAs at the nuclear membrane with NXF1, consistent with a model in wherein CTE-NXF1 interactions regulate a transition from the nuclear membrane to dynein-based MT transport. In sum, we describe a heterologous viral RNA element capable of directing mRNA trafficking to the MTOC, and find it compelling that centrosome targeting during mitosis may represent a novel NXF1-dependent mRNA nuclear egress pathway operating independently of the nuclear pore complex.

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22 Identification of the stress granule proteome and disassembly factors

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Stress granules are conserved mRNA-protein granules that form when translation initiation is limited. Stress granules, and related RNA-protein granules, are proposed to modulate translation, mRNA degradation, signaling pathways, and mRNA localization. In this work, we demonstrate that yeast and mammalian stress granules are stable in lysates and characterize their biochemical properties. We also purify stress granules and use mass spectroscopy and validation by microscopy to identify the yeast and mammalian stress granule proteomes, which includes multiple unanticipated components of stress granules such as multiple subunits of RNA polymerase II, tRNA synthetases and the protein chaperone CCT complex. Bioinformatics analyses of stress granule proteins revealed numerous protein-protein interactions amongst stress granule proteins that could provide the basis for their assembly, which suggests the dynamic nature of stress granules is dependent on active disassembly of protein-protein interactions. Consistent with active disassembly of stress granules, we demonstrate multiple ATP-driven machines are required for efficient stress granule disassembly including the CCT complex, RNA helicases and Hsp70/Hsp40 complexes. These observations suggest that stress granules are stable assemblies with redundant assembly interactions that utilize multiple ATP driven machines for disassembly.

23 Single-molecule imaging of mRNAs in living cells during stress

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When cells are stressed, a variety of signaling cascades are activated which results in the inhibition of translation and the formation of cytoplasmic mRNP granules (stress granules and P-bodies). Stress granules (SGs) have been found to contain mRNAs, 40S ribosomal subunits and translation initiation factors suggesting that these structures are formed by the aggregation of mRNAs stalled during initiation of translation. In contrast, P-bodies (PBs) are enriched for proteins involved in mRNA turnover, which suggests these structures may be involved in degradation. While much is known about the composition of these mRNP granules and the forces that drive their assembly, much less is understood about how mRNAs are trafficked to and within these compartments and how this regulates RNA metabolism during stress.

We have engineered a tetracycline-inducible HeLa cell line that allows us to monitor the fates of single mRNA molecules during oxidative stress (arsenite). A 5′-terminal oligopyrimidine (5′ TOP) reporter mRNA was observed to specifically cluster within PBs upon addition of arsenite. Using the TRICK (translating RNA imaging by coat protein knock-off) methodology, which enables untranslated mRNAs to be distinguished from ones that have undergone translation, we find that sequestering of 5′ TOP mRNAs within PBs allows their translation to regulated differentially compared to 5′ TOP transcripts that remain in the cytosol. These results highlight the importance of cytoplasmic mRNP granules in the spatial and temporal regulation of RNA metabolism during stress.

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24 Common effects of ALS-associated mutations on RNA localization: a role for cytoplasmic RNA inclusions

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Our lab is interested in the functional roles of localized RNAs in mammalian cells. We had previously described a localization pathway that targets a number of RNAs at protrusions of fibroblasts or neuronal cells. RNAs localized through this pathway associate with and depend for their localization on the APC tumor-suppressor protein[1]. These RNAs are specifically anchored at the plus ends of detyrosinated microtubules, i.e. a subset of stable microtubules that do not undergo dynamic instability[1]. An additional component and regulator of these localized complexes is the Fus RNA-binding protein[2]. Mutations in Fus have been identified in a spectrum of neurodegenerative diseases, including Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD). Apart from Fus, ALS and FTD pathogenic mutations have been mapped in another RNA-binding protein, TDP-43. Such mutations commonly lead to the formation of cytoplasmic inclusions containing either Fus or TDP-43 proteins. These inclusions are a major hallmark of disease and are thought to be recapitulated by expression of mutant Fus, carrying ALS-associated mutations, in cell culture systems. This leads to formation of Fus cytoplasmic granules. We have shown that, contrary to expectations, Fus granules are translationally active and also recruit APC[2]. We further show that mutant Fus expression leads to mislocalization of APC-associated RNAs from cell protrusions and to a specific disruption of detyrosinated microtubules. Interestingly, expression of ALS-associated TDP-43 mutants leads to similar disruption of localized RNAs and of the microtubule network. Strikingly, formation of cytoplasmic granules appears to be important, since disrupted phenotypes are observed only in cells exhibiting cytoplasmic Fus granules, but not in cells expressing non-aggregated forms of the mutant protein. Our results indicate that RNA mislocalization is a common effect of pathogenic mutations in Fus and TDP-43, and suggest that it is specifically manifested upon pathogenic protein aggregation. We are investigating the underlying mechanisms and are identifying globally the affected RNAs.

1. Mili S. et al. (2008) Nature, 453:115

2. Yasuda K. et al. (2013) J. Cell Biol., 203:737

25 U1 snRNP is mislocalized in ALS patient fibroblasts bearing NLS mutations in FUS and is required for motor neuron outgrowth in zebrafish

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Mutations in FUS cause Amyotrophic Lateral Sclerosis (ALS), but the molecular pathways leading to neurodegeneration are not yet known. We previously found that U1 snRNP, the SMN complex, and RNAP II are abundant FUS interactors[1, 2]. We now find that components of the U1 snRNP core particle (Sm proteins and U1 snRNA), but not the mature U1 snRNP-specific proteins (U1-70K, U1A, and U1C), co-mislocalize with FUS to the cytoplasm in ALS patient fibroblasts harboring mutations in the FUS nuclear localization signal (NLS). Similarly, the U1 snRNP core particle and FUS mislocalize to the cytoplasm in HeLa cells expressing the ALS-causing FUS R495X NLS-truncating mutation, and mislocalization of Sm proteins is RRM-dependent. Moreover, knockdown of any of the U1 snRNP-specific proteins results in a dramatic loss of SMN-containing Gems. Significantly, knockdown of U1 snRNP in zebrafish results in motor axon truncations, a phenotype also observed with FUS, SMN and TDP-43 knockdowns. Together, our results linking U1 snRNP to ALS patient cells with FUS mutations, SMN-containing Gems, and motor neurons indicate that U1 snRNP is a component of a molecular pathway associated with motor neuron disease. Linking an essential canonical splicing factor (U1 snRNP) to this pathway provides strong new evidence that splicing defects may be involved in pathogenesis.

1. Das et al., Mol. Cell, 2007, 26: 867

2.Yamazaki et al., Cell Reports 2012, 2: 799

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26 Structure of the 800 kDa budding yeast U1 snRNP: a perspective to understand alternative splicing

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Pre-mRNA splicing is catalyzed by the mega-dalton spliceosom, a highly dynamic machine consisting of five large RNA-protein subunits, the Uridine-rich small nuclear ribonucleoprotein complexes (U snRNPs). Involved in initiation and regulation of the spliceosome, the U1 snRNP recognizes the junction between a 5' exon and an intron in a pre-mRNA, the 5' splice site. The U1 snRNP in metazoans is a 10 subunit complex, composed of a single RNA and ten proteins. All components of the metazoan U1 snRNP are also found in the budding yeast (*S. cerevisiae)* U1 snRNP. The yeast U1 snRNP has an additional seven integral proteins members with homology to metazoan alternative splicing factors. Alternative splicing factors guide the U1 snRNP to its target pre-mRNA sequence. With more than 70% of human pre-mRNAs targets for alternative splicing and the misregulation of this process linked to many diseases, visualizing the structural interactions of these factors to the core, metazoan U1 snRNP will enhance our understanding of functional vs nonfunctional interactions.

To better understand how the U1 snRNP recognizes the crucial pre-mRNA 5' splice site, we have determined the structure of the 800 kDa, yeast U1 snRNP. Comprised of 18 subunits, the yeast U1 snRNP has been isolated from a native source, determined to be homogeneous, and shown by two complementary assays to be functional. We have obtained both negative stain and cryo electron microscopy (EM) structures of U1 snRNP. We are utilizing a multi-faceted approach, in part facilitated by yeast genetics, to interpret the EM density of the structures. By fitting the smaller ~250 kDa human U1 snRNP previously determined by x-ray crystallography (Pomeranz Krummel et al., Nature, 2009) and assigning possible positions for proteins guided by labeling in EM, a path can be suggested for the pre-mRNA. Our emerging structural model of the yeast U1 snRNP provides a platform to understand how protein factors, auxiliary in metazoans, aid pre-mRNA recognition to initiate spliceosome assembly.

27 Structural insights into the flexible parts of U1snRNP and identification of a novel molecular link between U1snRNP and U2snRNP

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Early stages of spliceosomal assembly require the recognition of U1snRNP at the 5'-splice site, of U2snRNP-U2AF at the 3'-splice and branch sites and the establishment of transient interactions between both RNPs. Crystal structures of U1snRNP have deciphered the molecular arrangement of this compact particle (1, 2) and recently provided high resolution details (3). However, in these structures, several protein segments were not observed either because of their dynamic behavior or because they were not included. We developed a protocol to reconstitute the U1snRNP particle including most of these tails. By means of solution NMR, we were able to observe the Sm tails, the C-terminal parts of U1-C and U1-A in the U1snRNP context. These data provided, for the first time, novel structural insights into the role of U1-C tail and the position of the U1-A C-terminal part. In addition, it was recently shown that U1-SL4 is involved in establishing direct contacts with U2snRNP (4); however, it remains unclear how other parts of the U1snRNA could be involved in such process. In order to identify new molecular linkers between U1snRNP and U2snRNP, we investigated whether RBM39, a protein which was already identified in early spliceosomal complex A (5), involved in the splicing of VGEF mRNA (6) and overexpressed in several cancer types (6, 7), could be involved in this function on given mRNAs. Although its capacity to bind U2snRNP via its U2AF homology motif was already established (8), we demonstrated *in vitro* that the N-terminal region of RBM39 binds U1-SL2 and U1snRNP. Altogether, our results support RBM39 as a linker between U1snRNP and U2snRNP during critical splicing events and reveals structural insights into early spliceosomal assembly.

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28 Spontaneous Toggling of the U2 snRNA between Stem IIa and IIc Conformations is Regulated by Magnesium and Cus2

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Changes in pre-mRNA and snRNA conformation are key to spliceosome function. The U2 snRNA basepairs to the branch site region of the pre-mRNA in early spliceosomal complexes and forms additional, extensive basepairing interactions with the U6 snRNA in the catalytic spliceosome. In addition to these intermolecular rearrangements, genetic and *in vivo* structure probing experiments support intramolecular changes in conformation of the U2 snRNA. Adoption of the stem IIa conformation has been proposed to facilitate duplex formation between the pre-mRNA and U2 snRNA. In contrast, the stem IIc conformation has been proposed to facilitate the catalytic steps of splicing. In this model, correct progression of the spliceosome through splicing requires that the snRNA toggles back-and-forth between stem IIa and IIc as the spliceosome is formed, activated, and the substrate is repositioned between 5′ splice site cleavage and exon ligation. We have used single molecule fluorescence resonance energy transfer (smFRET) to study the conformational dynamics of the stem II-containing core of the yeast U2 snRNA. Our results show that the U2 core spontaneously toggles between stem IIa and IIc. This equilibrium can be shifted towards favoring the IIa conformer by addition of magnesium or the Cus2p protein. Electrophoretic mobility shift assays (EMSAs) reveal that Cus2p shows a strong preference for binding to the stem IIa conformer of U2 in contrast with previously reported models for Cus2 function. Differences in the conformational landscape sampled by the RNA in the presence of Cus2 or magnesium suggest that the Cus2Δ U2 snRNA may not behave identically to the snRNA in the presence of this protein; this suggests caution is needed for deducing the function of proteins in Cus2-defecient backgrounds. We propose that conformational toggling of stem II is a property inherent to the snRNA. The spliceosome has evolved mechanisms to shift equilibrium towards either stem IIa or IIc by the use of proteins such as Cus2p and magnesium. Regulation of magnesium availability may be key for controlling U2 snRNA toggling during 5' splice site cleavage and exon ligation.

29 Efficient annealing of spliceosomal U4 and U6 RNAs by Prp24 requires an electropositive groove, the U6 telestem, and the Lsm ring

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The spliceosomal protein Prp24 facilitates U4/U6 annealing by unwinding the U6 intramolecular stem loop (ISL) and annealing it to U4 snRNA. Our prior studies identified a mutation that stabilizes the ISL and results in a cold sensitive phenotype and low levels of U4/U6 *in vivo*. Suppressors of this phenotype have been isolated, yet the mechanism of U4/U6 annealing has not been described. We have developed an *in vitro* annealing assay that recapitulates the *in vivo* phenotypes and allows for detailed investigation of the U4/U6 annealing mechanism.

Our previous structure of the U6-Prp24 complex (PDB: 4n0t) reveals an electropositive groove on the surface of the protein that does not contact U6. We proposed that this groove promotes annealing by recruiting U4 to a single stranded region of U6 to nucleate duplex formation, which proceeds via strand invasion of the ISL. Our data indicate that this process is gated by an unstable base pair at the base of the ISL, consistent with the previously observed phenotypes. We further demonstrate that mutation of the electropositive groove on Prp24 does not affect binding affinity for U6, but strongly inhibits U4/U6 annealing. We show that Prp24 binds to free U4 RNA and that mutations in the groove that impair annealing also reduce the binding affinity for free U4. Together, these observations support our proposal that the electropositive groove nucleates U4/U6 annealing by recruiting U4 to the U6/Prp24 complex.

We have reconstituted the entire U6 snRNP in vitro and find that the Lsm2-8 heptamer stimulates Prp24-mediated U4/U6 annealing, but cannot catalyze U4/U6 annealing on its own. The Lsm2-8 heptamer is predicted to bind adjacent to the U6 telestem, a helix that is distal to the electropositive groove. Surprisingly, mutations that are expected to stabilize the telestem increase the annealing rate by approximately 15-fold in the absence of Lsm2-8. We hypothesize that the U4/U6 annealing mechanism is stimulated by Lsm2-8 via its interaction with the telestem, which in turn stabilizes the overall architecture of the U6 snRNP, including the conformation of the electropositive groove in Prp24.

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30 Brr2 Retinitis Pigmentosa Mutations Reduce Helicase Processivity

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To determine how Retinitis Pigmentosa (RP) mutations in Brr2's Ratchet Helix lead to reduced U4/U6 unwinding in triple-snRNPs, reduced yeast growth, and degenerative blindness in humans we have characterized yeast Brr2 helicase activity using a minimal *in vitro* system. The N-terminal 400 amino acids of Brr2 do not yet have a known structure and the full length protein is prone to aggregation *in vitro*. Therefore, we truncated either the first 422 or 247 amino acids from Brr2 and were able to purify high concentrations of active protein. Interestingly, while the wild type Brr2 construct lacking the entire N-terminal region (D422-Brr2) has similar helicase properties to those published in the literature, D422-Brr2 containing the RP mutations cannot bind RNA at any appreciable level. This suggests that, at least in the absence of the U5 snRNP, the N-terminus of Brr2 contributes to its RNA binding affinity. The longer D247-Brr2 with the RP mutation (N1104L) has similar RNA affinity as the wild type protein but unwinds U4/U6 to a lower extent, suggesting a defect in helicase processivity. The mutations at position 1107 have a much stronger affect on RNA binding. D247-Brr2(R1107A) has a weak affinity for U4/U6 while still exhibiting a U4/U6 unwinding defect under saturating conditions. A mutation to Leucine at position 1107 further weakens the affinity for RNA. This gradient of U4/U6 affinity with (N1104L) > (R1107A) > (R1107L) matches the severity of their respective yeast growth defects previously reported. By performing the helicase assay using model duplex RNAs with increasing numbers of base pairs we show that indeed the RP mutants have reduced processivity compared to the wild type protein. The processivity of both the wild type and mutant proteins is increased by the presence of the C-terminal domain of Prp8. Despite the U4/U6 duplex only having 17 base pairs in its longest stem, D247-Brr2 is able to unwind a duplex of over 30 base pairs rapidly and to completion when the Prp8 C-terminal domain is present. Brr2 may require this level of mechanical power in order to displace the U4/U6 snRNP proteins that block U6 from joining the spliceosome.

31 The architecture of the yeast spliceosomal U4/U6.U5 tri-sRNP revealed by cryo-EM

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U4/U6.U5 tri-sRNP is the largest preformed component of the spliceosome (1.5MDa) consisting of the extensively base-paired U4/U6 snRNA, U5 snRNA and over 30 proteins, including the three key proteins Prp8, Brr2 and Snu114. Tri-snRNP joins the spliceosome after U1 and U2 snRNPs have assembled on the pre-mRNA. This is followed by a major conformational and compositional change triggered by the unwinding of U4/U6 snRNA duplex catalyzed by the Ski2 helicase Brr2, which results in catalytic activation of the spliceosome.

Taking advantage of recent advances in image processing algorithms and direct electron detectors for cryo-EM, we determined the structure of the yeast tri-snRNP at 5.9 Å overall resolution. This allows us to fit known crystal structures or homology models of most protein subunits and double stranded helices of U4/U6 and U5 snRNAs, revealing a nearly complete organization of its proteins and RNA. The structure provides crucial insights into the activation process and the active site of the spliceosome.

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32 Quaternary structure of the core splicing factor Prp19

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Splicing occurs on the spliceosome, a multi-Megadalton machine that assembles step-wise on the pre-mRNA substrate, first as an inactive particle. Subsequently, the spliceosome undergoes dramatic compositional and conformational changes that lead to the formation of an active center, able to catalyze the chemistry of splicing.

The highly conserved splicing factor Prp19 is a scaffold of the so-called Nineteen complex (NTC) - a large building block of the spliceosome that is essential for the formation of the spliceosomal catalytic center, both in yeast and human. Prp19 forms a tetramer of about 220kDa in vivo and in vitro, and its quaternary structure is essential for Prp19 function as a protein scaffold. In addition, owing to the U-box domains of the Prp19 component, NTC regulates splicing and DNA repair by its ubiquitin ligation activity [1; 2]. We demonstrate that the Prp19 homotetramer becomes inactive in the absence of the other NTC components. Crystal structure of Prp19 tetrameric core reveals how a long quadruple coiled-coil sequesters the four U-box domains, providing a snapshot of Prp19 in the inactive state. Further analysis of the full-length Prp19 as well as of its tetrameric core by small-angle X-ray scattering are consistent with the crystallographic analysis and the previously published EM analysis [3]. This work elucidates the quaternary structure of a core splicing factor and provides the first insight into the regulation of its ubiquitination activity.

[1] Song, E. J., et al., (2010) Genes Dev. 1;24(13): 1434-47

[2] Maréchal, A., et al., (2014) Mol. Cell. 23;53(2):235-46.

[3] Ohi, M. D., et al., (2005) Mol. Cell. Biol. 25, 451-460.

33 Electron-microscopic location of tagged proteins reveals the subunit architecture of the spliceosomal B complex

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Pre-mRNA splicing is catalyzed by the spliceosome, a highly dynamic ribonucleoprotein complex which assembles on the pre-mRNA in a stepwise manner. The A complex, formed by binding of the U1 and the U2 snRNPs to the pre-mRNA, is the first stable spliceosomal complex. Its association with the pre-formed U4/U6·U5 tri-snRNP results in the fully assembled pre-catalytic B complex. Massive rearrangements, involving inter alia displacement of the U1 and U4 snRNPs, then lead to the Bact complex and catalytic activation. In recent years it has become possible to stall spliceosome assembly at various stages of the splicing cycle and to purify the stalled complexes. Electron-microscopic studies have revealed the structures of several of these assembly intermediates. To shed light on the architecture of the complete B complex of Saccharomyces cerevisiae we have used the tandem affinity purification (TAP) tag and anti-TAP antibodies to locate selected proteins on the surface of the B complex by negative-stain electron microscopy. We located the largest U5 snRNP components (Brr2, Prp8 and Snu114), two U4/U6 di-snRNP proteins (Prp3 and Lsm4) and several proteins from the SF3a (Prp9 and Prp11) and SF3b (Hsh155 and Cus1) complexes (SF3a and SF3b are major building blocks of the U2 snRNP). These locations allowed us to assign distinct domains of the B complex to the respective snRNPs. Furthermore, we found that the SF3a complex proteins are close to the U4/U6 di-snRNP proteins; this agrees well with earlier findings that base-pairing between the U2 and U6 snRNAs is necessary for stable B complex formation. Moreover, the SF3b complex proteins are close to the part of the U5 snRNP that harbors Brr2 and Prp8, suggesting a second site of contact between the A complex and the tri-snRNP. These findings yielded, for the first time, a detailed picture of the subunit architecture and protein arrangements of a functional spliceosome. Together with similar analysis of the Bact complex these results provide a first insight into the global structural rearrangements that take place during catalytic activation.

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34 Small-molecule spliceosome inhibitors share a common mechanism and reveal functional roles for the core protein SF3B1 before and after splicing chemistry.

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SF3B1 is a U2 snRNP component and plays a role in the ATP-dependent recognition of the branch point in early spliceosome assembly. Exome sequencing revealed that specific SF3B1 mutations are frequent in cells from several cancers. Remarkably, SF3B1 is also the target of natural products that were first identified as highly potent growth inhibitors of tumor cells: FR901464 (and its derivative SSA), pladienolide B (PB), and herboxidiene (HB). All three compounds inhibit splicing *in vitro* and affect alternative splicing in cells. These observations have raised SF3B1 as a promising target both for studying the links between splicing and cancer and for new chemotherapeutics. We are investigating the structure-activity relationships of SF3B1 inhibitors to understand their mechanisms of inhibition, and are using the compounds to explore the role of SF3B1 throughout the splice cycle.

By examining competition between compounds, we discovered that addition of inactive analogs restores the function of spliceosomes inhibited by active compounds. Notably, all of the inactive analogs are able to complete with any of the three active inhibitors. This is the first evidence that the three structurally distinct compounds bind to the same site on SF3B1 and likely interfere with its function by the same mechanisms. Our results also suggest that inhibition does not result from steric hindrance, but that the active compounds induce or inhibit an allosteric change in SF3B1 structure.

To identify additional roles for SF3B1 in splicing, we bypassed the first early block in spliceosome assembly caused by the inhibitors. We find that the compounds inhibit assembly following stable A complex formation in tri-snRNP depleted extracts. Strikingly, they also inhibit exon ligation in a bimolecular splicing assay that separates 1st and 2nd step chemistry. In both cases, inhibition can be rescued with inactive analogs, suggesting that SF3B1 undergoes a similar conformational change. These data expand SF3B1 function in the splice cycle and open the road to determining how changes in that activity relate to cancer. Ultimately, our findings will help to answer how SF3B1 mutations impair splicing in cancer, and how we can use this knowledge to design novel therapeutics.

35 Circular RNA biogenesis can proceed through a lariat intermediate

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Circular RNA is a recently discovered feature of eukaryotic gene expression programs with largely unknown biological function. The presumed biogenesis of these RNAs involves a non-canonical 'backsplicing' event in which the branch point in an upstream intron attacks a downstream splice donor, generating an exonic RNA circle. Recent studies in mammalian cell culture posit that spliceosomal backsplicing is due to inverted repeats flanking the circularized exon(s) which create a topology appropriate for splice site pairing across the exon. Although such sequence elements are common in mammals, they are rare in lower eukaryotes, making current models insufficient to describe circularization. Using biochemical and genetic analysis, we show that circular RNA in the *S. pombe* gene *mrps16* is generated through an exon-skipped lariat intermediate and that the debranching enzyme, Dbr1, negatively regulates this process. While not sufficient for circular RNA production, utilization of a lariat intermediate is likely a widespread and general mechanism for circular RNA production in *S. pombe*. Furthermore, we have performed the first unbiased, large-scale mutagenesis of a circular RNA, which led us to discover a systematic effect of exon length on RNA circularization. Our results uncover a novel mechanism for circular RNA biogenesis that may account for circularization in genes that lack noticeable intronic secondary structure.

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36 Spliceosomal intronogenesis

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The presence of intervening sequences, termed introns, is a defining characteristic of eukaryotic nuclear genomes. Once transcribed into pre-mRNA, these introns must be removed within the spliceosome prior to export of the processed mRNA to the cytoplasm where it is translated into protein. Their origin and the mysterious nature of how and if they are still capable of propagating remain largely unproven. Although intron loss has been demonstrated experimentally, no demonstration of an intron gain event has been shown and has only been suggested by comparative phylogenetic analyses. We have used a reporter-based strategy that selects for both intron loss and intron gain events. We experimentally verified the first demonstration of intron gain in any organism. Although intron loss events with this reporter are seen relatively frequently (~1 in 4 x 10^7 cells), intron gain was only seen once in $>10^{10}$ cells screened. In the intron gain event, the yeast gene *RPL8B* was shown to have a selected, perfectly transposed intron added into the genome. Due to its large size, the splicing of the reporter intron is very inefficient; we show that when overexpressed, this novel allele is functional in a strain lacking the Rpl8 paralogue *RPL8A* demonstrating that the newly formed intron can be removed by the spliceosome and that the gene targeted for intronogenesis is functional. Although we have thus far only detected a single event, we provide evidence that this reaction is spliceosome-mediated and will present a model for spliceosomal intron propagation and homeostasis.

37 A hungry spliceosome reveals rapidly evolving auxiliary introns in transcribed non-protein coding regions of *Saccharomyces* genomes.

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We recently discovered that pre-mRNAs compete with each other for the splicing machinery. Treating yeast with rapamycin, an inhibitor of ribosomal protein gene (RPG) transcription, creates a condition in which ~90% of the pre-mRNAs are depleted, relieving competition, and allowing non-RPG pre-mRNAs to be more efficiently spliced (Munding et al. 2013). Here we report that transcripts whose splicing is normally not detected in growing yeast cells become spliced when competition is relieved, a phenomenon we attribute to a "hungry spliceosome." By RNA-seq and RT-PCR, splicing is evident at hundreds of unannotated locations; most all have GT-AG intron ends, however their 5' splice site and branchpoint sequences are more varied than those of annotated introns. Rarely found entirely within ORFs, these inchoate introns often span the 5'UTR or 3'UTR of known mRNAs, or appear in intergenic or antisense noncoding RNAs, often using alternative 5' and 3' splice sites. Most of these auxiliary introns have appeared in *S. cerevisiae* since it diverged from *S. mikatae* ~5 million years ago. Parallel experiments on *S. mikatae* and *S. bayanus* show that they each possess a distinct species-specific set of auxiliary introns that has arisen from genetic drift in their intergenic regions as well. Based on this we conclude that (1) in combination with genetic drift, the spliceosome acts broadly to create novel non-genomic sequence information as RNA that may be adaptive, (2) introns are frequently born at the edges of existing genes where they may create additional variation in protein sequence that could prove adaptive, (3) introns are often born with alternative splice sites, suggesting that alternative splicing is not always an evolutionarily advanced phenomenon, but reflects the intrinsic flexibility of substrate recognition by the spliceosome, and (4) the reputation of the yeast spliceosome as "hard-wired" and unable to recognize a variety of 5' splice sites and branchpoints is undeserved. Given the ~thousand-fold greater concentration of spliceosomes and the far larger mass and complexity of RNA in the mammalian nucleus it is almost certain that huge numbers of auxiliary splicing events with indecipherable potential for future function also occur in mammalian cells.

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38 Alternative splicing regulates the expression of G9A and SUV39H2 methyltransferases, and dramatically changes SUV39H2 functions

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Alternative splicing affects the sequence of mature RNAs and is a source of proteome diversity. It is now accepted that regulation of splicing is influenced by both transcription and chromatin, and conversely splicing is now proposed to locally affect chromatin properties. Alternative splicing is also expected to impact the expression and activity of chromatin factors, although it has rarely been considered in functional studies. Here, we have characterized protein isoforms of histone methyltransferases, G9A/EHMT2 and SUV39H2.

We show that exon 10 in G9A and exon 3 in SUV39H2 are alternatively included in a variety of tissues and cell lines, as well as in a different species. The production of these variants is likely tightly regulated because both constitutive and alternative splicing factors control their splicing profiles. SiRNA-mediated depletion of 50 different RNA binding proteins on a high-throughput RT-PCR screening platform allow to identify hnRNAP1, RALY, SRp20/SRSF3; TRA2β/SRSF10, Sam68, RBM9/RBFOX2 and RBM39/CAPER as regulators of these exons. Based on this evidence, we have assessed the link between the inclusion of these exons and the activity on K9 methylation of histone H3 of both enzymes. We document that these genes yield several protein isoforms, which are likely issued from alternative splicing regulation. We demonstrate that inclusion of SUV39H2 exon 3 is a determinant of the stability, the sub-nuclear localization, and the H3K9-methyltransferases activity. Genome-wide expression analysis further revealed that alternative inclusion of SUV39H2 exon 3 differentially modulates the expression of target genes. We found a close correlation between the transcription regulation and the presence of active SUV39H2 at the target promoters. Our data also suggest that a variant of G9A may display a function that is independent of H3K9 methylation. Our work emphasizes that expression and function of genes are not collinear; therefore alternative splicing must be taken into account in any functional study.

39 SR protein kinase phosphorylation of the branch point binding protein is required for efficient splicing of non-consensus introns

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Using a chemical-genetic approach, we determined that the conserved SR protein kinase (Srpk) in the fission yeast *Schizosaccharomyces pombe*, Dsk1, is required for the efficient splicing of ~40% of introns. Computational analysis revealed that affected introns were enriched for sub-optimal splice sites. Notably, mutation of suboptimal sequences at the 5'SS, BPS, and 3'SS to consensus sequences bypassed the splicing requirement for Dsk1 phosphorylation *in vivo*. Systematic identification of direct Dsk1 and human Srpk substrates revealed that these kinases act through two evolutionarily conserved phosphorylation sites in the branch point binding protein (Bpb1/SF1). Genome-wide splicing analysis of Dsk1 substrates in fission yeast identified that the two phosphorylated sites in Bpb1 are vital for efficient splicing of sub-optimal introns, which parallel results obtained for Dsk1 inhibition. In order to determine how phosphorylation of Bpb1/SF1 by SR protein kinases is required for the splicing of sub-optimal introns we turned to gel shift assays using recombinant SF1 protein. These assays indicated that SF1 binds more weakly to intron fragments that contain non-consensus branch point sequences and that phosphorylation by Srpk strengthens these interactions. More broadly, our data suggest that modulating non-consensus intron splicing efficiency via kinase signaling may afford the necessary flexibility to tune dynamic gene expression profiles that result from response to environmental and developmental cues.

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40 Structural investigation of several SR proteins containing two RRMs reveals imbalance between the contribution of each domain

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SR proteins, one of the major families of alternative-splicing regulators in Eukarya, have two types of RNA-recognition motifs: a canonical RRM (RRM1) and a pseudo-RRM (RRM2). We recently solved the structure of SRSF1 pseudo-RRM bound to RNA and discovered a very unusual and sequence-specific RNA binding mode that is centered on one α-helix and does not involve the β-sheet surface of the RRM [1]. The domain specifically interacts with a 5'-GGA-3' motif and this mode of recognition is conserved for all pseudo-RRMs we tested from human, yeast and fly. All pseudo-RRM containing SR proteins have in addition to this domain a canonical RRM at their N-terminal extremity, for which the binding to specific RNA sequences still needs to be demonstrated.

In this study, we used NMR to investigate whether these canonical RRMs interact specifically with RNA. Surprisingly, we found that SRSF1, SRSF4, SRSF5, SRSF6, SRSF9 (in human), B52 (in fly) and Npl3 (in yeast) RRM1 all preferentially bind to sequences enriched in cytosines. We then focused on the canonical RRMs of two human SR proteins, SRSF1 and SRSF5, and revealed that these domains recognize slightly different C-containing RNA motifs: 5'-CG/A-3' and 5'-GUCG-3', respectively. Solving the structure of SRSF1 RRM1 bound to RNA revealed a highly specific interaction with a cytosine nucleotide, thus explaining how the single C/U nucleotide difference at position +6 of SMN1/2 exon 7 ESE influences SRSF1 recruitment on RNA [2]. Interestingly, our data also suggests that the relative affinity of each RRM (canonical versus pseudo-RRM) for their RNA targets determines the extent to which SR proteins are recruited to specific and distinct targets. SRSF5 RRM1 binds RNA with a higher affinity than SRSF1 RRM1, whereas SRSF5 RRM2 interaction with RNA is about 10-fold weaker than SRSF1 RRM2. Therefore, we predict SRSF1 and SRSF5 to bind preferentially motifs recognized by RRM2 (GGA) and RRM1 (GUCG), respectively.

Together, our results suggest that canonical RRMs have a crucial function in the specific interaction of SR proteins with their RNA targets in cells.

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41 Whole-transcript SHAPE-MaP reveals alternative splicing events in a 5' UTR that conserve structure

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Alternative splicing has long been recognized as a powerful mechanism to create different proteins from a single gene thereby increasing the molecular diversity of the cell. A surprising number of alternative splicing events occur in non-coding regions of mRNAs. This is especially true in genes with high transcript complexity; i.e. those genes producing 10 or more transcripts from single loci. SERPINA1, the gene coding for α-1-antitrypsin, yields 11 transcripts, placing it in the top 1% of transcriptionally complex genes. SERPINA1 mRNA transcripts are particularly interesting from a post-transcriptional perspective because all alternative splicing occurs in the 5' untranslated region (UTR) of the message. Structured elements in 5' UTRs can affect translation efficiency, localization, and even stability. As such, we are interested in investigating whether UTR alternative splicing conserves structural domains in the RNA analogous to what is observed in protein coding sequences. We used selective 2′-hydroxyl acylation analyzed by primer extension and mutational profiling (SHAPE-MaP) to characterize the structure of the ~1.5-1.8 kb SERPINA1 mRNA. SHAPE-MaP data uses next generation sequencing to measure the chemical probe reactivity, greatly improving the reproducibility and throughput of the structure probing data. As a result we were able to study a majority of SERPINA1 transcripts thereby providing an unprecedented characterization of exon-exon junctions in an alternatively spliced UTR. Combined with SHAPE-directed ensemble modeling and a novel Shannon entropy-informed hidden Markov model, we identified conserved structural elements in the SERPINA1 5' UTR splice variants. The number of these structured elements and their proximity to the start codon is predictive of translation efficiency in luciferase reporter assays. Our data therefore support the idea that alternative splicing in UTRs conserves structural domains (in an analogous manner to protein domain architecture) and that this could modulate post-transcriptional regulation of the gene.

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42 Changes in serotonin receptor 2C alternative splicing deregulate food intake

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The serotonin receptor 2C pre-mRNA undergoes extensive processing including alternative splicing of exon Vb, which creates RNA1 (lacking exon Vb) and RNA2 (containing exon Vb). RNA1 encodes a truncated receptor protein isoform that is located in intracellular membranes and sequesters the full-length receptor internally. Exon Vb inclusion is promoted by SNORD115, an orphan snoRNA that is not expressed by patients with Prader-Willi syndrome. These patients are characterized by hyperphagia and low growth hormone levels. They exhibit an increased RNA1/RNA2 ratio, suggesting the physiological relevance of SNORD115-serotonin receptor 2C interaction.

Using the oligo walk method, we developed an oligonucleotide that promotes inclusion of exon Vb. In reporter cell lines, this oligonucleotide mimics SNORD115 as it reduced truncated receptor pre-mRNA levels and an increased full-length receptor mRNA levels, resulting in the accumulation of full-length receptor in the plasma membrane. After injection into the third ventricle of mice, the oligonucleotide accumulates in the arcuate nucleus, where it decreases the RNA1/RNA2 ratio, promoting the formation of the full-length membrane bound receptor. We observed a stimulation of c-fos and POMC, consistent with the activation of serotonin 2C receptors. Decreasing the RNA1/RNA2 ratio in mouse brain using oligonucleotide injection reduced food intake by 70%.

Similar to other seven transmembrane receptors, the serotonin receptor 2C heterodimerises with related receptors, including the ghrelin receptor that controls growth hormone secretion. Cell based studies show that the RNA1/RNA2 ratio controls ghrelin receptor localization.

Together, the data suggest that the ratio of serotonin receptor 2C isoforms, regulated by SNORD115, determines the activity of the full-length serotonin receptor 2C and related ghrelin receptor. Loss of SNORD115 can thus explain the hyperphagia and low growth hormone levels in patients with Prader-Willi syndrome.

43 Antisense Oligonucleotides that Correct Alzheimer's Disease-Associated Alternative Splicing Improve Learning and Memory in a Mouse Model

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Apolipoprotein E receptor 2 is a post-synaptic apolipoprotein E (apoE) and Reelin receptor involved in long-term potentiation, learning and memory. Signaling through *ApoER2* requires amino acids encoded by the alternatively spliced exon 19. The exon 19-encoded domain is important for synaptic neurotransmission and memory, and to protect against neuronal cell death in normal aging in mice. Here, we demonstrate that ApoER2 mRNA lacking exon 19 is more abundant than full-length ApoER2 in post-mortem brain tissue from Alzheimer's disease (AD) patients compared to non-diseased samples. A similar decrease in exon 19 splicing was observed in transgenic mouse models of AD relative to non-transgenic controls. We identified SRSF1 and SRSF7 as negative regulators of exon 19 splicing in human cells and used antisense oligonucleotides (ASO) to block predicted negative regulatory sequences recognized by these splicing proteins. We identified ASOs that induce exon 19 inclusion in cultured cells and in mice. Treatment of neonatal transgenic AD mice with a single dose of ASO improved their performance on learning and memory tasks for months after treatment, validating *ApoER2* exon 19 splicing as a candidate target for the treatment of AD. These results reveal an association between *ApoER2* exon 19 skipping and AD in humans and provide preclinical evidence for the utility of ASOs as a therapeutic approach to mitigate cognitive decline in Alzheimer's disease by improving *ApoER2* exon 19 splicing.

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44 A mammalian-specific alternative splicing event shapes evolutionary differences between vertebrate transcriptomes

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Alternative splicing (AS) is responsible for generating remarkable transcriptomic and proteomic complexity among vertebrate species. However, the mechanisms and functions associated with species- and lineage-specific splice variants remain largely unknown. To address this, we focused on characterizing exon 9 of the polypyrimidine tract binding protein 1 (PTBP1), which we previously showed to be skipped in the mammalian lineage but constitutively included in non-mammalian vertebrates (Barbosa-Morais et al. *Science*, 2012). Exon 9 encodes a 26 amino acid sequence located in an intrinsically disordered linker region that connects RNA Recognition Motifs (RRMs) 2 and 3 of PTBP1. Using a combination of genetic techniques and transcriptome sequencing, we show that skipping of exon 9 in PTBP1 alters its splicing regulatory activities and affects the inclusion levels of many PTBP1 target exons. During neuronal differentiation, increased skipping of PTBP1 exon 9 reduces its repressive activity so as to facilitate the activation of a brain-specific AS program. Remarkably, using homologous recombination to engineer skipping of the orthologous PTBP1 exon in chicken cells induces a large number of mammalian-like AS changes in PTBP1 target exons in this species. These results thus reveal that a single lineage-specific exon skipping event in an RNA binding protein is sufficient to direct numerous AS changes in a species. The results further suggest that these changes may have contributed to evolutionary differences in the development of vertebrate nervous systems.

45 The CSR-1 RNAi pathway promotes germline transcription and defines the chromatin landscape

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Argonaute proteins and their small RNA cofactors short interfering RNAs (siRNAs) are mainly known to inhibit gene expression by a variety of mechanisms, which include inhibition of mRNA translation, mRNA or pre-mRNA degradation, and inhibition of transcription by promoting heterochromatin assembly. Surprisingly, nuclear Argonaute proteins also localize on active euchromatic regions of metazoan genomes, including humans, and their functions on these active loci still remain largely unknown.

I will present the results of our work on the *C. elegans* nuclear Argonaute protein CSR-1. CSR-1 localizes to the nucleus and is exclusively loaded with endogenous siRNAs (endo-siRNAs), called 22G-RNAs, which are antisense to more than 4,000 active protein-coding transcripts expressed in the germline. Inactivation of the CSR-1 pathway components leads to specific germline defects and embryonic lethality. Despite these observations, the role of CSR-1 in gene regulation is unclear. We adapted the Global Run-On sequencing (GRO-seq) method for use in *C. elegans* to investigate a possible function of CSR-1-bound 22G-RNAs in controlling transcription genome wide. We discovered that CSR-1 globally promotes transcription of its target genes. Moreover, we demonstrated that CSR-1 interacts with RNA polymerase II (Pol II) through nascent RNAs in a siRNA-dependent manner. Finally, our analyses revealed that CSR-1 restricts the activity of Pol II to active genomic regions to avoid ectopic initiation of transcription of silent chromatin domains. Remarkably, the distinction between silent and active chromatin domains is lost in CSR-1 mutant embryos. Based on these observations, we propose a model whereby CSR-1-bound 22G-RNAs produced from the active locations of the genome reinforce germline transcription and help to propagate the distinction between active and silent chromatin domains across generations.

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46 Primary microRNA processing is functionally coupled to RNAP II transcription in vitro

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Previous studies in vivo reported that processing of primary microRNA (pri-miRNA) is coupled to transcription by RNA polymerase II (RNAP II) and can occur co-transcriptionally. Here we have established a robust in vitro system in which pri-miRNA is transcribed by RNAP II and processed to pre-miRNA in HeLa cell nuclear extracts. Both the kinetics and efficiency of pri-miRNA processing are dramatically enhanced in this system compared to that of the corresponding naked pri-miRNA. This enhancement is general as it occurs with multiple pri-miRNAs. Moreover, nascent pri-miRNA is efficiently processed before it is released from the DNA template. Together, our work directly demonstrates that transcription and pri-miRNA processing are functionally coupled and establishes the first in vitro model systems for this functional coupling and for co-transcriptional processing.

47 SERRATE: a key protein involved in the communication between microRNA biogenesis and splicing machineries in plants

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MicroRNAs (miRNAs) are small non-coding RNAs of about 21 nt in length, which regulates gene expression by cleavage or translation inhibition of target mRNAs. Most of plant *MIR* genes are independent transcription units that encode long primary miRNA precursors which usually contain introns. For two miRNA genes, *MIR163* and *MIR161*, we showed that introns were crucial for the accumulation of proper levels of mature miRNA163 and miRNA161. Removal of the intron in both cases led to a drop-off in the level of the mature miRNA. We also demonstrated that the stimulating effects of the intron mostly reside in the 5'ss rather than on a genuine splicing event. Our results have suggested that in the communication between the spliceosome and the miRNA biogenesis machinery U1 snRNP is involved. To characterize this molecular interplay we decided to test interactions of key factors of the plant miRNA machinery with all ten *A. thaliana* U1snRNP proteins using: the yeast two-hybrid system, pull-down assays as well as the FLIM-FRET method. We have found that the SERRATE protein (SE), a plant homologue of Ars2, interacts with PRP39b, PRP40a, PRP40b and LUC7rl of U1 snRNP. Moreover, we have demonstrated that two unstructured C- and N-terminal fragments of SE are necessary for the interactions described. On the other hand, we have characterized the core part of SE as a region involved in the interaction with both subunits of the nuclear cap-binding complex (CBC). Our results suggest that the interplay between CBC, SE and U1 snRNP is crucial for the crosstalk between the plant spliceosome and the miRNA biogenesis protein complex. Our findings on the functional connection between splicing and miRNA biogenesis in plants are biologically significant as the presence of functional splice sites in the *MIR163* gene appears mandatory for pathogen-triggered accumulation of miR163 and proper regulation of at least one of its targets.

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48 Mutations in Integrator complex subunit INTS5 and INTS8 lead to altered gene expression and abnormal brain development.

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Integrator (INT) is a multi-protein complex that is essential for the 3' end formation of the U-rich small nuclear RNAs (UsnRNAs) and has also been involved recently in RNA polymerase II (RNAPII) transcription initiation and pause release. Despite recent progress, most of INT subunits remain structurally and functionally uncharacterized while little is known about the detail of its role in UsnRNA processing or in RNAPII initiation and pause release.

We have identified mutations in two INT subunits, INTS5 and INTS8 that are associated with severe neurodevelopmental defects including cerebellar hypoplasia and periventricular nodular heterotopia (PNH). In INTS8 mutant cells, we detect reduced levels of functionally essential Integrator subunits while affinity purified INTS8 mutant complexes fail to associate with these same subunits, reflecting a loss of integrity of the complex. Importantly, we have uncovered a physical interaction between INTS5 and INTS8, suggesting a functional link that could underlie the pathological similarities exhibited between patients. While we identify a small but significant increase in UsnRNAs missprocessing, RNA-seq analysis in patient fibroblasts reveals mis-splicing events in several genes known to play a role in differentiation. Importantly, we also detect the dysregulation of the expression of a large number of genes, many of which could have an impact on neural development. Expression data during development show that INTS8 expression peaks prenatally in the areas of actively migrating neurons, in agreement with the observed PNH phenotype. Accordingly, morpholino oligonucleotide-mediated knockdown of INTS8 in zebrafish results in underdevelopment of the head and brain. Finally, the INTS8 mutation was introduced into P19 mouse embryonic carcinoma cells using CRISPR/Cas9-mediated genome editing. Upon retinoic acid treatment, INTS8 mutant P19 cells differentiate but fail to express several neural differentiation markers.

We propose that INT dysfunction, through altered transcriptional regulation or splicing, leads to impaired brain development in humans. It is the first time that INT mutations are found associated with a human disease. They represent an unprecedented opportunity to study the inner mechanics of the Integrator complex, its function and its impact on the physiology of the cell and on neural development.

49 The Swi/Snf chromatin remodeling complex is a key regulator of meiotic splicing in Saccharomyces cerevisiae

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Despite its relatively streamlined genome and modest number of introns, there are important examples of regulated RNA splicing in Saccharomyces cerevisiae. One of the most striking is the regulated splicing of meiotic transcripts. At the onset of meiosis, Saccharomyces cerevisiae, like other eukaryotes, undergoes a dramatic reprogramming of gene expression. In yeast, this includes regulated splicing of a number of RNAs that are only expressed during meiosis. Removal of the introns from these RNAs is dependent upon the splicing activator Mer1, which binds to a specific enhancer sequence in these RNAs and helps to recruit the spliceosome.

The discovery that spliceosome assembly occurs co-transcriptionally, while RNA polymerase is engaged with a chromatin template, has raised important questions about the mechanisms by which transcription and chromatin modification influence splicing. In fact, little is known about the way in which chromatin modification influences regulated spliceosome assembly in yeast, even in the relatively well-characterized example of meiotic splicing. Here we show a crucial role for the chromatin remodeler Swi/Snf in meiotic regulation of splicing. We find that the complex affects meiotic splicing in several different ways. First, meiosis-specific expression of the splicing activator Mer1 is dependent upon Swi/Snf. Additionally, Swi/Snf regulates meiosis-specific downregulation of ribosomal RNAs, leading to the redistribution of spliceosomes from this abundant class of intron containing RNAs to Mer1-regulated transcripts. Finally, we find evidence of a direct role for the Swi/Snf complex in co-transcriptional splicing. By regulating splicing at these different levels, the Swi/Snf complex coordinates a cascade of events to direct the regulated splicing of meiotic genes, establishing it is a master regulator of meiotic splicing in Saccharomyces cerevisiae. Intriguingly, these mechanisms for regulating gametogenesis appear to be conserved in other eukaryotes.

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50 Transcription elongation factor regulates polyadenylation and splicing in plasma cells

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ELL2 is a transcription elongation factor previously shown to be responsible for increased first poly(A) site use and exon skipping for the Immunoglobulin heavy chain(Igh) mRNA. ELL2 associates with pTEFb in a super elongation complex on the Igh gene and travels with RNA Pol II in plasma cells, driving them to produce large amounts of Igh secretory-specific mRNA (1). Studies using a B cell conditional knockout mouse have shown that in the absence of ELL2 in plasma cells, the Igh mRNA is not efficiently processed to secretory specific form. Expression of other induced genes in plasma cells is also influenced by absence of ELL2 in the knockout. These include: Ig light chain, XBP-1, cyclin B2, and other components of the unfolded protein response and secretory machinery (2). Therefore ELL2 controls not only Igh processing but also the means by which the Ig protein would be secreted from the cells (2).

Using ELL2 antibody and plasma cell chromatin, Chip seq studies revealed a number of direct targets of ELL2 including Igh, XBP-1, and small nuclear RNAs. Analysis of the RNA seq experiments performed with the ELL2 knockout vs wild type plasma cells reveals alternative splicing of a number of mRNAs including CCR6, important for homing of cells to the germinal centers. Expression of the small nuclear RNAs is also diminished by knocking out ELL2. The U4 and U6 atac snRNAs are especially vulnerable to ELL2 control. In addition, several of the genes containing an AT...AC intron are subject to ELL2 loss. Thus ELL2 has profound effects on plasma cell development via splicing and polyadenylation through both its control of transcription elongation and the expression of snRNAs.

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51 The Ccr4-Not complex plays a key role in defining gene expression homeostasis

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The Ccr4-Not complex is conserved in eukaryotes and consists of the Not1 scaffold and several associated proteins, including the Ccr4 and Caf1 deadenylase and the Not4 E3 ubiquitin ligase. We demonstrate that coordinated gene expression is achieved by imprinting of mRNAs with Not1 during transcription. This regulates RNA stability, RNA translatability, and translational output, such as solubility or stability of produced proteins. Indeed, Not1 imprinting of mRNAs in the nucleus permits the recruitment of chaperones or partner proteins via other subunits of the Ccr4-Not complex that associate with the Not1 scaffold. For instance Not5 is essential for the imprinting of Not1 on ribosomal and mitochondrial mRNAs. Another subunit of the Ccr4-Not complex, Caf130, then recruits the Btt1 chaperone to the polysomes translating ribosomal and mitochondrial mRNAs. In the absence of Caf130 levels of mitochondrial proteins are reduced and mitochondria are fragmented. In the absence of Not5, production of ribosomes is reduced and translation is globally reduced. Hence the Ccr4-Not complex plays a key role in defining gene expression homeostasis by connecting regulation of translation efficiency and protein abundance with mechanisms regulating RNA abundance.

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52 Determination of in vivo regulation kinetics of small non-coding RNA in bacteria

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Small RNAs (sRNAs) play important roles in regulating gene expression through two major steps: target search by base-pairing interactions with the target messenger RNA (mRNA) and downstream execution by modulating translation or the stability of the mRNA. Here we describe a new imaging and analysis platform based on super-resolution fluorescence microscopy, which enabled the first in vivo kinetic measurement of sRNA-mediated gene regulation. Specifically, this platform was used to investigate a sugar-phosphate stress-induced bacterial sRNA that induces the degradation of target mRNAs. The data reveal that the sRNA binds to a primary target mRNA in a reversible and dynamic fashion, and that formation of the sRNA-mRNA complexes is the rate-limiting step, dictating the overall efficiency of regulation in vivo; whereas the downstream co-degradation of sRNA-mRNA complex can kinetically compete with the fast complex disassembly. Examination of a secondary target of this sRNA indicated that differences in the target search kinetics contribute to setting the regulation priority among different target mRNAs. This super-resolution imaging and analysis approach provides a conceptual framework that can be generalized to other sRNA systems and other target search processes.

53 Two distinct DNA binding modes guide dual roles of a CRISPR-Cas protein complex: a single-molecule FRET study

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Small RNA-guided protein complexes play an essential role in CRISPR-mediated immunity in prokaryotes. While these complexes are known to flag cognate invader DNA for destruction, recent evidence has implicated their involvement in new CRISPR memory formation against mutated invader sequences. The mechanism by which the target recognition complex mediates these disparate responses-interference and priming-remains poorly understood. Using single-molecule FRET, we visualize how bona fide and mutated targets are differentially probed by E. coli Cascade. We observe that the recognition of bona fide targets is an ordered process that is tightly controlled for high fidelity. Mutated targets are recognized with low fidelity, which is featured by short-lived and PAM- and seed-independent binding by any segment of the crRNA. These dual roles of Cascade in immunity with distinct fidelities underpin CRISPR-Cas robustness, allowing for efficient degradation of bona fide targets and priming of mutated DNA targets.

Ref: Blosser et al, Molecular Cell (in press)

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54 Rational design of a split-Cas9 enzyme complex

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Cas9, an RNA-guided DNA endonuclease found in clustered regularly interspaced short palindromic repeats (CRISPR) bacterial immune systems, is a versatile tool for genome editing, transcriptional regulation, and cellular imaging applications. Structures of *Streptococcus pyogenes* Cas9 alone or bound to single-guide RNA (sgRNA) and target DNA revealed a bilobed protein architecture that undergoes major conformational changes upon guide RNA and DNA binding. To investigate the molecular determinants and relevance of the interlobe rearrangement for target recognition and cleavage, we designed a split-Cas9 enzyme in which the nuclease lobe and α-helical lobe are expressed as separate polypeptides. Although the lobes do not interact on their own, the sgRNA recruits them into a ternary complex that recapitulates the activity of full-length Cas9 and catalyzes site-specific DNA cleavage. The use of a modified sgRNA abrogates split-Cas9 activity by preventing dimerization, allowing for the development of an inducible dimerization system. We propose that split-Cas9 can act as a highly regulatable platform for genome-engineering applications.

55 Piwi-piRNA regulates association of linker histone H1 with target transposon loci in *Drosophila*

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The piRNA (PIWI-interacting RNA) pathway in *Drosophila* ovarian somatic cells represses transposons transcriptionally to maintain genome integrity. The Piwi-piRNA complex establishes heterochromatic H3K9me3 marks on transposons and their neighboring regions to regulate gene expression. However, the molecular link between the Piwi-piRNA complex and chromatin regulation has not been elucidated. Here, we propose that the linker histone H1 is necessary for this regulation. We identified H1 as a novel nuclear interactor of Piwi. We generated specific monoclonal antibody for H1, and analyzed the involvement of H1 in Piwi-piRNA-mediated transposon silencing. This revealed that H1 is required for transposon silencing but not for piRNA biogenesis. The C-terminal region of H1 physically interacts with Piwi, and is essential for silencing Piwi-regulated transposons. Additionally, ChIP-seq analysis revealed that depletion of Piwi decreases association of H1 with Piwi-regulated transposons. Moreover, generation of artificial Piwi-piRNA target loci results in recruitment of H1 to the loci along with H3K9me3 modification. These data suggest a model in which Piwi recruits H1 to specific chromatin regions to regulate transposons.

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56 dsRNA termini and Loquacious-PD modulate alternate reaction states of *Drosophila* Dicer-2 in an ATP- and helicase- dependent manner.

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In previous studies we reported that *Drosophila melanogaster* Dicer-2 (dmDcr-2) acts processively on dsRNA with blunt (BLT) termini and distributively on dsRNA with 3'-overhanging (3'ovr) termini (Welker et al., 2011). We now provide mechanistic insights into dmDcr-2's ability to discriminate between dsRNA termini. Using gel-shift assay conditions that allowed monitoring of binding to dsRNA termini, we observed that, in the absence of ATP, dmDcr-2 bound 3'ovr (29 ± 2 nM), but not BLT, termini. However, in the presence of ATP, dmDcr-2 bound both 3'ovr (18 ± 3 nM) and BLT (7 ± 1 nM) termini, with the highest affinity binding occurring with BLT dsRNA. Similarly, in the presence of ATP, single-turnover cleavage assays revealed that dmDcr-2 cleaved BLT dsRNA ($k_{obs} = 0.19 \pm 0.01$ min^{-1}) far more efficiently than 3'ovr dsRNA ($k_{obs} = 0.01 \pm 0.001$ min^{-1}). We also observed termini dependence in multiple-turnover cleavage, and ATP hydrolysis, assays, with optimal reactivity occurring with BLT dsRNA.

Using limited proteolysis assays we observed an ATP- and helicase- dependent protease-resistant fragment of dmDcr-2, indicative of a conformational change. Consistent with the idea that the protease-resistant fragment correlates with an optimal conformation, the protease-resistant fragment was far more abundant with BLT dsRNA. Assays with non-hydrolyzable ATP analogs indicated the conformational change requires nucleotide binding, but not hydrolysis. By analogy to the related helicase, RIG-I, we speculate the optimal conformation correlates with full closure of dmDcr-2's helicase domain and enables the processivity observed with BLT dsRNA. In contrast, in the presence of ATP and 3'ovr dsRNA, dmDcr-2 favors an intermediate, semi-closed conformation that correlates with distributive cleavage. We show that dmDcr-2's partner protein, Loquacious-PD (Loqs-PD), modulates catalytic activity and allows dmDcr-2 to react equally well with BLT and 3'ovr dsRNA; in the presence of Loqs-PD limited proteolysis patterns are identical with BLT and 3'ovr dsRNA, suggesting Loqs-PD stabilizes the optimal, closed conformation. Loqs-PD also enabled dmDcr-2 to process dsRNA with blocked, structured or frayed ends that are normally refractory to cleavage. Our data suggest Loqs-PD allows dmDcr-2 to tailor its activity to the diverse termini of naturally occurring substrates.

57 Dissection of RISC assembly and function by single-molecule imaging

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Small interfering RNAs (siRNAs) direct cleavage of their complementary target mRNAs via the formation of the effector ribonucleoprotein complex called RNA-induced silencing complexes (RISC), which contains Argonaute2 (Ago2) protein at the core. Previous biochemical studies have shown that loading of siRNA duplexes into *Drosophila* Ago2 requires the Dicer-2/R2D2 heterodimer and the Hsc70/Hsp90 chaperone machinery, and that assembled RISC cleaves the target mRNA at the phosphodiester bond between the target nucleotides across from the positions 10 and 11 of the siRNA guide strand. However, exactly how RISC is assembled and what happens after target cleavage remain unclear. Here, we utilized single-molecule imaging techniques to dissect RISC assembly and the RISC-mediated cleavage reaction. Our results define the role of the chaperone machinery in the multi-step assembly pathway of RISC as well as the release order of the 5′ and 3′ cleavage fragments by RISC.

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58 Human genetic variation affects miR-30c biogenesis in cancer

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MicroRNAs (miRNAs) are small non-coding RNAs that negatively regulate gene expression, influencing many biological processes. Their biogenesis is highly regulated at the transcriptional and post-transcriptional level. Our previous data, as well as evidence from others, suggested the existence of auxiliary factors for the processing of specific miRNAs.

Here, we investigated the role of sequence and secondary RNA structure in miRNA biogenesis. Screening of novel genetic variants in human pri-miRNAs linked to breast cancer identified a novel rare variant in the precursor of miR-30c-1 in patients that were non-carriers of BRCA1 or BRCA2 mutations, suggesting the possibility that familial breast cancer may be caused by variation in the levels of this miRNA [1]. Indeed, miR-30c is a good prognostic indicator of breast cancer progression. This single G to A substitution in pri-miR-30c-1 terminal loop, which was also later observed in gastric cancer patients, results in an increase in the abundance of the mature miRNA.

In order to understand the mechanism underlying miR-30c deregulation in breast cancer, we investigated how this polymorphism affects miRNA biogenesis. We show that the G-A substitution in pri-miR-30c-1 directly affects Drosha-mediated processing both *in vitro* and in cultured cells. Furthermore, SHAPE structural analysis of this variant revealed an altered RNA structure affecting several nucleotides away from the terminal loop, in the vicinity of the Drosha cleavage site. Interestingly, we observed that this RNA structure reorganization promotes the interaction with SRSF3, an SR protein family member that was demonstrated to facilitate pri-miRNA recognition and processing [2]. Our results are compatible with a model whereby a genetic variant in pri-miR-30c-1 leads to a secondary RNA structure rearrangement resulting in increased binding of SRSF3 and increased levels of miR-30c. Altogether, these data highlights that primary sequence determinants and RNA structure are key regulators in miRNA biogenesis and help explain the deregulation of an individual miRNA during breast cancer progression.

1. Shen et al. (2009) Novel genetic variants in microRNA genes and familial breast cancer. *Int J Cancer* **124**: 1178-1182.

2. Auyeung et al. (2013) Beyond secondary structure: primary-sequence determinants license pri-miRNA hairpins for processing. *Cell* **152**: 844-858.

59 ADAR1 is required for differentiation and neural induction by regulating microRNA processing in a catalytically independent manner

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Adenosine deaminases acting on RNA (ADARs) are involved in adenosine to inosine RNA editing and are implicated in development and diseases. Here we observed that ADAR1 deficiency in human embryonic stem cells (hESCs) significantly affected differentiation and neural induction with widespread mRNA and miRNA expression changes, including the upregulation of self-renewal related miRNAs, such as miR302s. Global editing analyses revealed that ADAR1 editing activity contributes little to the altered miRNA/mRNA expression in hESCs and upon neural induction. Genome-wide iCLIP studies identified that ADAR1 binds directly to pri-miRNAs to interfere with miRNA processing by acting as an RNA binding protein. Importantly, aberrant expression of miRNAs and phenotypes observed in ADAR1 depleted hESCs upon neural differentiation could be largely rescued by a non-enzymatically active ADAR1 mutant, but not by the RNA binding null ADAR1 mutant. These findings reveal that ADAR1, but not its editing activity, is critical for differentiation and neural induction by regulating miRNA biogenesis via direct RNA interaction. We are now investigating how ADAR1 involves in the biogenesis of other noncoding RNAs, such as circular RNAs.

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60 Dual role of Lin28a in regulating miRNA levels during neuronal differentiation.

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The abundance of miRNAs is subjected to tissue-specific and development-specific regulation by transcriptional and post-transcriptional mechanisms. One of the most characterised examples of post-transcriptional regulation of miRNA biogenesis involves interaction of Lin28a with the pre-let-7. In undifferentiated cells Lin28a binds to pre-let-7 and together with the 3' terminal uridylyl transferases (TUT4/7) adds poly(U) tail. Subsequently, 3'-5' Pearlman syndrome exonuclease Dis3l2, promotes degradation of poly(U)-tailed pre-let-7. Lin28a, expression is progressively switched off during cell differentiation, allowing for the subsequent derepression of let-7 biogenesis

We have recently shown that brain-specific miRNA-9 is regulated transcriptionally and post-transcriptionally during early neuronal differentiation. We revealed that Lin28a induces degradation of pre-miRNA-9 in uridylation-independent manner. Furthermore, constitutive expression of untagged but not GFP-tagged Lin28a decreases neuronal differentiation potential of P19 cells, which coincides with reduced miRNA-9 levels.

Here we demonstrate that pre-let-7a and pre-miR-9 are both degraded by Dis3l2 exonuclease. However, unlikely in the case of pre-let-7, sequence and structural features of pre-miRNA-9 allow formation of tertiary complex with Lin28a and Dis3l2. Furthermore, using small RNA-seq, we reveal that Lin28a has the capacity to regulate many other miRNAs during neuronal differentiation, contributing to correct timing of neurogenesis. Surprisingly, Lin28a exerts stabilising effect on the levels of numerous miRNAs. The primary transcripts of these miRNAs harbour a number of Lin28a binding motifs, which suggests a direct, positive role of Lin28a in regulating miRNA levels.

61 Uridylation of hairpin-RNAs by Tailor confines the emergence of miRNAs in *Drosophila*

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Uridylation of diverse RNA species represents an emerging theme in post-transcriptional gene regulation. In the microRNA pathway, such modifications regulate small RNA biogenesis and stability in plants, worms and mammals.

Here, we report the first uridylyltransferase that acts on small RNAs in *Drosophila*, which we refer to as Tailor. Tailor is the source for the majority of 3´ uridine-modifications in small RNAs and predominantly targets precursor-miRNAs. Uridylation modulates the characteristic two-nucleotide 3´ overhangs of miRNA hairpins, which regulates pre-miRNA processing by Dicer-1 and destabilizes RNA hairpins. Furthermore, Tailor preferentially uridylates mirtron-hairpins, thereby impeding the production of non-canonical microRNAs. Mirtron-selectivity is explained by unique primary sequence specificity of Tailor, selecting RNA substrates ending with a 3´ guanosine, a feature not previously observed for TUTases. In contrast to mirtrons, conserved *Drosophila* pre-miRNAs are significantly depleted in 3´ guanosine, thereby escaping regulatory uridylation.

Our data support the hypothesis that evolutionary adaptation to pre-miRNA uridylation shapes the nucleotide composition of pre-miRNA 3´ ends. Hence, hairpin-uridylation may serve as a barrier for the de novo creation of miRNAs in *Drosophila*.

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62 Sensing the end: how TUT7 controls the fate of precuror miRNAs by uridylation

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Terminal uridylyl transferases (TUTs) function as integral regulators of microRNA (miRNA) biogenesis. Using biochemistry, single-molecule analyses, and deep sequencing techniques, we here investigate the mechanism how human TUT7 (also known as ZCCHC6) recognizes and uridylates precursor miRNAs (pre-miRNAs) in the absence of Lin28. We find that the overhang of a pre-miRNA is the key structural element that TUT7 and its paralogues, TUT4 (ZCCHC11) and TUT2 (GLD2/PAPD4), recognize. For group II pre-miRNAs which have a 1 nt 3′ overhang, TUT7 restores the canonical end structure (2 nt 3′ overhang) through mono-uridylation, promoting miRNA biogenesis. Interestingly, however, once the 3′ end is further recessed into the stem (as in 3′ trimmed pre-miRNAs), TUT7 generates an oligo-U tail that leads to degradation. Unlike Lin28-dependent oligo-uridylation reaction which is processive, a distributive mode is employed for both mono-uridylation and oligo-uridylation in the absence of Lin28. The overhang length dictates the frequency (but not duration) of TUT7-RNA interaction, explaining how TUT7 differentiates pre-miRNA species with different overhangs. Our study reveals dual roles and mechanisms of uridylation in repair and removal of defective pre-miRNAs.

63 Distinct targeting by let-7 miRNA family members

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MicroRNAs (miRNAs) are important regulatory molecules for controlling post-transcriptional gene expression. These small, ~22 nucleotide-long RNAs bind to target transcripts through imperfect base pairing and, as part of the miRNA Induced Silencing Complex (miRISC), trigger mRNA destabilization and translational inhibition. Complementarity to nucleotides 2-7 of the miRNA (the seed region) is thought to be the primary indicator of target recognition. Highly related miRNAs that share a seed sequence are considered to be members of a family of miRNAs. In *Caenorhabditis elegans*, the let-7 miRNA has six family members, known as the let-7 sisters. Since three of these miRNAs share the same expression levels and patterns, in addition to identical seed sequences, with the let-7 miRNA, it is predicted that these miRNAs would have highly overlapping sets of target sites. This assumption was tested by analyzing chimeric reads that form due to the ligation of a miRNA to its target site during the isolation of Argonaute bound RNAs through individual nucleotide-resolution crosslinking immunoprecipitation (iCLIP). Surprisingly, these unambiguous biochemical datasets reveal that the majority of reproducible chimeras formed by let-7 family members contain target sites that are not bound by other let-7 family members. Using an unbiased classifier, we ascertained the most relevant nucleotide and base-pairing features that distinguish let-7 targets from let-7 sister targets. We find that 3' end-complementarity of the miRNA to a target is the most important feature to discern specific targets among miRNAs that share the same seed sequence . The prominent contribution of the 3' end of the miRNA to target specificity is unexpected and has been largely disregarded in current prediction approaches that focus primarily on the seed sequence. In support of the importance of 3' end complementarity, the minimum free energy (MFE) of hybridization of the let-7 family members to their specific target sites is most favorable for the specific family member that is found at each site. These findings suggest that, although the seed sequence is a predominant and reliable feature in target prediction, base pairing along the 3' end of the miRNA is critical for targeting fidelity, especially among miRNA families.

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64 Cotranslational microRNA mediated messenger RNA destabilization

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The mechanism whereby microRNAs (miRNAs) repress protein output from targeted mRNAs remains in question. Substantive evidence indicates that miRNAs function primarily, if not exclusively, by destabilizing their mRNA targets. Whether destabilization is the consequence of inhibition of initiation of translation is still debated. Other evidence has suggested that miRNAs inhibit translation at some step after initiation but mechanistic insight into how this could occur has been difficult to obtain. The notion that miRNAs impact translation post initiation derives largely from many observations that miRNA targets and miRNAs themselves appear to be associated with actively translating ribosomes. The vast majority of studies that had examined mechanism of miRNA-mediated repression have been carried out using transient transfection of both targets and miRNAs; most often with engineered 3' UTRs. Here, we made stable cell lines expressing the well characterized miRNA-targeted 3' UTRs of *Drosophila* Reaper and Hid. Lines expressing two versions of each UTR, one with wild type miRNA recognition sites, and one where the recognition sites were mutated, were studied. All detectable mRNAs from all four lines were associated with actively translating ribosomes as assessed by Harringtonine treatment and Northern blot. Half-life measurements showed that mRNAs containing wild type miRNA recognition sites were dramatically destabilized when compared to those with mutant recognition sites When transcription was arrested, we observed a rapid accumulation of mRNA fragments. These fragments resulted from progressive truncation from the 5' end of the mRNA and were ribosome associated. These data indicate that mRNA-mediated mRNA destabilization and degradation occur while targeted mRNAs are being translated and therefore are cotranslational. These observations may rationalize disparate views of miRNA mechanism. In reports examining subcellular location of targeted mRNAs, half-lives were not determined, and when half-lives were determined, subcellular localization was not.

65 Biosynthesis and Functions of circRNAs in Drosophila

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Tight regulation of RNA metabolism is essential for normal brain function. Recently, circular RNAs (circRNAs), a highly abundant new type of regulatory non-coding RNA have been found across the animal kingdom. Two of these RNAs have been shown to act as miRNA sponges but no function is known for the thousands of other circRNAs, indicating the existence of a widespread layer of previously unknown gene regulation.

Here we show that circRNAs are generated co-transcriptionally. We demonstrate that circularization and linear splicing compete against each other and that these mechanisms are tissue-specific and conserved from flies to humans. The second exon of the splicing factor muscleblind (MBL/MBNL1) is circularized in flies and humans. This circRNA (circMbl) and its flanking introns contain conserved muscleblind binding sites, which are strongly and specifically bound by MBL. Moreover, modulation of MBL levels strongly affects the biosynthesis of circMbl and this effect is dependent on the MBL binding sites. In addition, we show that circRNAs are highly expressed in the fly brain and their host genes are strongly enriched in genes with synaptic functions. By manipulating the levels of specific circRNAs in the brain we are determining functions of these new types of molecules at the physiological and behavioral levels. Last but not least we show that temperature dynamically alters the levels of circRNAs in the fly neural tissue.

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66 Natural Antisense Transcript from MALAT1 locus modulates the 3' end processing and maturation of MALAT1 lncRNA

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MALAT1 (Metastasis Associated Lung Adenocarcinoma Transcript1) is a highly abundant nuclear-restricted long noncoding RNA that promotes malignancy. The enhanced stability of MALAT1 has been attributed to its ultra-conserved 3' end, which harbors a tRNA-like structure, an A-rich tract and two upstream U-rich motifs, which are proposed to be the cellular homolog of the ENE (Expression and Nuclear Retention Element) found in viral lncRNAs. The presence of the tRNA-like structure serves as a cleavage signal for the tRNA-processing enzymes to produce a cytoplasmic tRNA-like small mascRNA (MALAT1-associated small cytoplasmic RNA), and a mature MALAT1 transcript lacking the canonical poly(A) tail, but is stabilized by a bipartite triple helix at its 3' end, formed among the A-rich tract and two U-rich motifs. This cleavage event is crucial in that it generates a blunt end in the bipartite triple helix, enabling the formation of this stabilizing structure. Despite the presence of these genome-encoded features, how this cleavage reaction is regulated, and subsequently how MALAT1 level is regulated, are unknown. Here, we identified a broadly expressed natural antisense transcript at the MALAT1 locus, designated as TALAM1, that positively regulates MALAT1 levels by promoting its 3' end cleavage and maturation. TALAM1 displays homogenous nucleus distribution and is also enriched at the transcription site of the MALAT1 locus. TALAM1 is required for the maintenance of cellular MALAT1 level, and depletion of TALAM1 results in the accumulation of the primary MALAT1 transcript with a concomitant decrease in mature MALAT1 and mascRNA levels. Conversely, overexpression of TALAM1 could *in trans* facilitate the cleavage reaction. *In vivo* decay assay revealed that TALAM1 overexpression could further stabilize a reporter gene with MALAT1 ENE+A+mascRNA sequence, illustrating the physiological significance of TALAM1 in modulating the stabilization effect of the MALAT1 ENE+A+mascRNA sequence. On the other hand, MALAT1 RNA positively regulates the transcription and stability of TALAM1, completing a feed-forward positive regulatory loop. Our results not only reveals how a positive regulatory loop is established to maintain the high cellular levels of MALAT1, but also proposes a sense-antisense mediated regulatory mechanism for the 3' end processing of the cellular lncRNAs containing ENE-like structures.

67 Pervasive, novel ncRNA transcription in histone H3 lysine 36 mutants links chromatin-mediated RNA processing with organismal viability and development

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Covalent, post-translational modification (PTM) of histone proteins is widely believed to facilitate epigenetic inheritance of gene regulatory information. This so-called "histone code" hypothesis has not been directly tested in animals, limiting our understanding of the contribution that histone PTMs make to organismal development and human disease. In particular, trimethylation of histone H3 lysine 36 (H3K36me3) has been implicated in suppression of cryptic transcription initiation in yeast and proper specification of alternative splicing in cultured human cells.. Furthermore, loss-of-function alleles in the H3K36 methyltransferase SETD2 are overrepresented in certain renal cell carcinomas, indicating a role for H3K36me3 in cell proliferation and tissue homeostasis.

We have developed a genetic platform in *Drosophila* to directly test the role of specific histone residues in metazoan development, wherein all the replication-dependent histone genes can be deleted and replaced with transgenic arrays of mutant histones. Using this method, we generated animals whose entire supply of H3 genes contained R substitutions at lysine 36 (H3K36R). These mutants are devoid of H3K36 methylation, and fail to develop fully to adulthood. However, proliferation and cell growth in induced mutant clones in late larval imaginal discs is unaffected, suggesting catastrophic loss of cellular integrity is not the primary source of lethality.

To determine whether the RNA targets of H3K36me3 might contribute to lethality, we isolated poly-A and nuclear short-capped RNA from 3rd instar larvae, carried out high-throughput sequencing (HTS) and analyzed differential gene expression and promoter usage. We found that H3K36R mutants exhibit increased transcription from a variety of ncRNA promoters that are normally expressed at very low levels, in addition to numerous transcription starts from previously unannotated promoters. Furthermore, we identified a subset of genes exhibiting antisense transcription in coding regions. We also found that dysregulated genes were clustered more closely with one another than would be expected by a random selection of genes, suggesting chromatin perturbation may impose cis-regulatory constraints on gene expression. These and other findings lead us to conclude that proper H3K36 methylation is required for metazoan development, serving to generally suppress suboptimal transcription initiation and enhance the fidelity of co-transcriptional RNA processing.

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68 Exploring the architecture of lncRNA RepA, a key player in X-chromosome inactivation

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Recent work has demonstrated that long non-coding RNAs (lncRNAs) are essential components of the mammalian transcriptome. They exhibit multiple structural and functional roles in various cellular processes and diseases, and their analysis has become a new frontier in biomedical research. One of the most extensively studied lncRNAs is the X-inactivation specific transcript (Xist, 17 kb in mouse). During X-chromosome inactivation (XCI) in female mammals, Xist localizes to chromatin, spreads over the future inactive X-chromosome (Xi), and causes transcriptional silencing of X-linked genes. It has been proposed that RepA, a lncRNA consisting of the same 1.6 kilobases as the 5' region of Xist, up-regulates the expression of Xist, and contributes to the initiation and spread of XCI. Despite the availability of numerous functional data, little structural work has been performed to characterize the molecular mechanisms of RepA or Xist. In order to study its secondary structure, a non-denaturing purification protocol was employed to obtain a homogeneous and monodisperse form of RepA, and by studying its compaction with biophysical hydrodynamic methods, the ionic requirements for RepA folding were assessed. Chemical probing experiments (SHAPE and DMS probing) on the uniform, co-transcriptionally folded RNA sample, in parallel with phylogenetic analysis, revealed that RepA has a complex structural organization, with a variety of secondary structural motifs. It is comprised of three independently-folding modules, one of which is the 5' domain, which is a potential protein binding region whose secondary structure elements are found to be evolutionarily conserved. Our results provide the first secondary structural map of RepA, and offer structural insights for understanding the evolution and functional properties of this important lncRNA.

69 Structural basis for RNA-mediated regulation of lysine specific demethylase-1

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Lysine specific demethylase 1 (LSD1) is an essential epigenetic regulator in mammals and influences chromatin structure by catalyzing the removal of mono- and dimethyl functional groups from histone 3 at the lysine 4/9 positions (H3K4/K9). LSD1 is a flavin adenine dinucleotide (FAD)-dependent amine oxidase and interacts with over 60 gene regulatory proteins (including CoREST, REST, p53, E2F1), key enzymes (DNMT1, MRE11, HDAC1/2), essential nutrients (tetrahdyrafolate (THF)), and lncRNAs (including HOTAIR and TERRA). Some of these lncRNAs are known to interact with DNA binding proteins, suggesting a regulatory role for RNA in LSD1 function. Here, we present a 2.8 Å crystal structure of the functional LSD1-CoREST complex in complex with a single stranded RNA (ssRNA). The ssRNA binds to the amine oxidase interface of LSD1, located on the opposing face of the histone 3 peptide-binding cleft. RNA also interacts with the N-terminal domain of LSD1, which contains a novel structured domain based on the analysis of our NMR data. Kinetic assays of LSD1-catalyzed demethylation show that a G-quadruplex forming RNA serves as a strong non-competitive inhibitor (IC_{50} ~ 0.5 uM) whereas a similar length ssRNA serves as a weak inhibitor (IC_{50} > 100 uM). These data suggest that a structured RNA can function as a negative allosteric effector in LSD1-mediated regulatory pathways.

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70 Towards a therapy for Angelman syndrome by targeting a long non-coding RNA to active UBE3A

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Angelman syndrome (AS) is a single gene disorder characterized by intellectual disability, developmental delay, behavioral uniqueness, speech impairment, seizures, and ataxia. It is caused by maternal deficiency of the imprinted gene UBE3A, encoding an E3 ubiquitin ligase. All patients carry at least one copy of paternal UBE3A, which is intact but silenced by a nuclear-localized long non-coding RNA, UBE3A antisense transcript (UBE3A-ATS). Murine Ube3a-ATS reduction by either transcription termination or topoisomerase I inhibition increased paternal Ube3a expression. Despite a clear understanding of the disease-causing event in AS and the potential to harness the intact paternal allele to correct disease, no gene-specific treatment exists for patients. Here we developed a potential therapeutic intervention for AS by reducing Ube3a-ATS with antisense oligonucleotides (ASOs). ASO treatment achieved specific reduction of Ube3a-ATS and sustained unsilencing of paternal Ube3a in neurons in vitro and in vivo. Partial restoration of UBE3A protein in an AS mouse model ameliorated some cognitive deficits associated with the disease. Although additional studies of phenotypic correction are needed, for the first time we developed a sequence-specific and clinically feasible method to activate expression of the paternal Ube3a allele.

71 An *in vivo* assay uncovers new specificities of enzymes that add nucleotides to the 3' end of RNAs

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Untemplated nucleotides are added post-transcriptionally to the 3' ends of a variety of RNAs. CCA-addition is critical for tRNA function, while polyadenylation and uridylation regulate numerous classes of RNAs, from mRNAs to miRNAs. CCA-adding enzymes, poly(A) polymerases (PAPs), and poly(U) polymerases (PUPs) catalyze these post-transcriptional nucleotide additions. They are classified as ribonucleotidyl transferases and are conserved from fungi to humans. Despite preferences for different nucleotides, PAPs and PUPs cannot be distinguished by inspection of their protein sequences.

We aim to globally characterize the mechanisms and biological roles of PUPs and PAPs in an organism, and have initially focused on nematodes and fungi. To identify the activities of uncharacterized ribonucleotidyl transferases, we developed an *in vivo* assay in which the candidate enzymes were tethered to a reporter RNA in *S. cerevisiae*. The number and identity of added nucleotides were determined by high-throughput sequencing. Our assay affords three main advantages: (1) activity is assayed without purifying either the enzyme or substrate, (2) enzymes are assayed quickly and in parallel, (3) thousands of tail sequences per sample are analyzed without bias, which enables quantification of both the efficiency of the enzyme and the precise identity of the nucleotides it adds.

Through these analyses, we identified previously uncharacterized PUPs, PAPs, and CCA-adding enzymes from *C. elegans*, *S. pombe*, *C. albicans*, *N. crassa*, and *A. nidulans*. We detected several proteins with unique nucleotide addition specificities including poly(C) polymerase activity, and have discovered unexpectedly complex nucleotide preferences of known enzymes. Our current work focuses on the evolution of fungal PUPs and PAPs, and elucidation of the roles of these enzymes in nematode development.

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72 Structural basis for the activation of the *C. elegans* non-canonical poly(A)-polymerase GLD2 by GLD3

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The *C. elegans* GLD2-GLD3 complex upregulates the expression of genes required for meiotic progression. GLD2-GLD3 acts by extending the short poly(A)-tail of germline-specific mRNAs, switching them from a dormant state into a translationally active state. GLD2 is a cytoplasmic non-canonical poly(A)-polymerase that lacks the RNA-binding domain typical of the canonical nuclear poly(A)-polymerase PAP. The activity of *C. elegans* GLD2 *in vivo* and *in vitro* depends on its association with the Bic-C homologue GLD3. We have identified a minimal polyadenylation complex that includes the conserved nucleotidyl-transferase core of GLD2 and the N-terminal domain of GLD3, and determined its structure at 2.3 Å resolution. The structure shows that the N-terminal domain of GLD3 does not fold into the predicted KH domain, but wraps around the catalytic domain of GLD2. The picture that emerges from the structural and biochemical data is that GLD3 activates GLD2 both indirectly by stabilizing the enzyme and directly by contributing positively-charged residues near the RNA-binding cleft. The RNA-binding cleft of GLD2 has distinct structural features as compared to the PAP and Trf4 poly(A)-polymerases. Consistently, GLD2 has distinct biochemical properties: it displays unusual specificity *in vitro* for single-stranded RNAs with at least one adenosine at the 3' end. GLD2 thus appears to have evolved specialized nucleotidyl-transferase properties that match the 3' end features of dormant cytoplasmic mRNAs.

73 Exploring the dark side of poly(A) tails: Diverse mechanisms control 3´-end processing and transcription termination genome-wide

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RNA polymerase II (Pol II) transcribes the vast majority of the eukaryotic genome. However, only a small fraction of the transcriptome accumulates as stable RNA, and various mechanisms have evolved to control the output of pervasive Pol II activity. Several non-canonical 3´-end processing mechanisms are coupled to the activity of the Trf4/5-Air2/1-Mtr4 polyadenylation (TRAMP) complex. This adds a destabilizing oligo(A) tail to target RNAs, tagging them for 3´ trimming or degradation by the nuclear exosome component Rrp6. We identified these destabilizing, "dark side" poly(A) tails genome-wide by direct single-molecule RNA sequencing (DRS) of 3´ ends of polyadenylated RNA in yeast cells lacking Rrp6. This revealed a global profile of destabilized 3´ ends arising from various non-canonical processing and termination mechanisms, including endonuclease cleavage by spliceosome-mediated decay, nuclear Rnt1 (RNase III), and roadblock transcription termination. We identified poly(A) tags at annotated 5´ splice sites, at known sites of spliceosome-mediated decay, as well as at novel 5´ splice sites in introns and UTRs. Furthermore, DRS sequencing of yeast cells lacking both Rrp6 and Rnt1 revealed a previously hidden class of long non-coding RNAs (lncRNAs) and ORF anti-sense RNAs (asRNAs) targeted by RNase III activity. We employed a combination of Rnt1 UV crosslinking and analysis of cDNAs (CRAC), computational RNA folding, and high-throughput sequencing of *in vitro* cleaved RNA by recombinant Rnt1 to validate these ncRNAs as *bona fide* substrates. We show that co-transcriptional cleavage of lncRNAs by Rnt1 plays an important role during the salt stress response, as *cis*-acting mutations that block cleavage by Rnt1 prevent induction of downstream genes. We also define the genome-wide locations of roadblock termination of Pol II by Reb1p and tRNA genes, and show that these mechanisms can cooperate with the Nrd1-Nab3-Sen1 (NNS) complex to promote efficient termination. Our results demonstrate how a diverse set of non-canonical 3´ processing mechanisms control pervasive transcription and regulate gene expression genome-wide.

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74 Global analysis of poly(A) tail changes during inducible gene expression

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It has long been widely accepted that all mRNAs, excluding histone mRNAs, leave the nucleus and receive their poly(A) tail of a uniform 200-250 adenosine residues long (Brawerman, 1981). Using thiouridine labelling as a method of capturing nascent mRNAs, we have been able to measure poly(A) tail sizes of newly synthesised individual mRNAs. Our data indicate that the initial poly(A) tail size can vary greatly depending on both the individual mRNA and the state of the cell.

In order to track changes of poly(A) tail length during rapid gene induction we have used the serum response as a model in NIH 3T3 cells. After serum starvation, the cells are serum stimulated and poly(A) tail changes measured in comparison to no serum stimulation. Using poly(A) fractionation technique developed in our laboratory (Meijer et al, 2007), we have been able to study global changes of the poly(A) tail length during the serum response and have observed striking increases in rapidly induced mRNAs compared to control levels. Mathematical modelling of transcriptional induction and polyadenylation indicates that transient induction of transcription, as observed in these genes, is enhanced by these changes in poly(A) tail length, enabling both rapid induction and removal of these mRNAs. In contrast, constitutively expressed mRNAs have less need of a poly(A) tail, and many may never obtain a long tail. Indeed, we found that the effects of the polyadenylation inhibitor cordycepin on gene expression closely resemble those of serum withdrawal, with both treatments preferentially affecting unstable mRNAs. We propose that the poly(A) tail has an especially important role in rapid transcriptional regulation, enabling timely changes in mRNA and protein levels.

Further analysis of the poly(A) fractionation microarray data, also indicates that certain mRNAs, change in poly(A) tail length but not abundance. When treated with Actinomycin D, these mRNAs still retain the increased poly(A) tail length during the serum response. This indicates that regulation of polyadenylation, nuclear as well as cytoplasmic, plays a significant role in the serum response.

75 The nucleic acid-binding protein FUS/TLS plays important role in replication-dependent histone gene expression by interaction with U7 snRNP

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Histone proteins synthesis in metazoans are cell cycle-regulated to accompany DNA replication at S phase. The histone genes expression and processing of their transcripts are regulated at three steps: transcription, mRNA maturation and transcript stability. All these processes must be tightly coordinated and recruit a number of specific factors. There is still not fully elucidated how histone gene transcription is coupled to 3'end processing of pre-mRNAs at S phase of the cell cycle.

The U7 small nuclear ribonucleoprotein (U7 snRNP) is an essential factor mediating the unique 3'end processing of non-polyadenylated histone mRNAs in metazoans. Recently, using affinity purification based on MS2-tagged U7 snRNA and biotinylated oligonucleotide complementary to U7 snRNA we identified FUS/TLS as novel U7 snRNP interacting protein. IP and RIP experiments confirmed that FUS co-immunoprecipitates U7 snRNP/U7 snRNA as well as replication-dependant histone gene transcripts: H2A and H2B, H3 and H4, but not H2A.Z variant histone transcript. These FUS:RNA interactions are the highest at S phase. The qRT-PCR analyses revealed that in FUS knockdown cells the level of correctly processed histone mRNAs decreases what corresponds with increased level of extended transcripts. The opposite effect was observed when FUS was overexpressed. Interestingly, FUS was also found to co-immunoprecitate transcriptional factor NPAT and transcriptional repressor hnRNP UL1, however only the interaction with NPAT was S-phase dependent. ChIP assay revealed that FUS binds to the promoter region of histone genes and enhances the binding of RNAPII through the histone gene at S phase. We conclude that FUS acts as a activator of histone gene transcription and is involved in correct processing of 3'end of their transcripts at S phase of the cell cycle thus linking both these processes by interacting with U7 snRNP and other factors involved in replication-dependent histone gene expression.

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76 The role of exosomes in telomerase RNA biogenesis

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Telomerase is a key enzyme that maintains and replenishes telomeric DNA by using part of an RNA subunit as a template for reverse transcription. Telomerase RNA is an essential component of telomerase and processing of its 3' end is required for maturation and stability in all species examined. The mechanisms underlying 3' end processing differ among different organisms. The *S. pombe* telomerase RNA (TER1) precursor contains an intron immediately downstream of the mature form. The 3' end is processed by spliceosomal cleavage, a reaction that corresponds to the first transesterification of splicing. After the cleavage, an Lsm site is generated. Lsm2-8 proteins protect the mature 3' end of TER1 and facilitate the assembly of telomerase holoenzyme. Both precursor and spliced forms of TER1 RNA fail to support telomere maintenance. We have now characterized the fates of the different forms of TER1 and found that the precursor and spliced forms are highly unstable. A series of deletions identified an RNA Destabilization Signal (RDS) that is critical for destabilizing the precursor and spliced forms. Using MudPIT, we identified exosome components and the TRAMP complex in association with RDS containing RNA. Mutation of exosome components stabilized all forms of TER1 and resulted in accumulation of oligo-adenylated mature forms. Adenylation was blocked by deletion of TRAMP components. Mutation of the Lsm site destabilized mature TER1 and dramatically enhanced the efficiency of polyadenylation. Consistent with a reduction in mature TER1 levels, Lsm site mutations caused telomere shortening, a defect that was rescued by deleting exosome components. We have also examined the roles of the exosome in human telomerase RNA (hTR) processing. Our preliminary data indicate that the exosome is required for the 3' end processing of hTR. Knockdown of exosome components stabilized hTR and resulted in the accumulation of longer forms of hTR. Our results demonstrate a conserved role of exosomes functioning in 3' end processing of telomerase RNA. Exosomes polish the 3' end of telomerase RNA, degrade inactive forms and function in quality control to eliminate faulty mature forms.

77 Transcriptome-wide mapping reveals pseudouridylation as a dynamic process in budding yeast mRNA.

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Pseudouridylation is the most abundant posttranscriptional modification playing critical roles in RNA structure and function in all kingdoms of life. While numerous pseudouridylation sites in noncoding RNAs have been mapped, quantitative assessment of pseudouridine transcriptome-wide has been technically impractical. We have developed a novel pseudouridine sequencing technique that maps and quantitatively measures pseudouridylation transcriptome-wide. Pseudouridine sequencing has identified hundreds of novel pseudouridines in mRNAs and snoRNAs. Interestingly, pseudouridine modification of snoRNAs lie predominantly in their targeting arms suggesting the distinct biochemical properties of pseudouridine play a role in mediating the base pairing interactions between the snoRNA and its rRNA binding partner. Furthermore, we have identified pseudouridine modifications in *S. cerevisiae* mRNA. These modifications are dependent upon specific pseudouridine synthases, and occur at consensus motifs. Previous data indicate that pseudouridylation is induced in *S. cerevisiae* snRNAU2 by stress. We used pseudouridine sequencing to assess pseudouridylation transcriptome wide under a variety of stress conditions, and found that pseudouridylation levels change significantly at a number of sites in noncoding RNAs during heat shock, cold shock, and nutrient deprivation. In addition, during heat shock pseudouridylation is induced at over two hundred sites in mRNA. The majority of these modification sites are dependent on the pseudouridine synthase Pus7, and occur at a Pus7 consensus motif. Furthermore, we find that Pus7 deletion renders *S. cerevisiae* heat sensitive, suggesting Pus7 facilitated pseudouridylation is important for heat shock survival. In addition, while Pus7 is predominantly nuclear at room temperature, Pus7 is primarily cytoplasmic during heat shock suggesting Pus7 localization plays a role mediating mRNA pseudouridylation during heat stress. In conclusion we have mapped pseudouridine transcriptome-wide, and we find pseudouridylation to be a dynamic process important for yeast stress response.

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78 Profiling ribonucleotide modifications at full-transcriptome level: a step towards MS-based epitranscriptomics

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The discovery of RNA silencing has prompted a reassessment of the significance of non-coding sequences, which has led to an increasing recognition of the role of RNA in cellular processes. This reassessment, however, will remain incomplete without a greater understanding of the functions performed by the over 100 post-transcriptional modifications (PTMs) that decorate natural RNA. Next-generation RNA sequencing (RNA-seq) affords unprecedented accuracy, sensitivity and throughput, but is typically blind to PTMs because the actual analysis employs a DNA copy of the RNA, not the genuine sample bearing the modifications. In contrast, approaches based on mass spectrometry (MS) can directly identify PTMs in any RNA sample by their unique mass and fragmentation signatures. Based on these distinctive features, we have developed a robust MS approach for the analysis of hydrolyzed ribonucleotide mixtures from whole-cell extracts, which enables both characterization and quantification of all PTMs in the transcriptome. Our approach involves initial assignment by database searching, followed by structure confirmation supported by gas-phase fragmentation data.

The analysis of total RNA from S. cerevisiae grown in rich medium enabled the detection of 41 unique PTMs, whereas samples grown in synthetic complete medium provided 8 additional ones. Of the 41 common PTMs, many displayed variations exceeding ±7.8% RSD%, which represents the typical biological reproducibility afforded by these analyses, thus indicating that their biogenesis was either up- or down-regulated. The fact that 13 of the common PTMs were previously unreported for this microorganism was likely due to the almost exclusive attention paid in the past to rRNA and tRNA analysis, whereas the comprehensive nature of our approach captured any type of PTM present in the total RNA extract. When samples treated with high salt were analyzed, we found unique PTMs absent in untreated cells, as well as others that were up-/down-regulated. We identified PTMs whose induction, like that of a discrete set of ~200 long non-coding RNAs (lncRNAs), is dependent on the stress-activated protein kinase Hog1, thus suggesting that PTMs may be involved in the activity of different classes of RNAs. Ongoing work is addressing the role of PTMs in stress response mediated by lncRNAs.

79 Modified Nucleosides in the tRNA Anticodon Accelerate Decoding to Maintain Protein Solubility

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Proteins begin to fold as they emerge from translating ribosomes. The kinetics of ribosome transit along a given mRNA can influence nascent chain folding, but the extent to which individual codon translation rates may impact proteome integrity remains unknown. Importantly, degenerative diseases like amyotrophic lateral sclerosis (ALS) or familial dysautonomia (FD) are linked to codons-specific translation rates through defects in tRNA synthetases or RNA modifying enzymes.

Here, we show that slower decoding of discrete codons elicits widespread protein aggregation *in vivo*. Using ribosome profiling, we find that loss of anticodon wobble uridine (U_{34}) modifications in a subset of tRNAs leads to ribosome pausing at their cognate codons in *Saccharomyces cerevisiae*. Yeast cells lacking U_{34} modifications exhibit gene expression hallmarks of proteotoxic stress and accumulate aggregates of endogenous proteins with key cellular functions. Moreover, these cells are severely compromised in clearing stress-induced protein aggregates. Overexpression of hypomodified tRNAs rescues stress sensitivity of modification mutants by alleviating ribosome pausing, protein homeostasis and concomitantly restoring transcriptional changes. Importantly, we similarly observe reduced decoding speed in *Caenorhabditis elegans*, which coincides with increased accumulation of protein aggregates as well as reduced life span in worm mutants with hypomodified U_{34}.

Our findings demonstrate that modified U_{34} is an evolutionarily conserved accelerator of decoding and reveal an unanticipated role for tRNA anticodon modifications in maintaining proteome integrity.

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80 5' phospho-methylation regulates fate of processed RNAs.

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The fate of cellular RNAs is determined by post-transcriptional modifications occurring at their 5' and 3' termini, such as capping and poly-adenylation of messenger RNAs. We recently described an unexpected RNA modification, consisting in the methylation of the 5' monophosphate (Xhemalce et al., Cell, 2012). In particular, we showed that this modification mediated by the BCDIN3D methyltransferase targeted the precursor of microRNA 145 and actively counteracted its processing into the mature microRNA by Dicer. Because many types of RNAs have 5' mono-phosphate intermediates, we sought to determine whether BCDIN3D had substrates other than premiR-145. To reach our goal we set up an unbiased approach combining biochemical and high-throughput sequencing methodologies that overcome the problem linked to the inability of presently available methods to amplify phospho-methylated RNAs. During the meeting, we will report our findings and discuss their functional and biological implications.

81 A new direction: Multiple roles for 3'-5' polymerases in Dictyostelium discoideum

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The synthesis of RNA and DNA in the 5'-3' direction is thought to be a universal feature of biological systems and is a hallmark of the central dogma of biology. However, the discovery of Watson-Crick dependent reverse (3'-5') polymerase activity catalyzed by members of the $tRNA^{His}$ guanylyltransferase (Thg1) enzyme family challenged this idea that the 5'-3' mechanism is the exclusive route for nucleic acid synthesis. In many eukaryotes, Thg1 enzymes use a non-templated 3'-5' addition activity to play an essential role in maturation of nuclear-encoded $tRNA^{His}$. Until now, however, any biological functions for the 3'-5' polymerase activity associated with this enzyme family have remained a mystery. Using in vitro and in vivo approaches, we investigated the functions of four Thg1 orthologs in the eukaryotic slime mold, Dictyostelium discoideum, and demonstrated that each of these four enzymes carries out a distinct cellular function that is important for viability. One of these Thg1-like proteins (TLPs) utilizes the 3'-5' polymerase activity to repair the 5'-ends of mitochondrial tRNA (mt-tRNA) during an unusual 5'-editing reaction that is widely found in lower eukaryotes. This study provides the first identification of a bona fide biological function for the Watson-Crick dependent 3'-5' polymerase activity of this enzyme family in any species. Moreover, we demonstrated that another D. discoideum TLP catalyzes a previously unknown 3'-5' polymerase reaction with cytosolic non-coding RNAs, providing the first evidence for the ability of 3'-5' polymerases to act on non-tRNA substrates. These data open the door to identification of additional roles for 3'-5' polymerases in biology and ongoing characterization of the mechanisms and biological functions of these unusual enzymes will provide insight into their roles in diverse RNA repair and processing reactions.

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82 Discovery of mitochondrial 3' Processome in *Trypanosoma brucei*

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Trypanosomes are parasitic protozoan hemoflagellates that cause serious diseases such as Chagas disease and African sleeping sickness. The trypanosome mitochondrion encloses an unusual DNA structure composed of a few maxicircles and thousands of minicircles. Mitochondrial genes are encoded in maxicircles, but 12 out of 18 genes are encrypted. Therefore, their transcripts require extensive uridine insertion/deletion RNA editing to produce open reading frames. RNA editing is directed by minicircle-encoded guide RNAs (gRNAs). Mature 50-60 nt gRNAs are generated from ~800 nt precursors by nucleolytic processing and subsequent 3' uridylation. We previously demonstrated that RET1 TUTase repression in *Trypanosoma brucei* leads to a loss of 3′ oligo(U) tails and accumulation of gRNA precursors (pre-gRNAs). The former observation is consistent with the uridyltransferase activity of RET1, while the latter finding remained puzzling because RET1 lacks nuclease activity. Here, we show that RET1 forms a stoichiometric complex with a 3'-5' exonuclease DSS1 and several proteins without discernible motifs, which we named RDS1-3 (RET1-DSS1). DSS1 RNAi knockdown led to a loss of mature gRNAs and accumulation of ~800 nt pre-gRNAs indicating that RET1 and DSS1 function in the same processing pathway. Furthermore, overexpression of enzymatically inactive DSS1 inhibited processing of gRNA, messenger and ribosomal RNA precursors. We propose that RET1, DSS1, RDS1, 2 and 3 constitute a mitochondrial 3' processome. This particle represents an ultimate example of coupling uridylation and 3'-5' degradation activities by confining TUTase and RNase R-type nuclease in the same protein complex. Purified processome processively degrades single-stranded RNAs to 5-6 nt. Remarkably, substrate uridylation by RET1 stimulates processome recruitments but not the ensuing processive degradation. Finally, we present evidence that antisense transcription participates in formation of mature gRNA 3' termini. Specifically, we show that short RNAs are produced from opposite strands of gRNA genes and more than 9000 of potential gRNA-anti-gRNA pairs exist in small mitochondrial RNA population. We also show that *in vitro* 3' processing partially stops 10-12 nt before double-stranded region, which is consistent with 3' overhangs of gRNA-anti-gRNA pairs observed *in vivo*. We will propose a model for gRNA transcription and maturation mechanisms.

83 The RNA-binding landscape of the human exosome-associated exonuclease hRRP6

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The exosome complex is the major RNA decay machinery in eukaryotes. In humans, the nuclear form of the exosome is composed of nine different inactive subunits, which form a ring structure and associate to two different catalytical subunits hDIS3 and hRRP6. The activity of the exosome is governed by binding to other cofactors and adapter complexes, which provide specificity for recruiting this complex to specific RNA substrates. However, the identification of the different RNA substrates for the alternative forms of the exosome still remains unknown on a global scale in higher eukaryotes.

We have performed iCLIP on endogenous and overexpressed hRRP6 (wild-type and a catalytically inactive form) to identify the cellular RNA targets of the human exosome. The use of a catalytically inactive form of hRRP6 stabilized important *in vivo* interactions that are highly dynamic and transient and also highlighted the role of hRRP6-mediated trimming of 3'flanks of immature non-coding RNAs. We will present a global view of the RNA-binding capacity of the hRRP6-form of the exosome.

The Microprocessor complex (DGCR8/Drosha) is required for microRNA biogenesis but also binds and regulates the stability of several types of cellular RNAs, including mRNAs, and lincRNAs [1]. We have recently observed that DGCR8 forms a nucleolar complex with the exosome, which is mutually exclusive with its association with Drosha. This led us to suggest that DGCR8 acts as a novel adaptor to recruit the exosome to structured RNAs and induce their degradation [2].

Analysis of the hRRP6-iCLIP revealed an extensive overlap of RNA targets with the DGCR8-CLIP, highlighting a role for the DGCR8-exosome complex in the turnover of mature snoRNAs. Altogether, we have established an unprecedented global view of the direct RNA targets of hRRP6 within human cells and identified the substrates of the DGCR8-mediated exosomal degradation.

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2. Macias,S., Cordiner,R.A. & Cáceres,J.F. DGCR8 functions as an adaptor for the exosome-mediated degradation of subset of nuclear RNAs. *in preparation*

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84 Selectivity in Substrate Binding by the Exonuclease Rrp6p

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The 3' to 5' exonuclease Rrp6p is associated with the nuclear exosome and plays key roles in nuclear RNA processing and degradation. While Rrp6p is thought to accommodate a broad range of substrates in the cell, its degradation efficiency varies for different RNAs. To understand the molecular basis for this substrate selectivity, we have quantitatively examined the exonuclease activity of Rrp6p *in vitro* at single nucleotide resolution.

We find that RNA substrate length and sequence markedly impact degradation rates. Detailed kinetic analysis of each degradation step shows that functional binding of Rrp6 depends on both, length and sequence of the substrate. Non-productive binding that also depends on substrate length impacts Rrp6p activity as well. However, cleavage rate constants are not comparably affected by substrate length and sequence. The four 3'-terminal nucleotides show the greatest energetic contribution to functional binding of Rrp6p and account for differences in functional binding constants between substrates by up to two orders of magnitude. We further show that the first and third positions from the 3'-terminus have the greatest effect on Rrp6p binding. Structural modeling reveals that measured differences in binding energy correspond to changes in the binding mode of the 3' terminus of the RNA. Of note, our data show that Rrp6p discriminates against CCA termini, which are found in tRNA.

Collectively, our data reveal and explain a complex mode of substrate recognition by Rrp6 and an unexpected degree of inherent selectivity of the enzyme towards the substrate sequence.

85 The C. elegans germ granule assembly protein, PGL-1, is a base-specific, RNA nuclease

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Germ granules are specialized RNA-protein assemblies required for gamete development in higher metazoans, from mammals to nematodes. Loss of P-granules, the *Caenorhabditis sp.* germ granule, leads to adult germline deterioration (reviewed in 1-2) and germ cell differentiation into somatic cell types (3), resulting in sterility. P-granule formation requires PGL-1 and PGL-3 (PGLs), *Caenorhabditis sp.* conserved protein paralogs capable of granule self-assembly (4-5). We set out to structurally characterize the PGLs to better understand their molecular function in P-granules. Our biochemical analyses of recombinant PGL-1 identified a dimerization domain in the central region of the protein, hence referred to as "PGL-1 DD." We determined the 1.6 Å and 3.6 Å crystal structures of *C. remanei* and *C. elegans* PGL-1 DD, respectively. PGL-1 DD has a novel fold consisting entirely of alpha helices. When dimerized, PGL-1 DD forms a central, positively charged channel with the appropriate dimensions to fit ssRNA. To our surprise, incubation of PGL-1 DD with oligonucleotides resulted in RNA cleavage. Additional *in vitro* results demonstrate PGL-1 DD to be a guanosine-specific, single-stranded RNase. The crystal structure allowed us to identify a central site in the dimer channel that brought together conserved glutamine and lysine side chains. Mutation of this glutamine to alanine abrogated cleavage activity without affecting either the dimerization or RNA binding of recombinant protein. We are currently creating this RNase mutant in the *C. elegans* genome to assess the role of PGL-1's enzymatic activity in germ cell development. Our results support the notion that PGL-1 may have a dual function in P-granules as an assembly protein and as an RNase. Our results bring to question whether other germ granule scaffold proteins, like Oskar and Bucky Ball, may also have enzymatic functions involved in RNA regulation.

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86 The eIF4E binding protein 4E-T is a component of the mRNA decapping machinery that bridges the 5' and 3' termini of target mRNAs

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Eukaryotic mRNA degradation often initiates with the recruitment of the CCR4-NOT deadenylase complex and decapping factors to the 3' untranslated regions of target mRNAs. A fundamental question that remains unresolved is how does the decapping machinery, which is recruited to the mRNA 3'-terminus, interact with the 5'-terminal cap structure in order to engender mRNA decay. Human 4E-T is an eIF4E-binding protein that has been reported to promote mRNA decay, albeit via an unknown mechanism(s). Here, we show that 4E-T utilizes an array of conserved motifs to interact with multiple components of the mRNA decapping machinery, including DDX6, LSM14, PATL1 and the LSM1-7 complex. We also provide evidence that 4E-T associates with and contributes to the decay of mRNAs targeted by the CCR4-NOT deadenylase complex, including microRNA targets. Importantly, we demonstrate that 4E-T must interact with eIF4E in order to enhance mRNA decay. Taken together, our data support a model where 4E-T promotes the circularization and decay of CCR4-NOT target mRNAs by physically linking the mRNA decapping machinery to the 5'-cap via its interaction with eIF4E.

87 Preference of the RNA pyrophosphohydrolase RppH for diphosphorylated RNA suggests a new mechanism of 5'-end-dependent RNA degradation in *Escherichia coli*.

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Primary transcripts in bacteria are commonly believed to have a 5'-terminal triphosphate. It has been postulated that 5'-end-dependent mRNA degradation begins with conversion of the 5'-terminal triphosphate to a monophosphate by the RNA pyrophosphohydrolase RppH, an event that triggers rapid attack by ribonucleases specific for RNAs with monophosphorylated 5' ends.

Escherichia coli RppH (EcRppH) requires at least two unpaired nucleotides at the RNA 5' end and prefers either G or A at the second position. To understand the mechanism of RNA recognition and catalysis by this enzyme, we have determined X-ray structures of EcRppH in apo and RNA-bound forms. EcRppH interacts with RNA by a bipartite mechanism involving specific recognition of the 5'-terminal triphosphate and the second nucleotide, thus enabling discrimination against mononucleotides as substrates. These structures and mutational studies suggest a catalytic mechanism for EcRppH and reveal the molecular basis for the modest selectivity of the enzyme for RNAs bearing guanine at the second position and the requirement for at least two unpaired nucleotides at the 5' terminus.

Surprisingly, our structural data also suggest that the gamma phosphate of triphosphorylated RNA may interfere with hydrolysis by EcRppH. Indeed, *in vitro* rate measurements show that EcRppH removes a single phosphate from diphosphorylated RNA ~75-fold more efficiently than it removes pyrophosphate from triphosphorylated RNA. This finding raises the possibility that the principal function of EcRppH in cells may be to produce monophosphorylated intermediates from diphosphorylated RNA rather than from triphosphorylated RNA. Using a new assay that we devised, we have discovered that 20-50% of the 5' termini of the cellular mRNAs we have tested are diphosphorylated in wild-type *E. coli* cells and that the relative abundance of disphosphorylated 5' ends is even higher in cells lacking RppH. These observations suggest that 5'-end-dependent RNA degradation in *E. coli* is triggered by a two-step process involving conversion of the 5'-terminal triphosphate to diphosphate by an unidentified enzyme followed by conversion of the resulting diphosphate to monophosphate by RppH. Together, these results challenge a common perception that bacterial mRNAs bear a 5'-terminal triphosphate or monophosphate and suggest a new mechanism for 5'-end-dependent RNA decay.

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88 Heat-Induced ribosome pausing triggers mRNA co-translational decay in *Arabidopsis thaliana*.

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The reprogramming of gene expression in heat stress is a key determinant to organism survival. Gene expression is downregulated through translation initiation inhibition and release of free mRNPs that are rapidly degraded or stored. In mammals, heat also triggers 5'-ribosome pausing preferentially on transcripts coding for HSC/HSP70 chaperone targets, but the impact of such phenomenon on mRNA fate remains unknown. Here, we provide evidence that, in *A. thaliana*, heat provokes 5'-ribosome pausing leading to the XRN4-mediated 5'-directed decay of translating mRNAs. We also show that hindering HSC/HSP70 activity at 20°C recapitulates heat effects by inducing ribosome pausing and co-translational mRNA turnover. Strikingly, co-translational decay targets encode proteins with high HSC/HSP70 binding scores and hydrophobic N-termini, two characteristics that were previously observed for transcripts most prone to pausing in animals. This work suggests for the first time that stress-induced variation of translation elongation rate is an evolutionarily conserved process leading to the polysomal degradation of thousands of "non-aberrant" mRNAs.

89 A conserved mechanism for protection of retroviral and cellular mRNAs from nonsense-mediated mRNA decay

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The nonsense-mediated mRNA decay (NMD) pathway degrades transcripts containing long 3' untranslated regions (3'UTRs). This activity allows the pathway to both recognize *de* novo nonsense mutations and regulate thousands of apparently normal human mRNAs. However, a large number of potential NMD targets, including retroviral mRNAs, are able to evade NMD via unknown mechanisms. Here, we identify a novel, conserved role for the polypyrimidine tract binding protein 1 (PTBP1) in protecting both Rous sarcoma virus (RSV) and cellular mRNAs from NMD.

Using an RNA-based affinity purification approach, we identified PTBP1 as a major binding partner of the well-characterized RNA stability element (RSE) of RSV. This large (~400nt) RNA segment resides immediately downstream of the viral *gag* termination codon, preventing it from being recognized as premature. Mutation of putative PTBP1 binding sites in the RSE abolish protection from NMD, while addition of PTBP1 binding sites to long 3'UTRs is sufficient to fully recapitulate RSE activity. Further, we find that PTBP1 binding reduces UPF1 association with long 3'UTR-containing transcripts, preventing accurate discrimination of potential decay targets.

Demonstrating a widespread role for PTBP1 in inhibiting NMD, RNA-seq studies of cells depleted of PTBP1 and UPF1 independently and in tandem identify hundreds of human mRNAs that are rendered susceptible to NMD in the absence of PTBP1's protective activity. Consistent with our model, the mRNAs shielded by PTBP1 have longer-than-average 3'UTRs and exhibit enrichment of putative PTBP1 binding sites near termination codons (TCs). Moreover, comparison of RNA-seq data with published CLIP studies of transcriptome-wide PTBP1 binding sites reveals that transcripts marked by TC-proximal PTBP1 peaks have long 3'UTRs but are less likely to be up-regulated by NMD inactivation than mRNAs lacking evidence of PTBP1 binding. Together, our data inform a model in which PTBP1 binds near genuine stop codons to prevent UPF1 recognition of long 3'UTRs and inhibit NMD.

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90 Antisense-oligonucleotide-directed inhibition of nonsense-mediated mRNA decay

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RNA-based therapeutic approaches have become increasingly promising, as demonstrated by recent progress in the development of antisense oligonucleotides (ASOs) and stop-codon read-through drugs as therapeutics for various genetic diseases. Nonsense mutations, which account for a large proportion of pathogenic genetic lesions, can be partially overcome by suppressing premature stop codons (PTCs) using read-through drugs, such as ataluren or aminoglycosides. Nonsense-mediated mRNA decay (NMD) is a cellular quality-control mechanism that degrades mRNAs that harbor PTCs. In the context of genetic diseases caused by nonsense mutations, NMD can worsen the clinical outcome by degrading transcripts that code for truncated but semi-functional proteins, or by reducing the efficacy of read-through therapy. NMD is highly dependent on the exon-junction complex (EJC), which assembles upstream of exon-exon junctions at the completion of pre-mRNA splicing. ASOs were designed to target presumptive EJC sites of *HBB* and *MECP2* transcripts with PTCs, and screened for suppression of NMD. Increases in gene expression were observed with a subset of ASOs, both at the mRNA and protein levels. These results illustrate the potential for applying this approach more broadly as targeted therapy for genetic diseases caused by nonsense mutations.

91 Glucose-Responsive Phosphorylation of the PUF Protein Puf3 Regulates the Translational Fate of its Bound mRNAs and its Association with RNA-Protein Granules

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PUF proteins are conserved post-transcriptional regulators that bind to the 3'UTRs of mRNA transcripts. Herein, we show how a yeast PUF protein Puf3p responds to glucose availability to switch the fate of its bound transcripts that encode proteins required for mitochondrial biogenesis. Upon glucose depletion, Puf3p becomes heavily phosphorylated within its N-terminal region of low complexity, associates with polysomes, and promotes translation of its target mRNAs. Such nutrient-responsive phosphorylation toggles the activity of Puf3p to promote either degradation or translation of these mRNAs according to the needs of the cell. Moreover, activation of translation of pre-existing mRNAs might enable rapid adjustment to environmental changes without the need for de novo transcription. Strikingly, a Puf3p mutant that prevents its phosphorylation no longer promotes mRNA translation but also becomes trapped in intracellular foci in an mRNA-dependent manner. Our findings suggest how the inability to properly resolve Puf3p-containing RNA-protein granules via a phosphorylation-based mechanism might be toxic to a cell.

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92 Molecular architecture of 4E-BP translational inhibitors bound to eIF4E

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The eIF4E-binding proteins (4E-BPs) represent a diverse class of translation inhibitors that are often deregulated in cancer cells. 4E-BPs inhibit translation by competing with eIF4G for binding to eIF4E through an interface that consists of canonical and non-canonical eIF4E-binding motifs connected by a linker. The lack of high-resolution structures including the linkers, which contain phosphorylation sites, limits our understanding of how phosphorylation inhibits complex formation. Furthermore, the binding mechanism of the non-canonical motifs is poorly understood. Here, we present structures of human eIF4E bound to 4E-BP1 and flie eIF4E bound to Thor, 4E-T and eIF4G. These structures reveal architectural elements that are unique to 4E-BPs and provide insight into the consequences of phosphorylation. Guided by these structures, we designed and crystallized a 4E-BP mimic that shows increased repressive activity. Our studies pave the way for the rational design of 4E-BP mimics as therapeutic tools to decrease translation during oncogenic transformation.

93 Control of embryonic cell fates by the Bicaudal-C translational repressor

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The temporal and spatial regulation of cell fates in vertebrate embryos is critical for normal development. We have focused on the Bicaudal-C (Bic-C) translational repressor protein and its role as a cell fate regulator in *Xenopus* embryos. To analyze development in the absence of Bic-C, antisense oligonucleotide ablation was used to create frog embryos that lack maternal Bic-C mRNA and protein. The resulting Bic-C deficient embryos exhibit a unique phenotype and develop with an expansion of anterior cell types and enlarged heads. Over-expression of Bic-C causes the opposite phenotype and treated embryos exhibit severe reductions of the head and trunk. These results suggest that Bic-C normally represses the translation of specific maternal mRNAs required for anterior development. To identify such mRNAs, Bic-C was immunoprecipitated from *Xenopus* embryos and the associated mRNAs analyzed by RNA-Seq. Several of the Bic-C targets encode key regulatory proteins, such as Ddx5 and Cripto-1 that are known to function in the Wnt and Nodal pathways. Signaling by these pathways is essential for the normal development of anterior structures in vertebrate embryos. In depth analysis of the Cripto-1 mRNA identified a 32 nucleotide Bic-C binding site in its 3'UTR. This site forms a stem-loop secondary structure, and mutational analyses supported the importance of this structure for Bic-C-RNA interactions and repression *in vivo*. Our results indicate that Bic-C guides cell-fate decisions during the maternal stages of vertebrate embryogenesis by directly repressing the translation of mRNAs that encode key cell-fate regulators.

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94 RNA topoisomerases are conserved in all domains of life and linked to neurodevelopment and mental disorders

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DNA Topoisomerases are essential to solve topological problems during DNA metabolism in all species. However, the prevalence and function of RNA topoisomerases remain largely unknown. We have previously shown that human Top3b contains a distinctive RNA-binding domain, directly binds mRNAs in vivo, and possesses RNA topoisomerase activity. Moreover, Top3b interacts with the Fragile X syndrome protein, FMRP, to promote normal synapse formation; and Top3b gene deletion is linked to schizophrenia and intellectual disability. Here we show that RNA topoisomerase activity is present in multiple Type IA topoisomerases from bacteria, archaea, and eukarya, but absent in Type IB topoisomerases. The same catalytic residue used in the DNA topoisomerase reaction is also essential for the RNA topoisomerase reaction, indicating that similar catalytic mechanisms are employed for both substrates. Furthermore, the distinctive RNA-binding domain is required for Top3b to bind mRNAs and to promote normal synapse formation. Finally, two de novo single nucleotide variants of Top3β identified previously in schizophrenia and autism individuals are both defective in association with FMRP; and one of them is also deficient in binding to mRNAs, in catalyzing RNA topoisomerase reaction, and in promoting synapse formation. These data suggest that RNA topoisomerases are conserved in all three domains of life, and are required for promoting neurodevelopment and preventing mental dysfunction in higher eukaryotes.

95 Comprehensive analysis of RNA-binding proteins and architectures from yeast to man

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In the past, we reported "mRNA interactome capture" as a method to chart "all" active RNA-binding proteins (RBPs) in mammalian cells leading to the identification of >800 RBPs from HeLa and mouse embryonic stem cells (1-3). We have now determined the mRNA interactome compositions of several additional cell types, including the human hepatocytic cell line Huh7 and the yeast Saccharomyces cerevisiae. Key insights emerging from the datasets include (a) the definition of a eukaryotic "core interactome" of ~230 RBPs conserved between mammals and yeast, (b) the realization that RBPs are far higher in number than previously anticipated, and exceeding 10% of all yeast genes, (c) the identification of dozens of metabolic enzymes as RBPs, including many that are conserved; and perhaps most surprisingly, (d) the identification of a large class of well-studied proteins for which their RNA-binding activities were unknown and their biological roles are enigmatic (termed 'enigmRBPs')(4). Applying a newly developed technique, RBDmap, to identify the RNA-binding domains of enigmRBPs, we uncovered new RNA-binding architectures yielding functional insights (5). Integrating all information, we propose a new function for genomes in addition to their classical role in driving protein biosynthesis via mRNAs, rRNAs, and tRNAs and their associated modifying and regulatory RNAs.

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96 Analysis of protein-RNA specificity enables targeted activation of an endogenous human transcript

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Targeted control of mRNAs by proteins requires an easily programmable scaffold. PUF domain proteins provide an attractive platform for that purpose. Like TALENs and zinc fingers, which target DNA via reiterated modules, PUF proteins possess simple repeated domains. These target RNA rather than DNA, and so provide an opportunity to control translation, decay and processing of mRNAs. PUF proteins bind to single-stranded RNA using eight repeated modules, each of which contributes three amino acids that contact an RNA base. Here, we identify the specificities of natural and designed combinations of these three amino acids. Our strategy to assay RNA-protein interactions (SEQRS) integrates *in vitro* **se**lection, high-throughput se**q**uencing of **R**NA, and **s**equence specificity landscapes [Campbell ZT et al. Cell Reports 2012]. The resulting compendium of specificities reveals the global RNA binding preferences of natural proteins and enables the design of new specificities. Using the recognition code, we design a protein to bind endogenous cyclin B1 mRNA in human cells. A chimeric protein consisting of the designed PUF protein fused to a translation activation domain specifically increases cyclin B1 protein levels, resulting in enhanced sensitivity to chemotherapeutic drugs. Our study provides a guide for rational design of engineered mRNA control, including translational stimulation.

97 Molecular mechanisms of siRNA recognition by tandem dsRBD proteins in *D. melanogaster*

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RNA interference (RNAi) is a defense mechanism against RNA viruses and retro-transposons that is triggered by duplex short interfering RNAs (siRNAs). One of the siRNA strands becomes incorporated into the effector protein Ago2 and confers sequence-specificity to the RNA induced silencing complex (RISC). Strand selection occurs according to the relative stability of base-pairing at either end of the duplex siRNA. In *Drosophila*, Dicer-2 and the double-stranded RNA binding domain containing protein R2D2 form the RISC loading complex (RLC). RLC binds asymmetrically to siRNAs and predetermines the incorporated strand. R2D2 and its paralog Loqs-PD form a complex with Dcr-2 during endo-siRNA maturation. Like R2D2, Loqs-PD has two double-strand RNA binding domains (dsRBD).

Studies of different homologues and of proteins involved in the micro-RNA (miRNA) pathway have revealed some insight for the RNA recognition and the biological function of dsRNA binding proteins. Important aspects of the dsRNA recognition of multi-domain dsRBD proteins are the limited sequence specificity and the presence of dynamics upon binding to dsRNA. However, structural and molecular details for these mechanisms are not well understood.

We present the solution structures of the dsRBDs of Loqs-PD/R2D2 and have analyzed the interaction of the single and tandem domains with siRNA. Biochemical and NMR studies of wild type and linker variants are consistent with a propensity of tandem dsRBD proteins to slide along dsRNA. NMR studies using optimized isotope labelling techniques and paramagnetic relaxation enhancements (PREs) as well as SAXS experiments reveal the RNA binding mechanism and indicate binding to the terminal ends of the siRNA. Cross-linking experiments show that Loqs-PD and Dcr-2 can interpret the base-pairing stability at the ends of duplex siRNAs. Furthermore, Loqs-PD has a moderate intrinsic preference to associate with the more stably base-paired end of an siRNA. Strand discrimination can thus be initiated by binding and diffusion of the tandem dsRBDs along an siRNA duplex. Subsequent engagement of Dcr-2 could then fix the orientation of all components and initiate hand-over of the mature siRNA to Ago2. Our results provide novel clues on the siRNA recognition in the RNAi pathway.

98 Sequestering and protein cofactor competition regulate a multifunctional RNA helicase in different pathways

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DEAD/H-box RNA helicases play key roles in all major pathways of RNA metabolism by regulating the structure and dynamics of RNA-protein complexes. A rapidly increasing number of RNA helicases are implicated in several distinct cellular processes, however, the modes of regulation of such multifunctional RNA helicases and their recruitment to different target complexes have remained unknown. Here we identify the orphan G-patch protein Cmg1 as a novel RNA helicase cofactor that alone does not contact RNA, but stimulates the RNA binding and ATPase activity of the DEAH-box protein Prp43. Cmg1 was found to localise to the cytoplasm and to the intermembrane space of mitochondria. Furthermore, overexpression of Cmg1 promotes apoptosis while its deletion increases cell survival, indicating that Cmg1 is a new pro-apoptotic factor. Prp43 predominantly functions in ribosome synthesis and nuclear pre-mRNA splicing, and our data demonstrate that in apoptosis Prp43 is no longer able to interact with RNA. Moreover, different G-patch protein cofactors compete for interaction with Prp43. Changes in the expression levels of Prp43-interacting G-patch proteins modulate the cellular localisation of Prp43 causing accumulation of the helicase in the cytoplasm or nuclear splicing speckles. G-patch protein overexpression also leads to defects in ribosome biogenesis that are consistent with the withdrawal of the helicase from the pathway. Together, these findings suggest that the interplay of cofactors and the sequestering of a helicase are novel means to regulate the activity of multifunctional RNA helicases and their distribution between different cellular processes.

99 Mechanistic dissection of the early phase of U snRNP biogenesis uncovers a role of ribosomes in assembly and RNP homeostasis

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The productive formation of macromolecular complexes within the crowded environment of cells often requires aid from assembly chaperones. An elaborate system of assembly factors united in PRMT5- and SMN-complexes has been shown to mediate formation of the common core structure of the pre-mRNA processing U snRNPs. The core of these abundant RNPs is composed of seven proteins of the Sm/Lsm protein family bound to snRNA. Core formation is initiated by the PRMT5-complex subunit pICln, which is an assembly chaperone that pre-arranges Sm/Lsm proteins into spatial positions occupied in the assembled snRNP. The SMN-complex then catalyzes snRNP formation by accepting these pre-arranged Sm/Lsm proteins and uniting them with snRNA. While the general function of the assembly factors have been understood in some detail it remained a conceptually unresolved question as to how newly synthesized proteins engage with the cellular assembly machinery to evade aggregation and/or mis-assembly. Here we report that newly synthesized Sm/Lsm proteins initially remain bound to the ribosome near the polypeptide exit tunnel upon translation termination. Release from the ribosome is dependent on pICln, which works in remarkable analogy to folding chaperones. Coincident with its release activity, pICln, as part of the PRMT5-complex ensures the formation of cognate Sm/Lsm heterooligomers and their chaperoned and ordered guidance into the late assembly stage mediated by the SMN-complex. Inactivation of the SMN-complex leads to a block of Sm/Lsm protein flow through the assembly line and to accumulation of Sm proteins on the assembly chaperone. Removal of pICln in contrast, leads to the retention of Sm/Lsm protein on the ribosome and eventually their translational down-regulation. Our results identify an elaborate assembly line for U snRNPs in which the ribosome plays a crucial part as a quality control hub and starting point for the chaperone-mediated assembly process. They further suggest that the coordinated hand-off of newly synthesized U snRNP subunits from the ribosome to specialized assembly chaperones safeguards individual subunits from mis-assembly and aggregation and determines the homeostasis of cellular snRNP levels. While shown here specifically for snRNPs, these principles may also find use in the assembly of other macromolecular complexes.

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100 U1-70K Hijacks the SMN Complex to Produce U1 snRNP Over-Abundance

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U1 snRNP (U1) telescripting - protection of nascent transcripts to ensure full-length transcription, requires great U1 abundance. However, how U1 over-abundance compared to other snRNPs (U2, U4, U5, U6) is achieved is unknown. The biogenesis of snRNP and their functions depend on assembly of a heptameric Sm core on each snRNA, a process mediated by the SMN complex. We discovered that U1-70K, a U1 snRNP-specific protein previously characterized for its function in pre-mRNA splicing, is a U1 snRNP assembly factor. U1-70K binds to a key Sm core assembly intermediate comprised of SMN/Gemin2-Sm pentamer, giving U1 snRNA a large competitive advantage and inhibiting other snRNAs' Sm core assembly. SMN deficiency causes spinal muscular atrophy and mutations in several hnRNP proteins that modify their binding to the SMN complex have been linked to neurodegenerative diseases. Our studies on U1-70K reveal how the SMN complex can be hijacked and suggest that it could be used with a variety of adaptors to assemble diverse RNPs. Additional implications for telescripting and for how one of nature's most ubiquitous, ancient and non-specific Sm/Lsm modules can be directed to bind specific RNAs will be discussed.

101 Dysfunction of U6 biogenesis protein causes incomplete splicing of neutrophil-specific genes in a zebrafish model of poikiloderma with neutropenia

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Recent studies have identified a new U6 snRNA biogenesis protein, USB1, and the loss of which leads to poikiloderma with neutropenia (PN; OMIM#604173), a unique genodermatosis characterized by poikiloderma, facial dysmorphism, pachyonychia, short stature, permanent neutropenia and skeletal defects. The USB1 functions as a 3'-5' exoribonuclease involved in the 3' end processing of U6 snRNA, a core component of the active spliceosome. However, the pre-mRNA splicing was normal in PN patients. These studies suggest that PN manifestations are not derived from common pre-mRNA splicing defects. Instead, they might have resulted from defective splicing of the genes expressed in the tissues that are highly affected by the disease.

In this study, we developed a zebrafish model of PN by the morpholino antisense oligo (MO) based loss-of-function strategy to suppress usb1 gene function. Injection of splice-MO that interrupts usb1 splicing and translation-MO that inhibit Usb1 protein expression, into one-cell-stage embryos induces abnormal development of neutrophils that recapitulated the PN defects. We also observed the severe morphological abnormalities, including a thin yolk extension, a bent tail and reduced body length in the Usb1-suppressed embryos (morphants). Interestingly, the splicing of genes involved in neutrophil differentiation and development, such as mpx, ncf1, ela3l and npsn, was aberrant in the morphants. However, the splicing of hematopoietic precursors and erythroid-specific genes was unaltered. Importantly, the co-injection of ela3l mRNA, the most aberrantly spliced transcript, rescued neutrophil defects in the morphants. Overall, our results demonstrated a possible existence of a regulatory mechanism by which USB1 modulates the tissue-specific gene splicing that might eventually causes tissue-specific defects. This zebrafish model could serve as a valuable tool to investigate the causative role of USB1 in PN pathogenesis.

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102 Specific selection of the HIV-1 genomic RNA by the $Pr55^{Gag}$ precursor: towards the assembly of viral particles

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Specific selection of the HIV-1 genomic RNA (gRNA) from >100 spliced viral RNA species (vRNAs) and from the bulk of cellular RNA is mandatory for viral particle assembly. This step relies on interactions between the $Pr55^{Gag}$ viral precursor and a series of highly structured domains in the 5'-untranslated region of gRNA, including SL1, a short palindromic stem-loop motif mediating gRNA dimerization located into the packaging signal (Psi). Up to now, a major difficulty in *in vitro* studies has been the expression and purification of intact full-length $Pr55^{Gag}$, and as a result most of the previous studies have been performed using $Pr55^{Gag}$ devoid of the C-terminal p6 domain ($Pr55^{Gag}\Delta p6$). Here, we purified full-length $Pr55^{Gag}$ and investigated the determinants of the selective binding of $Pr55^{Gag}$ to gRNA. Based on the intrinsic fluorescence signal of Trp residues in $Pr55^{Gag}$, we characterized the equilibrium binding constants and the stoichiometry of $Pr55^{Gag}$-RNA complexes. According to DLS data $Pr55^{Gag}$ is likely a trimer, and about two $Pr55^{Gag}$ trimers specifically associate with gRNA. According to our results, specific discrimination and selection of gRNA from cellular and spliced vRNAs is provided by a high affinity binding site which is absent in spliced vRNA species. Importantly the specific recognition motif for $Pr55^{Gag}$ was identified as the internal loop B of SL1. Our data on $Pr55^{Gag}\Delta p6$ indicated an alternative binding mode of this truncated protein to gRNA, suggesting that the p6 domain is required for specific $Pr55^{Gag}$ binding to gRNA. Moreover, further analyses on gRNA fragments of different length spanning Psi and/or its flanking sequences support the existence of a long-distance tertiary interaction involving sequences upstream and downstream of Psi that promotes the optimal binding of $Pr55^{Gag}$ to gRNA. Altogether our data highlight the importance of a proper gRNA conformation regulating its specific binding to $Pr55^{Gag}$ to achieve efficient genome packaging.

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103 RNA-Drug Target Analysis using Click Chemistry

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The unique identification of small molecule-RNA targets is a challenge in analyzing the impact of RNA-drug interactions on cellular pathways. Platinum anticancer therapeutics form long-lived crosslinks, providing an avenue for comprehensive target identification following *in vivo* treatment. Pt compounds, broadly used in frontline treatments of solid tumors, crosslink cellular DNA and RNA, and DNA-based apoptotic pathways have been explored. Despite prevalent use, comprehensive identification of cellular RNA targets that may lead to observed resistance, side effects, and alternative apoptotic pathways is not available, nor has the potential for these crosslinking compounds towards in vivo structural analyses been explored. We have shown that cisplatin treatment results in significant platinum accumulation on yeast ribosomal RNA [1], with specificity towards high-impact sites [2]. To further identify, isolate, and visualize such targets we have developed platinum compounds modified for post-treatment 'click' azide-alkyne cycloaddition reactions. Picazoplatin, an azide-modified picoplatin, readily undergoes binding and subsequent fluorescent labeling with DNA and RNA [3]. Post-treatment fluorescent labeling of ribosomal and tRNA extracted from *S. cerevisiae* treated with click-ready Pt compounds demonstrates utility of these compounds for *in vivo* exploration [3-5]. Recent post-treatment fluorescent labeling in cell culture shows distinct accumulation of Pt compounds in the nucleolus, linking treatment with potential defects in ribosome assembly.

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104 Profiling lariats in vivo reveals the second life of introns

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Pre-mRNA contains short sequences of protein coding exons interrupted by long non-coding introns. The process called splicing connects adjacent exons releasing the intron as a branched RNA lariat. The lariats are produced in an equal quantity to the exon junctions in mRNA, but almost all we know about splicing *in vivo* comes from exon junctions in mRNA. Hundreds of thousands of splice site choices have been mapped from mRNA/genomic alignments in many different organisms in many different tissues. Here, we report our genomic scale detection of the transcript branchpoints from deep sequencing data. We present more than 102,479 lariats mapped to human introns. This data demonstrates a central role for branchpoint location in alternative splicing. Remarkably, certain 5'ss splice to branchpoints hundreds of nucleotides from the 3'ss AG. These distal branchpoints are bound by U2AF65, conserved across multiple vertebrates and strongly associated with exon skipping. We present data from permissive and restrictive tissues that suggest distal branchpoints play a kinetic role by holding up the splicing machinery and allowing downstream introns to be synthesized to enable alternative splice choices. In addition to this genomic survey, we utilize biochemical methods to characterize several novel alternate modes of U2snRNA pre-mRNA interactions that occur in the processing of human introns. While most lariats are rare and transient, certain species appear to be stabilized in the cell. For example, approximately 3% of excised introns appear to circularize and travel to the cytoplasm. We consider the possible non-splicing roles these (and other) lariats could perform in the cell. This "second life" of introns as non-coding RNAs is not new. The function of lariats as an expression vehicle for RNA gene (e.g. snoRNA) expression is explored, for the first time at a genomic scale. In addition the data implicates lariats in less well established functions such as chromatin modification and influencing gene expression.

105 Targeted RNA Sequencing with the SeqCap RNA Enrichment System

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Sequencing-based whole transcriptome analysis (RNA-Seq) is a powerful tool to measure gene expression, characterize transcript isoforms and identify sequence polymorphisms. However, the transcriptome is largely comprised of a small number of highly abundant transcripts, so RNA-Seq can be inefficient when the goal is to study a fraction of the transcriptome. Here we describe the Roche NimbleGen SeqCap RNA enrichment system which can focus sequencing on a subset of genes, enrich rare transcripts, and enable the search for putative transcripts in targeted genomic regions.

We demonstrate the capabilities of the SeqCap RNA enrichment system by capturing 256 genes associated with neurological disorders from a human brain sample. Compared to RNA-seq, the SeqCap RNA panel requires 50 fold fewer reads to achieve the same sensitivity for transcripts of similar abundance. Use of the widely known ERCC RNA spike-in controls demonstrates that targeted control transcripts maintain expected relative abundance through the capture workflow. The SeqCap RNA workflow is also useful for study of non-protein coding RNAs. A catalog lncRNA design detects 5 to 20 fold more lncRNA isoforms compared to RNA-Seq, demonstrating the enhanced discovery capability of the system. The SeqCap RNA Enrichment System also enables researchers to create tissue-specific enrichment designs which focus sequencing on low and moderately expressed genes in their particular biological samples, and avoid highly abundant, non-informative transcripts. Tissue-specific captures in esophagus and salivary gland-derived RNA detect significantly more transcript isoforms at all FPKM levels in both tumor and normal samples when compared to RNA-Seq.

The SeqCap RNA Enrichment System focuses sequencing output on targets of interest, dramatically increasing the efficiency of next-generation sequencing approaches for analyzing target RNAs.

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106 Withdrawn

107 Increasing Sensitivity of Next Generation Sequencing-based Transcriptome Profiling by Selectively Depleting Abundant RNAs

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Introduction: Next Generation Sequencing has become the method of choice in research and clinical diagnostics for transcriptome profiling and biomarker discovery. Sequencing the entire transcriptome is inefficient since few extremely abundant transcripts with minimal biological interest can dominate readouts, masking detection of more informative lower expressed transcripts. Here, we present a method to enrich for RNAs of interest by eliminating unwanted RNAs before sequencing. **Methods:** This method is based on hybridization of single-stranded DNA probes to the targeted RNA and subsequent degradation of the selected RNAs using RNase H endoribonuclease enzyme. We optimized this method to remove cytoplasmic (18S, 28S, 5S, 5.8S) and mitochondrial (12S, 16S) ribosomal RNA (rRNA) from different eukaryotic total RNA samples (human, mouse and rat). We applied this method to remove ribosomal RNA from highly degraded formalin-fixed paraffin-embedded (FFPE) RNA extracted from breast cancer biopsies. Additionally, we expanded the method to eliminate hemoglobin transcripts from RNA extracted from blood-derived samples (e.g. whole blood, umbilical cord and bone marrow cells). We evaluated the depletion efficiency and off target effect using strand specific RNA high-throughput sequencing. **Results**: We achieved excellent ribosomal RNA (>99% for human; >95% for mouse and rat) and hemoglobin (>99%) depletion efficiency, regardless of the input amount (1µg or 100 nanograms), or degradation level (intact or FFPE RNA). An excellent correlation (R^2>0.93) comparing the expression level (FPKM values) of non-targeted transcripts between rRNA depleted and non-depleted samples was found. **Conclusions:** The depletion of targeted transcripts by this method is not altering the expression level of the non-targeted transcripts and it produces increased coverage of less abundant transcripts. This method offers a robust and simple solution for transcriptome analysis of a variety of samples, including low quality and low quantity clinical samples such as FFPE RNA and it is also amenable to high-throughput sample preparation and robotic automation.

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108 Tracking RNA-RNA interactions by PLASH.

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Although fascinating, the RNA interactome remains uncharted due to a lack of adequate experimental tools. To face this problem, we are developing a new method called PLASH (Psoralen crosslinking, Ligation And Sequencing of Hybrids) for tracking direct RNA-RNA interactions within cells. PLASH is based on the use of biotinylated Psoralen (Ps), a compound that easily enters cells and intercalates into double stranded nucleic acids. Psoralen has an ability to form a covalent bond with bases and this process can be reversed. Unlike other methods for RNA structure analysis, PLASH will allow to freeze the interaction between thousands of interacting RNA molecules genome-wide. Ligation of crosslinked RNAs forming specific chimeras, followed by subsequent adaptor ligation will allow to step into Next Generation Sequencing. Currently we are optimizing the method and analysing first in vitro and ex vivo PLASH sequencing data. Our approach could be used for analysing differences in RNA structure profile in varied conditions, detection of different levels of dsRNA and finding new interacting RNAs.

109 Single-nucleotide resolution mapping of m6A throughout the transcriptome

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N^6-methyladenosine (m6A) is the most abundant modified base in eukaryotic mRNA, but the precise position of m6A in mRNAs cannot be identified on a transcriptome-wide level because there are no chemical methods to distinguish between m6A and adenosine. Here we show that anti-m6A antibodies can induce a specific mutational signature at m6A sites after ultraviolet-induced antibody-RNA crosslinking and reverse transcription. Using this mutation signature, we map m6A in human mRNA and identify snoRNAs as a novel class of m6A-containing ncRNAs.

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110 Enzymatic Synthesis of High-Density RNA Arrays

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The ability of a particular RNA to perform a function is governed by its ability to bind a given target. As with proteins, RNA binding and function is intimately linked with its sequence, making our understanding of this connection a crucial aspect of RNA biology. Here we present a technology to produce high-density arrays of RNA. We approach this by using a series of steps which subject photochemically produced DNA arrays to enzymatic transcription. RNA primers are covalently attached across the entire surface of a DNA array and then extended by using the DNA features as templates, copying these sequences into the RNA(s) of interest. Enzymatic removal of the DNA leaves behind only these newly formed RNA sequences, with each localized to the region of the original templating DNA. This technique allows over 786,000 different RNA sequences to be patterned within a ~1.5 cm^2 area. We expect these arrays to be useful for characterizing aptamer candidates, studying RNA binding protein specificity, constructing tiling arrays of viral genomes, studying ribozyme activity, examining miRNA binding, and in the engineering of RNAs which exhibit novel characteristics.

111 Directional qRNA-Seq: Combining the Power of Stranded RNA-Seq with the Quantitative Precision of Molecular Labels

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RNA-Seq is a powerful tool for transcriptome analysis. However, data generated from standard RNA-Seq protocols lack two important pieces of information about RNA expression - the DNA strand from which RNA transcripts were derived and the precise abundance of those transcripts. Here, we describe the development of a unique quantitative directional RNA-Seq protocol with companion analytical software. Using this protocol we identified new non-coding RNAs (ncRNA) and determined differences in mRNA expression including ncRNAs in normal colon, colon tumor and matched non-tumor surrounding tissues.

With over 80% of the genome transcribed non-uniformly, our human transcriptome is ultimately complex, within which ncRNAs play major roles in precise regulation of transcriptional and/or translational events. Although ncRNAs can be readily detected by Next Generation Sequencing, it is still difficult to determine from which strand they were derived, especially in non-spliced transcripts. The quantification of transcript abundance is another challenge in RNA-Seq. Gene expression is typically measured as the proportion of reads mapped to the transcript among all mapped reads. PCR amplification, however, introduces bias as some fragments are preferentially amplified, so that the relative abundance of the transcripts in the original RNA sample is not faithfully reproduced in sequencing reads.

Here we present effective solutions to both RNA strand ID and quantification. To enable stranded RNA-Seq, dUTPs are incorporated during second strand synthesis, followed by degradation of this strand by uracil-glycosylase prior to PCR amplification. To correct amplification bias, we randomly ligate 96 molecular labels (STL) to both ends of each cDNA fragment prior to PCR amplification, allowing for 9,216 unique combinations. For the deconvolution of unique read fragments we developed a special script which generates total read pairs per transcript after STL and transcript start/stop correction.

This new quantitative gene expression method combines stranded selection with unique molecular labeling based fragment calls. It offers biologists a new tool to carry out precision RNA biology.

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112 Oligo‑Stop‑Seq: Detection of binding sites of Locked Nucleic Acid oligonucleotides using massive parallel sequencing

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Antisense oligonucleotides (ASOs) form a novel class of promising drug candidates. They function by hybridizing to target RNA molecule and exploiting various biological mechanisms to trigger a therapeutic effect. Challenges in their design include ensuring efficient delivery, avoiding non-hybridization toxicity or off-target hybridization. We have developed a method (Oligo-Stop-Seq) for experimental detection of oligonucleotide binding sites within multiple long transcripts simultaneously and applied it for ApoB-targeting ASOs modified with Locked Nucleic Acids. The poly(A) fraction of liver RNA was hybridized with the ASO carrying a 4-thiothymidine at its 5' end, followed by crosslinking with long range UV light, which induces 4-thiothymidine to form covalent bonds with RNA within a close range. After washing away the non-crosslinked ASOs, the RNA samples were used as template for randomly primed reverse transcription, with primers carrying an adapter at their 5' side. Since reverse transcription terminates upon reaching the crosslinked ASO, the 3' end of cDNA carries the information on ASO binding site. An adapter was ligated to the cDNA 3' end, the construct was PCR amplified and pooled samples were sequenced using Illumina HiSeq system. Reads were mapped to transcripts and counts at each position were log-normalized to non-ASO sample using the RNAprobR Bioconductor package. This approach detected the intended target site at the ApoB transcript as well as multiple other binding sites. Motif analysis of the sequences just upstream from the sites with high Oligo-Stop-Seq signal allows recovering all except flanking nucleotides of the used ASO. Finally, we show that the predicted binding energy between RNA and ASO is much lower for the detected sites than for other locations. We expect this method to facilitate rational design of ASOs by providing direct evidence of off-target binding events. What is more it allows for disentangling observed RNA expression changes from actual ASO hybridization, as Oligo-Stop-Seq does not depend on activating effector molecules. Finally, we are currently working on establishing a map of transcripts accessibility by coupling Oligo-Stop-Seq with a library of random oligonucleotides.

113 RNA Tagging identifies and reveals new features of RNA regulatory networks

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Protein-RNA networks are essential to RNA biology. We have developed a method to identify the RNAs bound by any protein *in vivo*, which we call "RNA Tagging." In this approach, we express an RNA-binding protein of interest fused with a poly(U) polymerase. The chimera "tags" the RNAs it binds *in vivo* with 3' terminal uridines. Tagged RNAs are identified from the pool of total RNA using an adaptable high-throughput sequencing protocol. The RNA Tagging strategy eliminates crosslinking and protein purification steps required in other techniques. It thus provides a simplified approach to identify protein-RNA interactions and should be amenable to tissues that are less tractable with other approaches.

We implemented RNA Tagging in *S. cerevisiae* to examine four mRNA regulatory proteins: the PUF proteins Puf3p, Puf4p, and Puf5p, as well as the P-body component, Bfr1p. Our analyses lead to three main conclusions. First, RNA Tagging reproducibly identified transcriptome-wide targets of each protein, and the PUF protein targets were highly enriched for their expected biological functions. Second, RNA Tagging discriminated the regulated targets from those that are merely sampled, which correlates with how well the relevant RNA sequences are bound *in vitro*. Third, RNA Tagging revealed new biological roles of an RNA-binding protein, as evidenced by analysis of Bfr1p. Its targets were highly enriched for secreted proteins and those translated at the ER - enrichments that are consistent with prior genetic and localization studies but were undetected in a prior high-throughput study.

We conclude that RNA Tagging is a simplified approach to globally interrogate *in vivo* protein-RNA interactions and yields new biological and biochemical insights into how proteins bind and regulate RNA.

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114 Affinity purification of *in vitro* transcribed RNA with homogeneous ends using the ARiBo method

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In vitro synthesis using the T7 RNA polymerase and purification of RNA are essential and basic tools for the structural and functional characterization of RNA. However, traditional approaches for RNA preparation generally denature the RNA, can be time-consuming and often produce RNAs with chemically-heterogeneous 5'- and 3'-ends. Over the past few years, our laboratory has developed the ARiBo method, an efficient protocol for affinity purification of *in vitro* synthesized RNAs that exploits the **A**ctivatable *glmS* **Ri**bozyme and the high-affinity interaction between the ***Bo****xB* RNA and the N peptide from bacteriophage λ. Importantly, our ARiBo procedure rapidly generates (~3 hrs) highly pure native RNA with very good yields. We will describe the basic ARiBo procedure and its application to purification of RNAs with different sequences, secondary structures and sizes (29 to 614 nucleotides). Although the basic ARiBo procedure ensures 3'-sequence homogeneity of the purified RNA, it does not always produce RNA with 5'-sequence homogeneity. Thus, we will also present three complementary approaches that ensure 5'-homogeneity of the affinity-purified RNA: 1) selection of the starting sequence; 2) Cse3 endoribonuclease cleavage of a 5'-CRISPR tag; and 3) self-cleavage of a 5'-hammerhead ribozyme tag. Based on our recent results, we will discuss the advantages and limitations of these current approaches to achieve 5'-homogenity of affinity-purified RNA, including how one can select the best strategy to purify a particular RNA of interest.

115 Next-generation tools for RNA enzymology: Determination of rate and equilibrium constants for large populations of RNA substrate variants using high throughput sequencing.

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Structure-function studies of RNA binding and RNA-processing reactions, in which the effects of specific variations in sequence on specific reaction parameters such as binding kinetics, equilibrium binding affinity and catalytic rate, have provided deep insights into biological function to be gained. Nonetheless, our perspective is severely limited by the relatively small number of sequence variants that can be analyzed. Using RNase P processing of pre-tRNA as an experimental system are developing a set of tools based on high-throughput sequencing and competitive kinetic analysis to accurately and simultaneously determine kinetic and equilibrium binding constants for large RNA substrates. The resulting high-density structure-function data sets are providing unique insights into patterns of molecular recognition and the nature of specificity in RNA-protein interactions. Although powerful, an inherent limitation of competitive multiple turnover kinetics is that product inhibition, inactive substrate populations and multiphasic kinetics can limit precision. Using single turnover reactions which conform more directly to simple exponential kinetics should allow high resolution data sets to be gained for both binding kinetics and effects on catalysis. A similar approach is being developed to determine equilibrium binding constants by analyzing the distribution of sequences in free and bound populations separated by EMSA using simple competitive binding models. Since the high throughput kinetics gives us the entire affinity distribution, the classical methods such as sequence logos and position weight matrix provide restricted views of specificity that ignore information contain the affinity distribution accordingly. We develop a new multivariable regression method which considers the interaction between different positions. In combination these approaches are providing a comprehensive understanding of how substrate sequence and structure affect binding affinity, association kinetics and catalysis.

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116 Using recombinant Cas9 RNPs to assess locus modification in genome editing experiments

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Determining the extent to which specific loci are modified in genome editing experiments is important for characterizing tissues, mixed pools or isolated colonies of edited cells. This study compared *in vitro* digestion of PCR amplicons with recombinant Cas9 ribonucleoproteins (RNPs), to T7 Endonuclease I and other mismatch detection assays for determining the extent of locus modification in genome editing experiments.

We examined detection sensitivity, useful range of input, and activity on unpurified PCR products in targeting efficiency determination assays using model substrates. In addition, we compared these methods to high-throughput amplicon sequencing of libraries prepared from cell lines transfected with Cas9 and sgRNA targeting specific loci.

We find *in vitro* digestion with recombinant Cas9 RNPs to be a more streamlined workflow for determining the extent of locus modification that enables the direct digestion and analysis of DNA without cleanup steps. *In vitro* digestion of PCR amplicons with Cas9 RNPs is as sensitive as mismatch detection assays, but unlike mismatch detection assays, Cas9 has the additional advantage of allowing for determination of targeting efficiencies above 50%. This is of value as targeting efficiency in genome editing experiments increases and for detection of biallelic editing in isolated cell colonies or tissues, and was previously only achievable using specialized PCR or amplicon sequencing approaches.

117 New methods for RNA-Seq analysis of whole-cell, exosomal, and human plasma RNAs utilizing thermostable group II intron reverse transcriptases (TGIRTs)

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We have developed new methods for next-generation RNA sequencing (RNA-Seq) that exploit advantageous properties of thermostable group II intron-encoded reverse transcriptases (TGIRTs) and demonstrate their use in analysis of whole-cell, exosomal, and human plasma RNAs. TGIRTs have higher thermostability, processivity and fidelity than conventional retroviral reverse transcriptases, as well as a novel template-switching activity, which is minimally dependent upon base pairing and enables attachment of RNA-Seq adaptors to target RNA sequences during cDNA synthesis without RNA ligation. Advantages of TGIRT-Seq include the ability to comprehensively profile protein-coding gene transcripts and lncRNAs together with small ncRNAs in the same RNA-seq run; facile and less biased RNA-Seq library construction due to the absence of an RNA-ligase step; and the ability to obtain full-length reads of structured ncRNAs, such as tRNAs and snoRNAs, which are not readily analyzed by conventional methods. TGIRTs also make it possible to identify post-transcriptional modifications by distinctive patterns of misincorporated nucleotides and to distinguish aminoacylated from non-aminoacylated tRNAs. We show via a number of examples that TGIRTs enable the identification and analysis of non-coding RNAs, including a previously undetected miRNA species, that cannot be studied readily by conventional methods due to factors such as stable secondary structure, nucleotide modifications, 3'-end occlusion, or length. For the analysis of small amounts of human plasma RNA, we developed a streamlined method in which cDNAs with attached RNA-Seq adaptors generated via TGIRT template-switching are minimally PCR amplified without size-selection or phenol-extraction steps, permitting the preparation of RNA-seq libraries from 1-2 ng of plasma RNA in <5 h. TGIRT-seq of RNAs present in 1 ml of plasma mapped to >19,000 protein-coding genes and >6,000 lincRNAs and identified numerous small ncRNAs, including miRNAs, tRNAs, snoRNAs, Y RNAs, Vault RNAs, and 7SL and 7SK RNAs, that are present in plasma as mature full-length transcripts, suggestive of RNP complexes. With collaborators, we are applying TGIRT-Seq to clinical samples for potential applications in diagnostics.

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118 Novel preservation and storage method maintains RNA integrity at room temperature without chemical intervention for up to three months as evidenced by multiple analytical techniques

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Handling and maintenance of biological samples for RNA analysis has long been a challenge because of the perceived instability of these macromolecules at room temperature if not preserved and processed. Structural damage and compromised integrity of aforementioned biomolecules subsequent to preservation have also posed difficulties in their use in research. The development of technologies employing nonfixative methods with the capability to store at room temperature have been of growing interest. Herein, we report the results of quantitative and qualitative analyses of RNA from samples that were desiccated and stored at room temperature for up to 3 months. Our results indicate that viable RNA can be obtained from dehydrated ex vivo tissue samples and purified aliquots that have been stored at room temperature.

119 Capturing primary RNA transcripts, a novel strategy for analyzing transcriptomes

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We have developed a novel method, Cappable-Seq, to distinguish 5' termini of primary transcripts from 5' RNA termini generated by processing or degradation. Cappable-Seq directly and specifically modifies the initial 5' triphosphorylated nucleotide incorporated by RNA polymerase. To achieve this modification, the RNA is enzymatically capped with a modified guanosine triphosphate containing a desthiobiotin moiety. Desthiobiotinylated RNA is selectively bound to streptavidin. The bound fraction is eluted and decapped leaving the RNA with ligatable 5' monophosphate end. Coupled with direct ligation-based library preparation and sequencing, this technique for the first time provides an enrichment based solution for transcription start site determination at single base pair resolution. As a proof of concept, we applied Cappable-seq genome-wide to *E. coli* and found an unprecedented number of transcription start sites (TSS). Ribosomal RNA sequence reads are down from 95% to 3% providing deeper sequencing of the informative transcriptome. Cappable-Seq is species agnostic and can be applied to all prokaryotes as well as eukaryotic pol I and pol III transcripts. Beyond TSS determination, this methodology is ideally suited for microbiome studies by providing a unique solution for digital profiling of gene expression in complex bacterial communities while universally removing the contaminating ribosomal RNA that constitute the major burden of metatranscriptomes.

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120 Mutational Interference Mapping Experiment (MIME) for studying the interaction between $Pr55^{Gag}$ and the HIV-1 genomic RNA

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A common challenge encountered by viruses is the selective packaging of their genome into the progeny viral particles. In HIV-1, this selection is mediated the $Pr55^{Gag}$ precursor protein through specific interactions with a packaging region termed Ψ. This region maps to the 5' untranslated region (5' UTR) and the beginning of *gag* and is comprised of several independent structural domains (TAR, PolyA, PBS, SL1, SL2, SL3, SL4) that have all been implicated in the packaging of HIV-1 RNA. Remarkably, there is no consensus concerning the relative importance of each these signals and their interaction with $Pr55^{Gag}$.

We have developed mutational interference mapping experiment (MIME) to identify at single nucleotide resolution the primary and secondary structures of the HIV-1 genomic RNA that are crucial for $Pr55^{Gag}$ binding *in vitro*. MIME is based on random mutagenesis of the RNA target followed by functional selection and next generation sequencing. Our approach allows the recovery of quantitative binding parameters for thousands of mutants in a single experiment, their statistical properties and the identification of interacting partners directly from the sequencing data.

We used MIME to precisely define the $Pr55^{Gag}$ binding site within the first 532 nucleotides of the HIV-1 genome. We identified a single contiguous region, encompassing nucleotides 227 to 337, that significantly and strongly impaired $Pr55^{Gag}$ binding when mutated. This core-binding domain includes SL1, SL2 and SL3 as well as a portion of the PBS domain and the AUG start codon. Of the 10 positions with the greatest effect on $Pr55^{Gag}$ binding, we found that 9 mapped to SL1, indicating that SL1 is the primary binding site. Mutations to the palindromic GCGCGC sequence within the SL1 apical loop strongly impaired binding consistent with the idea that dimerization is an important determinant of genome packaging. To a lesser extent, SL3, the basal portion of SL2, and the single stranded regions between these stem-loops were also required. Interestingly, mutations to SL4 improved rather than inhibited $Pr55^{Gag}$ binding. Finally, MIME provides both indirect and direct information on interacting nucleotides, which we demonstrate through the *de novo* prediction of structural motifs important for $Pr55^{Gag}$ binding.

121 Selective Single Cell Isolation and RNA Sequencing Using Microraft Arrays

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Background: The recent development of single cell RNA-seq protocols enabled genomewide investigation of organismal systems at the cellular level, opening many new biological questions for study. However, it remains unclear how common cell isolation techniques such as microfluidic devices, flow sorting, and immune-labeled magnetic bead selection affect the gene expression profiles of single cells.

Approach: We present a novel method for single cell selection, isolation and sequencing based on a microfabricated "microraft" array platform. Cells are plated on the array in the same manner as a Petri dish and allowed to adhere to individual microrafts. Individual rafts and their cells are subsequently collected using a magnet. This platform enables selective isolation of single cells based on a variety of phenotypes, not just surface markers. The isolation process does not subject cells to harsh manipulations that modify cell morphology and physiology. Furthermore, cells can be cultured on the array, allowing examination of morphology and growth over time or in response to treatments.

Results: We assess our method by sequencing single cells from a human pancreatic tumor cell line comparing the microraft arrays and the Fluidigm C1. Raft and C1 samples show comparable numbers of sequencing reads, genomic read distribution, 3' bias profiles, and number of genes detected, indicating that our method produces high quality sequencing data. In addition, we find systematic differences in gene expression profiles between C1 and raft samples. Gene set enrichment analysis reveals upregulation in cell growth and division genes among raft cells, while C1 cells show upregulation in cell adhesion and cell migration genes. This suggests that processing cells using the C1 alters gene expression levels, especially among cell cycle related genes. Using the raft cells, we identify genes that vary more than expected from technical noise, including genes involved in cell growth, epithelial differentiation, and a cancer cell surface marker. We also isolate single cells that do not proliferate after gemcitabine treatment and small clonal populations of cells that arise from single cells after treatment, demonstrating the flexible selection abilities of microrafts. We have extracted cDNA from these cells and will present the sequencing results.

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122 An improved, sequencing-based approach for quantifying global changes in pre-mRNA splicing

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The advent of next generation sequencing technologies has revolutionized biology over the past decade. With constant improvements in read fidelity, resolution and depth, gene expression studies have become both more precise and less expensive than ever before. Simultaneously, the proliferation of RNA-seq experiments has dramatically increased in our understanding of the prevalence of alternative splicing through detection of novel exon-exon junctions. Nevertheless, the precision with which many splice isoforms can be quantitatively measured is poor, owing to the relatively low frequency with which they are sampled in a standard experiment and as well to the noise associated with counting these numbers of reads. Here we present a complementary sequencing approach that is designed to give high precision measurements of global pre-mRNA splicing by specifically targeting sequencing efforts at splicing boundaries. Briefly, primers which have been designed to specifically anneal immediately downstream of every annotated 3' splice site in a genome are used as the basis for generating sequencing libraries. Use of these primers allows for specific enrichment of the splice junctions of every transcript. For every given splice site, mapping of the reads generated from these libraries allows for a precise determination of the quantities of unspliced, canonically spliced, or alternatively spliced isoforms present in a sample. As a proof of concept, we compared results obtained by splicing-sensitive microarray, standard RNA-seq, and our targeted splicing-sequencing, when examining a canonical splicing mutant in budding yeast. The results of these experiments support the notion that our approach can significantly improve the precision with which changes in splicing efficiency are detected, and argue that this approach can be a powerful complement to standard RNA sequencing. Importantly, complex pools of discreet oligonucleotide sequences can now be readily and inexpensively synthesized, allowing this approach to be applied to nearly any organism of interest.

123 Efficient and quantitative high-throughput transfer RNA sequencing

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Transfer RNAs (tRNAs) are essential for cells and are under stringent cellular control. tRNAs are overexpressed in many types of cancer, and tRNA overexpression can directly increase the tumorigenic potential for cells. Accumulating evidences show that tRNA expression and mutations are associated with various diseases such as neurological pathologies and cancer development. Despite its biological importance, tRNA could not be adequately sequenced due to the presence of abundant post-transcriptional modifications and stable secondary structure, which interfere with cDNA synthesis and adapter ligation. We achieve efficient and quantitative tRNA sequencing by using engineered demethylases to remove base methylations such as N1-methyl-A (m1A), N1-methyl-G (m1G), and N3-methyl-C (m3C) and a highly processive thermostable group II intron reverse transcriptase without the need for adapter ligation (DM-TGIRT-seq). Our method should be applicable to biological investigations of tRNA abundance, isodecoder distribution and modification dynamics in all organisms.

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124 Detained introns couple splicing kinetics to gene expression

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Under steady-state conditions, most pre-mRNA splicing is believed to occur cotranscriptionally. Using molecular analyses and a novel computational approach, we identified thousands of introns in mouse and human-many of which are evolutionarily conserved-that are present in polyadenylated and otherwise fully-spliced transcripts, indicating they are not cotranscriptionally spliced. We refer to this class as "detained introns" (DIs) because they are detained in transcripts that are themselves detained in the nucleus. Ultimately DIs undergo post-transcriptional splicing, or the messages containing them are destroyed. However, although DIs invariably contain premature termination codons, they are not subject to nonsense-mediated decay (NMD). This is likely due to the fact that they are trapped in the nucleus and therefore not translated, distinguishing DIs from retained introns (RIs)-introns that are retained in messages that are exported to the cytoplasm and either encode a peptide or target the mRNA for NMD. This difference in nuclear export indicates that DIs and RIs are produced by distinct mechanisms. We hypothesize that this distinction stems from prespliceosome formation across DIs, which is known to cause nuclear accumulation, that is absent from RIs. We find a common subset of DIs present in mouse embryonic stem cells and mouse liver that are differentially abundant in each cell type, indicating that these introns are dynamically regulated rather than simply poorly spliced. To obtain further evidence that DIs are dynamically regulated, we performed the first global transcriptome analysis following drug inhibition of the SR protein kinase Clk. We found that Clk affects, likely directly, a specific subset of about 300 DIs of which some undergo increased splicing while others are detained at a higher rate. Clk inhibition induced a p53 transcriptional response, possibly though a direct effect on the splicing of the negative p53 regulator Mdm4. DIs also responded to DNA damage, indicating that they play a role in physiological stress responses. These data suggest that DIs are a widespread, previously unappreciated post-transcriptional control point-by regulating the rate at which specific introns are cotranscriptionally spliced, the cell can modulate the amount of coding mRNA that reaches the cytoplasm in response to environmental signals or stresses.

125 Transcriptome analysis reveals thousands of targets of nonsense-mediated mRNA decay that offer clues to the mechanism in different species

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Many alternatively spliced isoforms contain a premature termination codon that targets them for degradation by the nonsense-mediated mRNA decay RNA surveillance system (NMD). Some such unproductive splicing events have a regulatory function, whereby alternative splicing and NMD act together to impact protein expression. The "50nt rule" is the prevailing model for how premature termination codons are defined in mammals, and requires a splice junction downstream of the stop codon. There is evidence that this rule holds in Arabidopsis but not in other eukaryotes. There is also evidence that a longer 3' UTR triggers NMD in yeast, plants, flies, and mammals.

To survey the targets of NMD genome-wide in human, zebrafish, and fly, we have performed RNA-Seq analysis on cells where NMD has been inhibited via knockdown of UPF1, a critical protein in the degradation pathway. We found that hundreds to thousands of genes produce alternative isoforms that are degraded by NMD in each of the three species, including over 20% of the genes alternatively spliced in human HeLa cells. These genes, potentially subject to regulation through NMD, are involved in many functional categories and, in human and fly, are significantly enriched for RNA splice factors. We also found a significant enrichment for ultraconserved elements in the human NMD targets, often overlapping a poison cassette exon.

We were also able to gain insight into what defines NMD targets. We found that the 50nt rule is a strong predictor of NMD degradation in human cells, and also seems to play a role in zebrafish and in fly. In contrast, we found little correlation between the likelihood of degradation by NMD and 3' UTR length in any of the three species. In fly, we see no enrichment for longer 3' UTRs in isoforms degraded by NMD, unless they have an intron. We also found that thousands of human transcripts have uORFs that seem to affect their likelihood of degradation. Ultimately, our findings demonstrate that gene expression regulation through NMD is widespread in human, zebrafish, and fly, and that NMD is strongly predicted by the 50nt rule but not by 3' UTR length.

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126 The interconnection between splicing and chromatin state in *S. cerevisiae* - the role of Prp45

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Dynamic modifications of chromatin histones, such as acetylation and methylation, contribute to the regulation of gene expression. At the same time, ongoing transcription and transcript processing can affect the chromatin landscape. Prp45/SNW1/SKIP is a conserved part of the NTC/CDC5 spliceosomal sub-complex that is required for splicing. In higher eukaryotes, it associates with sequence specific transcription factors as well as components of the elongating RNA Pol II complex.

We have shown previously that Prp45 aids the recruitment of 2nd step helicase Prp22 to spliceosomes and that C-terminally truncated Prp45 (*prp45*(1-169)), while still capable of supporting splicing, causes defects in both splicing steps. Co-transcriptional recruitment of BBP and the snRNPs which follow in the assembly pathway is impaired, indicating that Prp45 affects spliceosome formation prior to NTC incorporation. Remarkably, alleles of chromatin regulators, such as gcn5, also hamper efficient co-transcriptional spliceosome recruitment.

Here, we show that *prp45*(1-169) genetically interacts with effectors of chromatin modifications, including factors which affect H2B ubiquitylation and H3 acetylation and methylation. We observed that *prp45*(1-169) partially suppresses the telomeric silencing defect of *set1*Δ cells monitored in a strain with *URA3* reporter positioned within a telomeric region. Truncating Prp45 thus impairs reporter gene expression positioned in a derepressed heterochromatin. In euchromatin, we observed that *prp45*(1-169) causes delays in the induction of intronless genes such as GAL or PHO. Combining *prp45*(1-169) with null alleles of several chromatin modifiers revealed a synthetic defect in PHO induction after phosphate starvation. We also found synthetic interaction between *prp45*(1-169) and *gcn5*Δ on the level of pre-mRNA accumulation of intron containing genes. Our results suggest that Prp45 and Gcn5 both contribute by non-overlapping pathways to the regulation of chromatin accessibility and pre-mRNA processing.

127 A conserved function for alternative splicing-induced translation into the 3'UTR

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The 3' untranslated regions (UTR) of mRNAs are involved in controlling mRNA stability and, through binding of miRNAs, translation. However, some 3'UTRs are longer than their coding regions, suggesting that they fulfill other, as of yet unknown, functions. We have recently uncovered a circadian splicing switch leading to exclusion of exons 6 and 7 from the mouse U2AF26 mRNA. Exclusion of these exons induces a frame shift that allows translation past the canonical stop codon in exon 8 into the sequence supposedly representing the 3'UTR. In our present work, we show that this mechanism, a splicing-induced frame shift and translation into the 3'UTR, can be used in hundreds of other pre-mRNAs and that it is conserved across species.

We have first used U2AF26 as a model system and addressed the question whether an alternative, prolonged reading frame in its 3'UTR is present in other species. We find that this indeed is the case for every species harboring a U2AF26 gene. Although the resulting new C-termini show little sequence homology, they show functional conservation, as they act strongly destabilizing in all cases tested. Furthermore, destabilization of U2AF26 of different species leads to destabilization of interacting Period1. This suggests a function of U2AF26 alternative splicing in controlling the molecular clockwork throughout mammalian species.

We have then addressed the question whether skipping of the penultimate exon leading to a frame shift and translation into the 3'UTR is also found in other genes. Using bioinformatics, we identify numerous mouse and human genes in which an extended reading frame in the 3'UTR can be accessed through alternative splicing. We find many of these splicing switches to be regulated in a tissue specific manner, suggesting functional relevance. First analyses indicate that, as observed for U2AF26, the alternative C-termini lead to reduced protein levels, which may indicate a common regulatory principle.

Together, our data suggest that frame shift-inducing alternative splicing leading to translation into the 3'UTR is a prevalent gene regulatory mechanism that is conserved across mammalian species.

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128 Linking the C-Terminal Domain Code of RNA Polymerase II to Modulating Chromatin States in *Schizosaccharomyces pombe*

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Regulation of gene expression is essential for all living organisms. One critical step in modulating gene expression is altering the ability of the transcriptional enzyme, RNA Polymerase II (RNAPII), to access DNA packaged in chromatin. The carboxy-terminal domain (CTD) of RNAPII, is believed to play a critical role in chromatin remodeling through its recruitment of factors that modify histones. Conserved throughout evolution, the RNAPII CTD contains a repeated $Y_1S_2P_3T_4S_5P_6S_7$ heptapeptide sequence that undergoes dynamic posttranslational modifications. The capacity of each serine in the sequence to undergo phosphorylation and dephosphorylation creates a readable 'code' for recruiting factors that can influence when processing events such as chromatin remodeling occur. To characterize how specific phosphorylation marks in the CTD affect gene expression, mutants of fission yeast *Schizosaccharomyces pombe* were rendered defective for phosphorylation by substituting a nonphosphorylatable alanine in place of each serine 2 (S2A), serine 7 (S7A), or both serine 2 and serine 7 (S2A/S7A). In addition, a fourth mutant was created in which each serine 7 was substituted for the phosphomimetic glutamic acid (S7E). We have performed microarray experiments with these mutants to study the genome-wide effects of eliminating and altering these phosphorylation events. Interestingly, analyses of our microarray data reveals an upregulation of positionally related clusters of genes, specifically at both ends of chromosomes 1 and 2, but not chromosome 3. Further qPCR analyses of genes in these subtelomeric regions confirm a significant upregulation of gene expression in these regions spanning approximately 50-100kb.

To investigate our hypothesis that chromatin modifications may be affected by our RNAPII CTD mutants, we examined the levels of the repressive chromatin mark H3K9 methylation by ChIP-Seq analyses. Our data show that the levels of H3K9 methylation are generally reduced near the ends of chromosome 1 and 2, but not chromosome 3 in the RNAPII CTD mutants, indicating that the increased gene expression that we observe and reduced repressive chromatin states in these subtelomeric regions overlap. This suggests a role for the dynamic phosphorylation and dephosphorylation of serines within the CTD code in modulating chromatin for large subtelomeric regions of *S. pombe*.

129 Splicing inhibition decreases phosphorylation level of Pol II CTD Ser2

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Phosphorylation of C-terminal domain of the largest subunit of RNA polymerase II (Pol II), especially Ser2 and Ser5 residues, plays important roles on transcription and mRNA processing, which includes 5' end capping, splicing and 3' end processing. It is well established that these phosphorylations stimulate mRNA processing events, however, it has remained unanswered whether splicing activity affects the phosphorylation status of Pol II or not. In this study, we found that splicing inhibition by potent splicing inhibitors, spliceostatin A (SSA) and pladienolide B (Pla-B), or by antisense oligos against snRNAs decreased phospho-Ser2 level but not phospho-Ser5 level. Interestingly, SSA treatment caused early dissociation of Pol II from DNA, which is dephosphorylated for the next round of transcription. In addition to the early dissociation, decrease in phospho-Ser2 level of chromatin-bound Pol II, which is engaged in on going transcription, was observed. These results indicate that splicing inhibition causes down-regulation of phospho-Ser2 through two mechanisms: early dissociation of Pol II and decrease in phospho-Ser2 of chromatin-bound Pol II during transcription.

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130 Slow vs. inefficient splicing of U12-type introns

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U12-type introns are a rare class of nuclear introns and it is thought that they are removed more slowly by a dedicated U12-dependent spliceosome. Earlier studies have proposed that U12-type introns regulate the expression of their target genes because of their rate of splicing is slower than the U2-type introns. The key supporting argument for this model is that RT-PCR analyses have consistently shown approximately 2-fold higher intron retention levels for U12-type introns compared to the U2-type introns. However, the mechanism of slow splicing has remained elusive. Here our data suggests an alternative hypothesis, namely that instead of being slower the splicing of U12-type introns may rather be less efficient than U2-type introns. In experiments where we block transcription elongation with DRB we observe very slow decay of mRNAs containing unspliced U12-type introns. In contrast, the splicing of U2-type introns in the same genes showed significantly faster decay kinetics. The decay was further exacerbated after a knockdown of RRP41, a core component of the exosome. Thus our data is consistent with an alternative hypothesis where a subpopulation of U12-type introns fail splicing, even after extended time periods. Our RNAseq data indicate that these introns have been recognized by the U11/U12 di-snRNP, suggesting that splicing defect reflects a failure in later stages of the spliceosome assembly. To ask if transcription or transcription rate is linked to inefficient splicing, we investigate the interconnections between RNA polymerase regulation by CTD kinases and the activity of U12-type spliceosome.

131 Sec16 alternative splicing dynamically controls COPII transport efficiency

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Signal-induced alternative splicing is one of the major mechanisms to control gene expression upon changes in cellular conditions. In response to T cell activation hundreds of exons were shown to be alternatively spliced, however, how these splicing switches impact on cellular functionality is largely unknown. T cell activation is a complex and dynamic process leading, amongst others, to dramatic changes in the production and secretion of effector molecules. Therefore, an increase of the protein secretion capacity is required, but the mechanism for this adaptation remains elusive. Here we show that activated T cells increase their secretory capacity by increasing the number of COPII-coated vesicles and that this adaptation is controlled by alternative splicing.

Protein secretion begins at ER-exit sites, where secretory proteins are packed in COPII-coated vesicles. We show here that T cells increase the efficiency of ER export upon activation and find an increase of COPII-coated vesicles as the underlying mechanism. Sec16 is a protein involved in COPII vesicle formation; it contains two exons (29 and 30) in its C-terminal domain that are alternatively spliced upon T cell activation. Using splice site blocking Morpholinos we show that increased inclusion of exon 29 upon activation is required to increase the number of COPII-coated vesicles and ER export efficiency. Interfering with exon 29 inclusion completely blocks the increase of COPII vesicles upon activation, suggesting that Sec16 alternative splicing is strictly required for this adaptation. To provide a mechanistic basis for this observation, we investigated the interaction of Sec16 splice variants with COPII components. Complex formation differs between Sec16 isoforms, suggesting the C-terminal Sec16 domain to be a splicing-controlled protein-interaction platform that controls COPII vesicle formation. To confirm the crucial role of Sec16 exon 29 in regulating COPII transport we have used CRISPR/Cas9 to generate cell lines lacking this exon. In line with our model, these cells show a strongly reduced number of COPII-coated vesicles and decreased ER export efficiency.

Together, our work connects the dynamic adaptation of protein export efficiency with alternative splicing, providing a compelling new example for a functionally important splicing switch.

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132 Poly(ADP-ribose) regulates microRNA activity of AGO2

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Poly(ADP-ribose) (PAR) is a polynucleotide molecule consisting of 2-200 ADP-ribose subunits as well as a post-translational modification. PAR is covalently attached to its target protein via a family of poly(ADP-ribose) polymerases (PARPs) and modifies its activity/function.

Our laboratory found that overexpression of PARP-13 reduces microRNA (miRNA)-mediated repression, correlating with an increase in PARylation of the core miRNA binding Argonaute (AGO) proteins. Although PARP-13 has PARP domain that is conserved in all PARP family members, the catalytic activity of ADP-ribosylation is inactive. How PARP-13 modulates AGO2 PARylation remains unclear. Besides the PARP domain, PARP-13 has 5 zinc-finger domains and 1 WWE domain responsible for RNA-binding and PAR-binding, respectively. We found that the RNA-binding of PARP-13 is critical for the reduction of miRNA-mediated repression and one of PARPs could modify AGO2. Under stress condition (heat shock, viral infection, etc.), stalled translation complexes aggregate to form cytoplasmic RNA-rich structures called stress granules. These cytoplasmic structures are enriched with PARylated proteins, five PARPs and AGO proteins.

In this presentation, we will discuss the possible mechanisms on how this inactive PARP PARylates AGO2. Our research potentially yields new insights into how PARP-13 and PAR regulates post-transcriptional mechanism as a general stress response.

133 Withdrawn

134 The reaction mechanisms of human Argonaute 2 characterized via single-molecule measurements

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Argonaute is a key enzyme of various RNA silencing pathways. We use single-molecule fluorescence measurements to characterize the reaction mechanisms of the core RISC (RNA Induced Silencing Complex) composed of human Argonaute 2 and a small RNA, and reveal the followings. Target binding of RISC starts at the seed region, resulting in four distinct reaction pathways: target cleavage, transient binding, stable binding, and Argonaute dissociation. The target cleavage requires extensive sequence complementarity and dramatically accelerates RISC recycling. The stable binding of RISC is efficiently established with the seed-match only, providing a potential explanation for the seed-match rule of miRNA (microRNA) target selection. Target cleavage on perfect match targets sensitively depend on RNA sequences, providing an insight into designing more efficient siRNAs (small interfering RNAs).

135 Loquacious-PD modifies the termini-dependence of Dicer-2 to facilitate endo-siRNA production.

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In *Drosophila melanogaster*, Dicer-2 produces siRNAs from a diverse set of long dsRNA precursors. In vitro, Dicer-2 senses dsRNA termini, such as blunt or 3' overhanging, to govern its reaction mode in an ATP- and helicase-dependent manner. Endogenous siRNA (endo-siRNA) precursors are predicted to have highly variable termini, including loops and frayed ends, which are normally thought to preclude cleavage. The dsRNA-binding protein (dsRBP), Loquacious-PD (Loqs-PD), interacts with Dicer-2 and is required for the biogenesis of a subset of endo-siRNAs in vivo, yet its exact function is unknown. We hypothesized that Loqs-PD might influence the termini discrimination of Dicer-2 to facilitate endo-siRNA biogenesis from dsRNA substrates with sub-optimal termini. Using single-turnover cleavage assays we observed that Loqs-PD minimizes the termini dependence of Dicer-2, allowing blunt and 3'overhanging dsRNA to be cleaved with similar efficiency in the presence of ATP. We next tested whether Loqs-PD could enable Dicer-2 to cleave a dsRNA with 2'3'-cyclic phosphate blocked termini, which precludes Dicer-2 binding and cleavage. In the presence of Loqs-PD and ATP, Dicer-2 was able to cleave the cyclic phosphate blocked dsRNA. We tested two additional dsRNA substrates that better mimic endo-siRNA precursors and only observed cleavage by Dicer-2 in the presence of Loqs-PD and ATP.

To understand how Loqs-PD enables Dicer-2 to become termini independent, we have begun biochemical characterization of Loqs-PD and its interaction with Dicer-2. First, using purified recombinant Loqs-PD we performed gel filtration and sedimentation equilibrium to determine that Loqs-PD is a stable homodimer. Next, using gel-shift assays we confirmed that Loqs-PD is a bona fide dsRBP that binds dsRNA with high affinity and independently of termini. Preliminary pull-down assays using purified Loqs-PD and Dicer-2 suggest that the two proteins directly interact. Ongoing experiments are aimed at determining the stoichiometry of the complex. We propose a model in which Loqs-PD enables Dicer-2 to initiate cleavage on endo-siRNA precursors with termini that otherwise would prevent Dicer-2 from binding and cleaving. This work provides insights into the role of accessory dsRBPs in Dicer-mediated small RNA biogenesis.

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136 Structure-based Insights into Alignments and Perturbations at Bulge Sites Within *T. thermophilus* Ago Silencing Complexes

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We have undertaken a systematic structural study of *Thermus thermophilus* Argonaute (*Tt*Ago) ternary complexes containing single-base bulges either within the seed segment of the guide or target strands. Our studies establish that single- base bulges at 7T8, 4A5 and 5A6 steps within the seed segment on the guide strand are stacked-into the duplex, with conformational changes localized to the bulge site, thereby having minimal impact on the cleavage site. By contrast, single-base bulges at 6'U7' and 6'A7' steps within the seed segment on the target strand are looped-out of the duplex, with the resulting conformational transitions positioning the 11'-12' rather than the 10'-11' phosphate opposite the catalytic acidic tetrad at the cleavage site. We observe a stable alignment for the looped-out 6'N7' bulge base on the target strand, which stacks on the sheared first base of the guide strand, with the looped-out alignment facilitated by weakened Watson-Crick and reversed non-canonical flanking pairs.

137 Fitness advantages conferred by RNA *cis*-regulators of ribosomal protein synthesis in bacteria

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In most bacteria, ribosomal proteins autogenously repress their own expression by interacting with RNA structures typically located in the 5'UTRs of their mRNA transcripts. This regulation is necessary to maintain a balance between ribosomal proteins and ribosomal RNA to ensure proper ribosome synthesis and assembly. Many such RNA structures have been identified and extensively characterized in *Escherichia coli*, four of which are highly conserved across the bacterial world. Moreover, structure-based homology searches and phylogenetic analyses have recently uncovered several distinct, independently derived RNA structures that perform analogous functions in different bacterial phyla. Despite the increasing amount of data on the functional and structural natures of these regulatory interactions, our understanding of the selective pressures that give rise to, and allow the maintenance of, these regulatory elements is nearly non-existent. While studies have demonstrated that coordinated ribosomal protein production and accurate ribosome composition correlate with robust bacterial growth, how these RNA *cis*-regulators of ribosomal protein synthesis directly contribute to overall bacterial fitness remains unknown. To measure the fitness advantages these *cis*-regulatory RNAs confer on an organism, we introduced point mutations into several RNA regulatory sequences in the *Bacillus subtilis* genome that disrupt ribosomal protein binding and consequent regulation. Our studies indicate that removing this regulation results in cold temperature sensitivity and growth defects. This suggests that the fitness advantages conferred by these RNA regulatory elements may lie within an organism's ability to transition between growth stages or adapt to environmental changes. Such work emphasizes the importance of the stoichiometric production of ribosomal components for proper ribosome composition, assembly, and overall organismal viability. While the bacterial ribosome itself is a common antibiotic target, this study also demonstrates the potential for identifying novel antimicrobials that target ribosomal protein production and ribosome assembly.

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138 Global-scale mapping of RNA-RNA interactions *in vivo* by 'LIGR-Seq'

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Transcriptome profiling technologies have revealed tens of thousands of non-coding (nc)RNAs that lack known functions. Moreover, among the small fraction of ncRNAs that have known functions, the full scope of their biological interactions and associated activities are not well understood. These challenges have been addressed in part by the development of methods enabling the case-by-case detection of interactions between individual ncRNAs and proteins, chromatin and/or other RNAs. However, what has been lacking is a method that allows the systematic, unbiased mapping of RNA-RNA interactions in cells. Such a method would have the potential to uncover novel trans- (and cis-) RNA-RNA interactions that provide 'guilt-by-association' insight into function of ncRNAs, analogous to methods that have yielded global-scale information on gene function and regulation by mapping protein-protein, protein-DNA and protein-RNA interactions.

To address this goal, we have developed a method for detecting RNA-RNA interactions *in vivo*: LIGation of interacting RNA followed by high-throughput Sequencing (LIGR-Seq). This method involves chemical crosslinking of RNA-RNA duplexes *in vivo*, ligation of free ends adjacent to cross-linked RNA duplexes, followed by high-throughput sequencing and subsequent computational detection and characterization of chimeric sequences formed from the ligated products. Supporting the efficacy of LIGR-Seq, the most highly enriched chimeras comprise sequences from well-established interactions involving U2+U6 and U4+U6 snRNAs, snRNA+scaRNA, and RNaseP+tRNA. Several thousand additional inter- and intra-molecular interactions were also detected, including surprising and informative interactions involving minor and major snRNAs, 7SL RNA, and previously uncharacterized ncRNAs. LIGR-Seq thus represents a powerful method for uncovering new and unexpected RNA-RNA interactions that serve as a basis for furthering our understanding of the extensive yet underexplored functional roles of RNA-RNA interactions in cells.

139 Participation of androgens and microRNAs in fetal lung development during a period important for the respiratory distress syndrome (RDS) in neonates

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Introduction: During the neonatal period, respiratory distress syndrome (RDS) occurs more frequently in male premature babies than in the female**.** The major cause of RDS is a delay in the surge of surfactant synthesis by the Type II cells (PTII). The etiology is the presence of androgens in fetal lung. Indeed, androgens in the fetal lung delay lung maturation and the surge in surfactant synthesis in PTII cells. How do androgens regulate fetal lung maturation, is unknown. Although, there is no evidence that microRNAs play a role in this mechanism, it has been shown that the expression profile of microRNA changes in androgen-dependent tissues namely the prostate. To understand how androgens participate into the prevalence of RDS in premature babies, my project aims to highlight the potential relationship between microRNAs and androgens during fetal lung maturation.

Hypothesis: During fetal lung maturation and especially during the period that overlaps the surge in surfactant synthesis, there is a regulatory mechanism involving microRNAs and androgens.

Objective: To study the mechanism by which microRNAs and androgens regulate fetal lung maturation. As first step, we have determined the expression profile of microRNAs and how they are modulated by the presence of androgens during the period that overlaps the surge in surfactant synthesis.

Methodology: We have studied the expression profile of microRNAs in male fetal lung at gestational days 17 and 18 (GD17, GD18) in the Balb/C mouse. Pregnant female were treated between GD 10 and GD 16 or 17 with the pure anti-androgen flutamide and expression profile of microRNAs were analyzed in lung pups by microarray, expression of some microRNAs was validated by q-PCR.

Results: The expression profile of microRNAs in fetal lung during the surge in surfactant synthesis is modulated by androgens. Some microRNAs have putative targets known as important regulators of lung development.

Discussion: Some microRNAs were identified as putative candidates of androgen action.

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140 MicroRNA (miRNA) deregulation upon ER stress is cell-type specific and confers robustness to the Unfolded Protein Response (UPR)

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MicroRNAs (miRNAs) are small non-coding RNAs that negatively regulate the expression of target mRNAs and affect a myriad of biological processes, including the activation of cellular responses to different types of stress. There is increasing evidence suggesting that miRNAs may play protective roles during the unfolded protein response (UPR)[1]. As the protein folding capacity of the Endoplasmic reticulum (ER) differs from one cell type to another and many miRNAs are cell-type specific, it is likely that miRNAs may contribute to the UPR in a cell-type specific manner.

Here, we have assessed the cell-type specificity of the UPR performing microarray-based profiling of miRNAs upon thapsigargin-induced ER stress in three human cell lines of different origin: RPE-1 (epithelial), HepG2 (hepatic) and HEK-293T (embryonic kidney). This analysis unveiled a highly cell-type specific deregulation of a significant number of miRNAs, with only 6 miRNAs commonly deregulated across all 3 cell lines. Further assessment of their respective primary miRNA transcripts, revealed that most of the ER-stress-induced miRNAs are activated at the transcriptional level.

Interestingly, these cell-type specific ER-stress-mis-regulated miRNAs are predicted to target overlapping sets of genes and pathways -many of them involved in cell cycle and cell growth- that may act to confer robustness to the UPR response. Subsequent functional studies inducing either overexpression or depletion of cell-type specific ER-stress-induced miRNAs, revealed that these miRNAs, not only can affect basal viability and cell growth across the different cell types, but can also modulate the susceptibility to ER stress. Depletion of cell-type specific miRNAs increased ER stress susceptibility, as measured by an increase in cell death. By contrast their overexpression, results in a reduction of ER stress susceptibility, which seems to arise from the induction of cell cycle arrest at G0/G1.

Altogether, these results demonstrate that the miRNA response linked to ER stress is mainly cell-type specific, and that these cell-type specific miRNAs converge downstream, regulating common targets and pathways within the UPR, which may exert a protective effect.

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141 RNA targets mediate nuclear retention of mature miRNAs

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miRNAs in association with the RNA induced silencing complex (miRISC) typically silence target mRNAs in the cytoplasm of higher eukaryotes. However, recent reports suggest that many mature miRNAs, miRISC factors and miRISC guided, site-specific RNA cleavage activity are also present in the nucleus. Yet, the mechanism by which these mature miRNAs localize to the nucleus is still unclear. Here, we report the nucleo-cytoplasmic transport kinetics and nuclear targets of mature miRNAs at single-molecule resolution. Specifically, the extent of miRNA nuclear retention is directly proportional to the cellular abundance of its predicted mRNA targets and antimiR mediated inhibition of target binding reduced nuclear localization. Live cell imaging suggests that miRNA mobility is much higher in the nucleus than in the cytoplasm. Moreover, nuclear localization of miRNAs can be inhibited by the addition of any double stranded RNA, strongly suggesting that nuclear import and retention is mediated by a saturable RNA binding factor. Put together, our data extends the role of miRNAs to mammalian cell nucleus and suggests that miRNAs interact with RNA targets via different modes in the nucleus and cytoplasm.

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142 A functional tRNA-derived RNA fragment fine-tunes gene expression in *Escherichia coli*

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During evolution, bacteria developed mechanisms that allow them to adapt to different environmental stresses (e.g. temperature, nutrient starvation, oxidative stress). Among those mechanisms, small regulatory RNAs (sRNA) are widely used by bacteria to efficiently regulate their metabolism in stressful conditions. To this day, more than 100 sRNAs have been identified in *Escherichia coli* but their mechanisms of action and their targets are not well known. For this reason, we were interested in the identification of diverse sRNA targetomes to better understand the complexity of those RNA molecules. To identify new interaction partners of sRNA, we used an in vivo capture technology which consists of an MS2-tagged RNA affinity purification followed by RNA sequencing (MAPS). To validate this approach, we applied MAPS to a well characterized sRNA, RyhB, which modulates iron homeostasis. Using this technique, we were able to co-purify known targets of RyhB, as well as identifying new ones.

Moreover, this technique allowed us to identify a new class of regulatory RNA in bacteria: functional RNA fragments derived from pre-tRNA (tRF). Here, we demonstrate that one tRF acts as a sRNA sponge. Indeed, the 3' external transcribed spacer of the pre-tRNA *glyW-cysT-leuZ* (3'ETS^{leuZ}) is able to pair with RyhB to sequester transcriptional noise in repressive conditions. This sRNA sponge system allows bacteria to fine-tune sRNA-dependent regulation of gene expression. We also demonstrate the physiological importance of the 3'ETS^{leuZ} as it actively contributes to maintaining bacterial fitness in presence of an antibiotic.

New evidence suggests that the 3'ETS^{leuZ} is not the sole representative of functional tRF. MAPS results indicate that the internal transcribed spacers (ITS) of pre-tRNA *metZ-metW-metV* could also interact with a sRNA, RybB, which is implicated in envelope stress response. Such data reinforce the idea that functional tRF may play important roles in fine-tuning gene expression in bacteria.

143 Novel functions for Sm-class RNAs in the regulation of gene expression

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Sm-class RNAs constitute a group of non coding RNAs (ncRNAs) with sizes ranging from ~60 to ~190 nucleotides (nt). The most studied members of this group of ncRNAs have essential roles in fundamental cellular processes such as pre-mRNA splicing and 3'-end processing. However, novel splicing-independent functions have been recently described for the U1 small nuclear RNA (snRNA), suggesting that this class of ncRNAs is more versatile in their molecular functions than previously thought.

Herpesvirus saimiri (HVS) is a member of the oncogenic gamma-Herpesvirus family that infects T cells in New World primates. HVS encodes seven Sm-class RNAs called HSURs that are expressed in latently infected cells. The molecular function of these spliceosomal-like viral snRNAs remained obscure for decades until recently, when we described for the first time an unexpected role for HSUR 1 in the degradation of a host-encoded miRNA, miR-27. In addition, HSURs 1 and 2 mimic the 3' untranslated region (3'UTR) of mRNAs by binding to host miRNAs (miR-16 and miR-142-3p) and also to AU-rich element (ARE)-binding proteins. However, expression of HSURs 1 and 2 does not impair the activities of the targeted miRNAs and ARE binding proteins. We hypothesized that HSURs 1 and 2 might recruit host miRNAs and ARE binding proteins to repress the expression of specific mRNAs in latently infected cells. In agreement with our model, we found that HSURs 1 and 2 can be crosslinked in a psoralen-dependent manner to mRNAs, indicating RNA-RNA base-pairing between HSURs and mRNAs. We also found that HSURs 1 and 2 associate with actively translated mRNAs. Using an unbiased method developed in our laboratory to identify RNA-RNA interactions, we identified specific mRNAs associated *in vivo* with HSURs 1 and 2. Transient knockdown of HSURs 1 and 2 resulted in upregulation of specific target mRNA levels, whereas transient overexpression of HSURs 1 and 2 resulted in downregulation of such mRNA levels. Taken together, our results assign novel functions to Sm-class snRNAs and unravel a fundamentally new mechanism for post-transcriptional regulation of gene expression.

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144 Elucidating the molecular basis for mechanism of a TBOX regulatory RNA switch.

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A riboswitch is a cis-regulatory RNA segment found in the untranslated region of an mRNA. Riboswitches can modulate their own transcription or translation based upon binding of a specific effector and can regulate gene expression based on changing cellular environment. These RNA switches play a central role in cellular adaptation and are found in all kingdoms of life. A TBOX is a special class of bacterial riboswitch that binds to a tRNA as its effector and can regulate its own transcription or translation based on the aminoacylated state of the bound tRNA. The bacterial TBOX serves as a model system to study the molecular basis for RNA-RNA interactions that are important for riboswitch mechanisms. TBOX riboswitches are found exclusively in gram positive bacteria which also include pathogens like the Staphylococcus aureus. This makes them an attractive drug target for development of new classes of antibiotics. The main focus of this study is to elucidate the molecular basis for the mechanism of a specific TBOX riboswitch that can recognize and bind to a type 2 tRNA molecule.

145 GAL lncRNAs Target Genes via RNA-DNA Hybrids and Facilitate Gene Looping to Enable Faster Gene Expression and Cellular Adaptation

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Long non-coding RNAs (lncRNAs) are a newly recognized class of molecules that regulate the expression of protein-coding genes through multiple mechanisms. Previous work from our lab uncovered a role for the *GAL* lncRNAs in promoting transcriptional induction of the galactose metabolism (GAL) genes in the budding yeast *S. cerevisiae*. Furthermore, we found that yeast strains lacking the RNA helicase DBP2, exhibited enhanced, lncRNA-dependent induction, suggesting that Dbp2 attenuates the function of the *GAL* lncRNAs. However, the precise mechanism by which the *GAL* lncRNAs facilitate rapid transcriptional induction was unknown. We now report that *dbp2*Δ strains accumulate lncRNA-DNA hybrids, termed R-loops, across the *GAL* cluster *in vivo* and that these hybrids are required rapid transcriptional induction. Moreover, we find that the *GAL* lncRNAs promote gene looping at the *GAL10* locus within the *GAL* cluster and that the *GAL* lncRNAs function in *trans*. Finally, we find that expression of the *GAL* lncRNAs promotes faster growth of cells in culture following a nutritional shift from glucose to galactose as a carbon source. We propose that the *GAL* lncRNAs are targeted to the *GAL* genes by direct base-pairing, via lncRNA-DNA hybrid formation, to promote gene looping and faster environmental adaptation.

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146 Testing the Competing Endogenous RNA or microRNA Sponge Hypothesis Using intracellular Single-Molecule, High-Resolution Localization and Counting (iSHiRLoC)

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microRNAs (miRNAs) are short non-coding RNA molecules that, when bound by the RNA-induced silencing complex (RISC), will down-regulate the expression of genes that bear partially complementary sequences. A single miRNA can regulate up to 200 different protein-coding messenger RNAs (mRNAs), which creates competition among the targets for the miRNA-guided RISC machinery. Consequently, changes in the intracellular concentrations and locations of either target mRNAs or targeting miRNAs can alter the expression profiles of other genes. Evidence has accumulated that the cell exploits this effect by expressing competing endogenous RNA (ceRNA) decoys, such as pseudogenes and circular RNAs, to "sponge" miRNA-guided RISC away from specific targets, but the lack of a system with controlled concentrations and locations of the RNA species involved has led to contradictory results. To overcome this challenge, we use intracellular Single-Molecule, High-Resolution Localization and Counting (iSHiRLoC). Microinjection introduces known quantities of Cy5-labeled sponge (ciRS-7 RNA and non-coding Luc2) and/or coding genes (Luc2 RNA) together with singly Cy3-labeled miRNA (miR-7 and miR-21, respectively) into cultured cells at time zero. We specifically use cells that are devoid of endogenous miR-7 (HeLa) and miR-21 (mouse primary-mesangial miR-21 mutant cells), respectively. The spatiotemporal distributions, kinetics of interactions, and extent of target site occupancy are measured in time-lapse experiments employing intracellular single-molecule particle tracking in live cells and particle counting in fixed cells. From these parameters and through systems biology modeling we will determine if miRNA sponges are kinetically and/or thermodynamically preferred targets over coding constructs. The most recent results of this approach will be presented.

147 AFP anti-sense transcripts in mouse liver and their potential role in gene regulation

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We have been studying mouse liver gene regulation to better understand mechanisms by which changes in gene expression contribute to liver development, homeostasis and disease. We identified Zinc Fingers and Homeoboxes 2 (Zhx2) as contributing to down-regulation of alpha-fetoprotein (AFP), a plasma protein that is highly expressed in the fetal liver but shut off after birth. AFP expression is elevated in regenerating adult liver and hepatocellular carcinoma (HCC) and has been used extensively as a diagnostic marker of liver cancer. Interestingly, all gene targets of Zhx2 that we have identified to date, including H19 and Glypican 3, are also known to be misregulated in HCC. Thus, a better understanding of the mechanism by which these genes are regulated by Zhx2 will likely lead to new insights into gene regulation during HCC progression. Previous results from our lab have suggested that AFP mRNA repression by Zhx2 occurs at a post-transcriptional level; nuclear run-ons from mice that differ in Zhx2 expression show similar levels of transcription, even though accumulated mRNA differs by ~20-fold. In studying the mechanism of AFP regulation by Zhx2, we have identified novel unannotated antisense transcripts that partially overlap the 3' half of the AFP gene. We characterized the asAFP transcripts in mouse liver, measuring the extent of the antisense transcription, characterizing several alternative isoforms and using 5' and 3' RACE to identify the asAFP RNA ends. These antisense transcripts were also detected in mouse liver RNA-seq data. The abundance of this antisense AFP (asAFP) RNA inversely correlates with AFP mRNA levels in adult mouse liver. asAFP RNA levels are high and AFP mRNA levels are low in the adult liver in mouse strains containing a wild-type Zhx2 gene. BALB/cJ mice, which have dramatically reduced Zhx2 levels due to a natural hypomorphic mutation, exhibit elevated AFP mRNA levels and reduced asAFP RNA levels in the adult liver. My central hypothesis is that asAFP RNA contributes to the post transcriptional regulation of AFP mRNA mediated by Zhx2, possibly through an RNA-RNA interaction. We are designing experiments to test this hypothesis and to determine at what level the regulation may occur.

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148 Diverse Phenotypes and Specific Transcription Patterns in Twenty Mouse Lines with Ablated lincRNAs

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In a survey of 20 knockout mouse lines designed to examine the biological functions of large intergenic non-coding RNAs (lincRNAs), we have found a variety of phenotypes, ranging from perinatal lethality to defects associated with premature aging and morphological and functional abnormalities in the lungs, skeleton, and muscle. Each mutant allele carried a *lacZ* reporter whose expression profile highlighted a wide spectrum of spatiotemporal and tissue-specific transcription patterns in embryos and adults that informed our phenotypic analyses and will serve as a guide for future investigations of these genes. Our study shows that lincRNAs are a new class of encoded molecules that, like proteins, serve essential and important functional roles in embryonic development, physiology, and homeostasis of a broad array of tissues and organs in mammals.

149 Structural and mechanistic insights into the role of repeat-derived non-coding RNAs in epigenetic regulation of mammalian heterochromatin

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A significant fraction of mammalian genomes is composed of repetitive elements. Repetitive elements present a critical challenge to genome stability because these regions are prone to mutagenic recombination. To safeguard genome integrity, repetitive sequences are maintained in an epigenetically silent heterochromatic state. Paradoxically, however, repetitive genomic regions are actively transcribed into noncoding RNAs (ncRNAs). Furthermore, these repeat-derived transcripts are themselves important to establish and maintain the heterochromatic structure at repetitive regions. How these repeat-derived ncRNAs precisely function in epigenetic regulation of heterochromatin is poorly understood. We are investigating ncRNAs transcribed from mouse pericentric major satellite repeats as a paradigm to understand the mechanism by which ncRNAs regulate heterochromatin formation at repetitive genomic loci. The regulatory functions of ncRNAs are tightly linked with their ability to form complex structures. We are thus employing a structure-driven strategy to determine how folding of major satellite ncRNAs influences the local recruitment of chromatin complexes to pericentric loci. Here, we present the recent progress of our *in vitro* and *in vivo* analyses.

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150 Evolutionary conservation of a long non-coding RNA, *COOLAIR*, supports a regulatory function

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Long non-coding RNAs are rarely conserved at the DNA sequence level, lending support to the argument that they are of little functional importance. We explored the relationship between function and conservation further, using the long non-coding RNAs at an important flowering time gene in the reference plant, *Arabidopsis thaliana*, as our model.

Sense transcripts at *Flowering Locus C* (*FLC*) encode a MADS box transcription factor which quantitatively delays flowering. Long non-coding RNAs, collectively known as *COOLAIR*, are transcribed in the antisense direction. The alternate use of a proximal or distal antisense polyadenylation site is able to influence sense expression levels. PRP8, a core spliceosome sub-unit, promotes use of the proximal site via efficient splicing of proximal isoforms, ultimately contributing to *FLC* down-regulation and an earlier flowering phenotype. The switch to the proximal site is additionally promoted by other components of an autonomous floral promotion pathway. Co-transcriptional coupling of chromatin modifications, transcription and *COOLAIR* processing results in feedback loops that reinforce low or high *FLC* expression states.

FLC homologues are wide-spread in flowering plants. From DNA sequence analysis we found that the protein-coding transcript was highly conserved, whereas (non-overlapping) regions of *COOLAIR* were not. Rather than ruling out functional significance we explored other aspects of conservation. Chemical probing revealed that *COOLAIR* has a complex secondary structure, and covariant base-pair mutations predict strong conservation of this secondary structure across flowering plants. We confirmed that *COOLAIR* was transcribed *in vivo* in several of these species by RT-PCR. The architecture of *COOLAIR* transcripts were remarkably similar: we observed conservation of the proximal and distal variants, splicing isoforms and polyadenylation sites. Syntenic transcription provides further evidence for evolutionary selection, and we are now checking for conservation of function.

COOLAIR is an example of an evolutionarily conserved long non-coding RNA with low DNA sequence identity. This challenges canonical views on how we identify conserved RNAs. This work additionally opens up the exciting possibility of a post-transcriptional role for *COOLAIR* in sense regulation. Deciphering the secondary structures of long non-coding RNAs could help us to understand how they function.

151 Spatiotemporal dissection of miR-122-mediated protection of Hepatitis C Virus (HCV)

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MicroRNAs (miRNAs) are evolutionarily conserved, genomically encoded, small non-coding RNAs that regulate gene expression during normal cellular homeostasis. In the cytoplasm, mature miRNA duplexes are incorporated into Argonaute-protein containing RNA-induced silencing complexes (RISCs), wherein the guide strand is selected to bind mRNA targets with imperfect complementarity to inhibit translation and promote mRNA degradation. Some viruses have evolved mechanisms to hijack human miRNAs to support the viral life cycle. Hepatitis C virus (HCV) is an example of a positive-sense, single-stranded RNA virus that binds and is protected by liver-abundant miR-122. To examine HCV-miR-122 interactions in miR-122-free HepG2 human liver cells, we are using a method developed in our lab termed intracellular Single-molecule High-Resolution Localization and Counting (iSHiRLoC) to determine the localization, diffusion constant and assembly state of single HCV and miR-122 complexes inside living cells at 30 nm spatial and 100 ms temporal resolution. We have extended the capabilities of iSHiRLoC to simultaneously detect two colors to examine the assembly of co-microinjected Cy3-labeled HCV and Cy5-labeled miR-122. To measure the baseline assembly of Cy5-labeled miR-122 in HepG2 cells, we have performed iSHiRLoC to count miRNA particle photobleaching steps in fixed cells at 4 h post-injection. We determined that a majority of particles photobleach in one step (55 ± 16%), while a minority photobleach in two or more steps (9.1 ± 7.2%). Particles categorized as non-determinable as monomer or multimer make up the remaining 36 ± 19%. A majority of single step photobleaching events is expected, as it is unlikely that microinjected miR-122, a miRNA that is not normally expressed in this cell type, will engage endogenous targets and form multimeric miRNA-mRNA P-body aggregates. Live and fixed cell iSHiRLoC analyses are in progress to examine miRNA localization, diffusion constants and assembly in HepG2 cells co-microinjected with Cy5-labeled miR-122 and HCV. Together, these preliminary data provide proof-of-principle that iSHiRLoC can be used in HepG2 cells to examine miR-122 protection of HCV.

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152 Angiogenin Promotes Cell Proliferation by a Novel Signal Transduction Mechanism

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Canonical growth factors deliver epigenetic information to DNA indirectly via receptor-mediated signal transduction pathways. Here we report on the discovery of a direct pathway in which a growth factor is internalized, has its localization regulated by phosphorylation, and ultimately uses intrinsic catalytic activity to effect epigenetic change. Angiogenin (ANG), a secreted vertebrate ribonuclease, is known to promote cell proliferation, leading to neovascularization as well as neuroprotection in mammals. Upon entering cells, ANG encounters the cytosolic ribonuclease inhibitor protein, which binds with femtomolar affinity. We find that protein kinase C and cyclin-dependent kinase phosphorylate ANG, enabling ANG to evade its inhibitor and enter the nucleus. After migrating to the nucleolus, ANG cleaves pRNA, which prevents the recruitment of the nucleolar remodeling complex to the rDNA promoter. The ensuing derepression of rDNA transcription promotes cell proliferation. These findings reveal an unprecedented mechanism of signal transduction, and suggest new modalities for the treatment of cancers and neurological disorders.

153 Regulation of the Arachidonic Acid Pathway by miR-146a in Lung Cancer Cells

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Arachidonic acid can be metabolized through two different biochemical pathways. In one, prostaglandin production is mediated by cyclooxygenases (COX). The other pathway involves the production of leukotrienes through the activity of 5-lipoxygenase (5-LO) and its activating protein (FLAP). These pro-inflammatory molecules are frequently dysregulated in cancers. We show that increased expression of cyclooxygenase-2 (COX-2), 5-LO, and FLAP is characteristic of A549 cells, a lung cancer cell line. The mRNA 3' untranslated region (UTR) of these molecules contains numerous sites for potential post-transcriptional regulation, including putative binding sites for microRNAs (miRNAs). Interestingly, miR-146a is predicted to target COX-2 and FLAP, suggesting there is a unified network of control over both sides of the arachidonic acid pathway. There is significantly lower expression of miR-146a in lung cancer cells compared to normal lung cells. We demonstrate that miR-146a directly regulates COX-2 via its 3' UTR. Remarkably, our data also suggest that synthetic miR-146a can repress expression of both FLAP and 5-LO in lung cancer cells. Taken together, our work suggests a role for miR-146a in a novel regulatory mechanism of arachidonic acid metabolism.

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154 Short RNAs derived from multiple RNA classes are as abundant as miRNA in human cells infected with hepatitis C virus

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The spectrum of known non-coding RNAs has significantly expanded in the recent years with the discovery that various constitutively expressed RNA classes (including tRNA, rRNA and snoRNA) give rise to a broad repertoire of stable fragments. These fragments have been identified in all kingdoms of life and some of them were proven to have regulatory functions. It has been shown that in mammalian cells RNA fragments are involved in stress response and are capable of guiding the silencing of target gene expression. In view of these data, it seems plausible that such molecules can also modulate the course of viral infections. The current state of knowledge in this matter is very limited.

To gain insight into the role of RNA fragments in host-virus interaction, we focused on hepatitis C virus (HCV), a model human (+)RNA virus capable of inducing persistent infections. We characterized the non-coding RNA repertoire in human hepatoma Huh-7.5 cells, which are widely used as HCV cell culture system. Both non-infected and infected (72 hours post infection) cells were examined. To this end, RNA molecules ranging from 15 to approx. 80 nucleotides were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and next generation sequencing (NGS).

The data demonstrated that Huh-7.5 cells contained a broad spectrum of short RNAs derived from multiple RNA classes. In general, these RNA fragments were as abundant as miRNAs. They originated from clearly defined regions of parental molecules, which indicates that they arose through a specific cleavage. The fraction of high-copy number RNA in the examined pool did not change considerably upon viral infection. Non-coding RNAs that differentially accumulated in infected and non-infected cells occurred at lower levels. Most of these molecules were up-regulated during the infection. The analysis permitted the selection of candidate molecules derived from various RNA classes for further bioinformatic and biochemical studies of their functional potential.

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155 Determination of the secondary structure of lincRNA cyrano

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Long intervening noncoding RNAs (lincRNAs) are RNAs longer than 200 nucleotides (nt) that do not encode protein, but are known to regulate key biological processes such as transcription and chromosomal inactivation. While thousands of lincRNAs have been discovered, few have been structurally characterized due to their long lengths and lack of primary sequence conservation. However, it is likely that many lincRNAs will fold into conserved complex structures that are required for function. The 4.5kb lincRNA *cyrano*, discovered in zebrafish and responsible for nasal and eye development; contains a highly conserved 300nt region that is found in many mammalian orthologs, including human and mouse. Mutating bases in the conserved region of *cyrano* caused developmental defects in zebrafish embryos, indicating that it plays a significant role in embryogenesis. Using Selective 2'-Hydroxyl Acylation and Primer Extension (SHAPE) and comparative sequence analysis, we aim to determine the secondary structure of the 300nt conserved region of *cyrano.* We hypothesize that determining the secondary structure of this conserved segment will help us to identify interaction partners and further understand *cyrano*'s functional role in embryogenesis.

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156 A Dynamic Search Process Underlies MicroRNA Targeting: A Single-Molecule FRET Study

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Argonaute proteins play a central role in mediating post-transcriptional gene regulation by microRNAs (miRNAs). Argonautes use the nucleotide sequences in miRNAs as guides for identifying target messenger RNAs for repression. Here we used single-molecule FRET to directly visualize how human Argonaute-2 (Ago2) searches for and identifies target sites in RNAs complementary to its miRNA guide. Our results suggest that Ago2 initially scans for target sites with complementarity to nucleotides 2-4 of the miRNA. This initial transient interaction propagates into a stable association when target complementarity extends to nucleotides 2-8. This stepwise recognition process is coupled to lateral diffusion of Ago2 along the target RNA. The combined results reveal the mechanisms that Argonaute likely uses to efficiently identify miRNA target sites within the vast and dynamic agglomeration of RNA molecules in the living cell.

157 SWI/SNF chromatin-remodeling complexes function in noncoding RNA-dependent assembly of nuclear bodies.

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Paraspeckles are unique subnuclear structures that form around the specific long noncoding (lnc) RNA, nuclear paraspeckle assembly transcript 1 (NEAT1). Recently, paraspeckles were shown to be functional nuclear bodies involved in stress responses and the development of specific organs. Paraspeckle formation is initiated by transcription of the NEAT1 chromosomal locus and proceeds in conjunction with NEAT1 lncRNA biogenesis and a subsequent assembly step involving >40 paraspeckle proteins (PSPs). Paraspeckles are considered as huge among ribonucleoprotein complexes, however, the mechanism of paraspeckle construction remains partially elucidated. In this study, catalytic subunits of SWI/SNF chromatin-remodeling complexes are identified as paraspeckle components that interact with PSPs and NEAT1 lncRNA. Electron microscopic observations revealed that SWI/SNF complexes were enriched in paraspeckle subdomains depleted of chromatin. Knockdown of SWI/SNF components resulted in paraspeckle disintegration, indicating that SWI/SNF complexes are essential for paraspeckle formation. SWI/SNF complexes play canonical ATP-dependent nucleosome remodeling functions, however, mutation of the essential ATPase domain of the catalytic subunit BRG1 did not affect paraspeckle integrity, indicating that the essential role of SWI/SNF complexes in paraspeckle formation does not require their canonical nucleosome remodeling activity. We previouly discovered that 7 PSPs are essential for the processing and stabilization of structural core NEAT1 lncRNA. Knockdown of SWI/SNF complexes barely affected the levels of known essential paraspeckle components, but markedly diminished the interactions between the essential PSPs, suggesting that SWI/SNF complexes facilitate organization of the PSP interaction network required for intact paraspeckle assembly. The interactions between SWI/SNF components and essential PSPs were maintained in NEAT1-depleted cells, suggesting that SWI/SNF complexes not only facilitate the PSP interactions, but also recruit PSPs during paraspeckle assembly. Another type of RNA-dependent nuclear bodies, nuclear stress bodies (nSB), are formed around Satelite III (Sat III) lncRNA which transcribed from pericentric tamdem repeats of Sat III sequence under heat shock condition. Electron microscopic observations revealed that SWI/SNF complexes localize to nSB. Furthermore, we discovered that SWI/SNF complexes were also required for nSB formation without affecting Sat III lncRNA level. Our data suggest the existence of a common mechanism underlying the formation of lncRNA-dependent nuclear body architectures in mammalian cells.

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158 RNA oligonucleotide-based strategy for controlling microRNA biogenesis

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During the last decade several types of regulatory RNAs have been discovered. Their significant role in the control of many important biological processes has been well documented. In our studies we focus on human ribonuclease Dicer, the enzyme that excises functional regulatory RNAs from longer perfectly or partially double-stranded RNA precursors. Although Dicer substrates and products have already been quite well characterized, our knowledge about cellular factors regulating the activities of this enzyme is still limited. We hypothesized that RNA molecules can function not only as Dicer substrates but also as its regulators.

To verify the above presumption we used the SELEX method to identify RNA oligomers that bind human Dicer. We demonstrated that some of the selected oligomers affected Dicer's ability to process pre-microRNAs (pre-miRNAs) into mature miRNAs. Among these identified RNAs we also found oligomers which operated as specific inhibitors; they decreased formation of certain miRNA species, whereas hardly influenced the production of other miRNAs. More detailed studies revealed that the selected RNA oligomers can simultaneously bind both Dicer and miRNA precursors. This new class of bifunctional riboregulators interferes with miRNA maturation by interacting with specific RNA precursor and by sequestering Dicer. Interestingly, the performed bioinformatic analysis also suggests that sequences similar to those of the selected oligomers can be found both, within long non-coding RNAs and in the coding parts of the human genome.

The results of our studies suggest that the mutual interactions between the miRNA precursors and other RNAs can form a very complex regulatory network which controls miRNA biogenesis and afterwards gene expression.

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159 The Human 3'UTRome V1: A Modular Resource for Exploring mRNA Regulation

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3'Untranslated Regions (3'UTRs) have recently emerged as central regulators of cellular function. They contain important, but as yet poorly characterized, *cis*-regulatory elements targeted by a multitude of factors, such as microRNAs (miRNA) and RNA-binding proteins (RBPs). The vast majority of these targets are still unknown, limiting our understanding of the mechanisms by which post-transcriptional gene regulation contributes to diseases. Yet it is clear that each of these molecules can regulate a large number of gene networks. Informatics tools have proven insufficient at predicting both targets and outcomes of 3'UTR regulation.

Recently, visionary high-throughput (HT) cloning efforts such as the ORFeome Collaboration, coupled with the development of tools to exploit them, have demonstrated the utility of genomic libraries as HT resources to probe protein function and find biomarkers and targets in diseases. HT tools are equally needed to identify and map targets of post-transcriptional regulation. A library of functional human 3'UTRs would foster studies into 3'UTR gene regulation and characterize the roles of 3'UTRs in disease with a level of precision and scale unattainable with today's tools. Currently there is no publically available repertoire of human 3`UTRs.

To overcome this limitation we are presenting the first release of a publically available human 3'UTR clone library (h3'UTRome). The h3'UTRome is: 1) produced to a single high quality standard; 2) produced in the same recombinational cloning technology used for the human ORFeome, enabling universal operating methods and high throughput experimentation; 3) thoroughly sequenced and annotated, and simple online access to information; and 4) made universally available through our DNASU repository at low cost to all scientists with minimal restriction.

The first release of the h3'UTRome library comprises 1,569 3`UTRs cloned in Gateway vectors, ready for downstream analyses. It contains 3'UTRs for 1,245 transcription factors, 257 kinases, 211 RNA binding proteins and 134 other genes involved in disease.

In parallel with building the resource, we are developing several complement vectors that enable the analysis of mRNA localization, and the systematic detection and characterization of miRNAs and RNA binding protein target elements in 3'UTRs.

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160 Integrator mediates the biogenesis of enhancer RNAs

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Integrator is a multi-subunit protein complex stably associated with the C-terminal domain (CTD) of RNA polymerase II (RNAPII). This complex displays catalytic RNA endonuclease activity with close homology to cleavage and polyadenylation specificity factor (CPSF) 73 and 100 subunits. We have already shown that Integrator is required for the 3'-end processing of non-polyadenylated RNAPII-dependent uridylate-rich small nuclear RNA genes (UsnRNAs) and implicated the catalytic endonuclease activity of INTS11 in processing of UsnRNAs primary transcripts.

Enhancers are distal regulatory elements that mediate tissue- and temporal-specific gene expression during development. Recent evidence has revealed that active enhancers are transcribed and that such enhancer RNAs (eRNAs) play an important role in transcriptional activation. Here, we show that the Integrator protein complex is recruited to enhancers and super-enhancers in a stimulus-dependent manner. Functional depletion of Integrator subunits diminished the signal-dependent induction of eRNAs at enhancers and super-enhancers. Global nuclear run-on and RNAPII profiling demonstrated that Integrator regulates the maturation of eRNAs. We show that the production of mature eRNAs require the catalytic activity of Integrator complex. We propose a role for Integrator in biogenesis of eRNAs in metazoans.

161 Delta-like 4 mRNA is regulated by adjacent natural antisense transcripts

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Delta-like 4 (Dll4) is a Notch ligand, specifically expressed on artery endothelial cell surface. Adjusting DLL4 protein levels, whose mechanism is largely unknown, has a significant impact on artery development. We identified natural antisense transcripts in the Dll4 gene locus in murine endothelial cells, referred to as Dll4 Anti-Sense (Dll4-AS). Dll4-AS comprises of three isoforms that map proximal to the Dll4 promoter region. Expression patterns of Dll4-AS isoforms vary among different endothelial cell lines, but are always congruent with those of Dll4. We have identified a dual promoter element in the Dll4 locus that controls the expression of all the transcripts. Both Dll4-AS and Dll4 are sensitive to cellular density in that higher cellular density favors their expression. Exogenous Dll4 stimuli such as VEGF, FGF and Notch signaling inhibitor alter both DLL4-AS and DLL4 expression suggesting co-regulation of the transcripts. Also, knocking down of Dll4-AS results in down-regulation of Dll4 expression. As a consequence, endothelial cell proliferation and migration decreases in vitro, and sprout formation increases. The regulation of Dll4 by Dll4-AS is also conserved in vivo. This study suggests a novel form of non-coding RNA-mediated regulation at the Dll4 locus contributing to vascular developmental processes such as cell proliferation, migration and sprouting.

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162 Functional dissection of microRNAs by Large-scale STTM and Artificial miRNA in Solanum lycopersicum

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MicroRNAs (miRNAs) are proved to play a critical role in plant development and may have functional conservations among different species. Unlike Arabidopsis and rice, tomato (*Solanum lycopersicum*) is a unique model for the development of its fleshy fruits, compound leaves, and sympodial shoot. To investigate the functional conservation and diversification of miRNAs between tomato and other plant species, we applied technologies of short tandem target mimic (STTM) and artificial miRNAs (amiRNAs) to down-regulate and over-express specific miRNAs, respectively, for their functions in tomato. In total, 25 conserved and non-conserved miRNAs have been selected to construct their STTMs and amiRNAs and finally to generate their transgenic tomato plants. Northern blot and stem-loop qPCR were applied to determine miRNA level changes in transgenic plants. Our results showed that not all of the STTM transgenic plants displayed decreased miRNA levels but exhibited an up-regulation of their target genes, indicating that some STTMs may function as sequesters to block the functions of miRNAs. In contrast, amiRNA transgenic plants displayed significant increases in the expression levels of miRNAs and the corresponding decreases in the expressions of their target genes. Furthermore, some miRNAs, such as miR166, have conserved functions in tomato, showing smaller statures and bushy aerial parts. Interestingly, some evolutionally conserved miRNAs, such as miR169, acquired new functions in tomato fruit development, showing a higher ratio of length to width of the fruits in miR169 over-expressed tomato plants. Finally, a tomato specific miRNA, miR1917, was revealed, by STTM/amiRNAs, to have a function related to flower development. Our study established effective approaches and a valuable resource for functional study of miRNAs in tomato.

163 Differential effect of the members of miR-34 family on SiHa cell proliferation

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MicroRNAs (miRNAs) play pivotal roles in controlling cell proliferation, apoptosis and invasion. Aberrant miRNA expression is now recognized as a molecular mechanism for many human tumors including cervical cancer. Infection with high-risk human papillomavirus (i.e. HPV-16 and 18) has been causally associated with the onset of cervical cancer. Furthermore, expression of cellular miRNAs has been linked to cervical cancer independently or associated to HPV expression. HPV-16 E6 protein inactivates and destroys p53 leading to a p53-null phenotype. P53 induces the transcription of the members of miR-34 family formed by miR-34a-5p, miR-34a-3p, miR-34b-5p, miR-34b-3p, miR-34c-5p and miR-34c-3p. This family is transcriptionally regulated by p53 in response to cell damage and oncogenic stress. The ectopic expression of the miR34 family members recapitulates the biological effects of p53. In this work we analyze the function of miR-34 members on SiHa cell proliferation. Over-expression of miR-34a-5p and miR-34a-3p in cervical carcinoma cells causes 30% inhibition of SiHa cell proliferation. MiR-34b-5p mimic causes 30% inhibition of SiHa cell proliferation, however, miR-34b-3p mimic recorded no effect. MiR-34c-5p and miR-34c-3p reach 85% inhibition of SiHa cell proliferation. Our results show that the miR-34 family regulates cell proliferation at different extent and this knowledge could be used as therapeutic or diagnostic/prognostic tool in cervical cancer.

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164 Non-coding RNA BC200 (BCRYN1) interacts with quadruplex resolving RNA helicase DHX36 and stabilizes unwound quadruplex through its cytosine rich region.

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Nucleic acid sequences, both DNA and RNA, which are rich in guanine bases can adopt non-canonical four stranded secondary structures known as G4-quadruplexes. In recent years bioinformatic analysis of various genomes have shown an enrichment of potential G4 quadruplex forming regions in non-coding/promoter for many genes, implying a regulatory role. Furthermore experimental data is beginning to show key roles for quadruplexes in disease states like cancer and neurodegenerative diseases. The discovery of so many putative RNA quadruplexes raises the need for the elucidation of their biological roles. The protein RHAU is an ATP dependent RNA helicase that has demonstrated RNA quadruplex unwinding activity *in vitro* as well as a strong (sub nano-molar) affinity for parallel quadruplex. Through immunoprecipitation experiments we have identified multiple RNAs that bound to RHAU in cell lysates; in high abundance was the primate specific non-coding neural RNA BC200 (BCYRN1). BC200 has been putatively classed as a modulator of translation, interestingly its expression has been observed to be increased in both the brains of Alzheimer's disease patients and various carcinomas. The RNA comprises an ALU like element similar to that of the 7SL RNA of the signal recognition particle followed by an adenine rich stretch and then a cytosine rich stretch. Herein we confirm the RHAU-BC200 interaction in a number of human cell lines, show that BC200 does not form quadruplex structures itself and binds to RHAU independent of the RHAU's quadruplex binding motif via its adenosine rich region. Finally we show that BC200 does not inhibit RHAU's quadruplex unwinding activity but rather the cytosine rich tract can act as a binding partner to unwound quadruplex, thus stabilizing quadruplex forming nucleic acids in a double stranded state. This final result is suggestive that BC200 may exert its regulatory role against sequences rich in guanines with the aid of the protein RHAU.

165 *In vitro* selection of pre-miRNA loop mutant molecules that bind to the restrained naphthyridine dimer

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Small molecules that bind to specific RNA secondary structures would be valuable tools for modulating gene expression and studying RNA functions. Previously, we have reported a series of naphthyridine derivatives that can bind to single nucleotide bulges in DNA complexes. These DNA binding molecules have long linkers and the heterocycles of these could stack each others.

This kind of molecules can be appropriate for DNA (which has orderly-lined bases), but not appropriate for RNA (which has a complicated structure). So new design strategies for RNA binding molecules are needed.

In this work, we designed and synthesized RND (the restrained naphthyridine dimer) as a new RNA binding molecule. RND is characterized by as follows. First, RND is a non-planar molecule; two naphthyridine rings are connected with a two-atom linker whose length is not long enough to permit intramolecular stacking of two heterocycles. The non-planar conformation would prevent non-specific intercalation into sequences of RNA duplex. Second, RND would bind to RNA by an entropy-driven manner. Many water molecules restrained by the hydrophobic surface of RND would be released when two heterocycles in RND are stacked with nucleotide bases of RNA. To determine the optimal RNA sequences that can bind to RND, pre-miRNA was selected as a target RNA. MiRNA is known to act as either oncogenes or tumor suppressors and produced from pre-miRNA by Dicer-catalyzed processing. We hypothesized that Dicer-catalyzed processing of pre-miRNA could be interfered by molecules binding to the loop of pre-miRNA. *In vitro* selection of pre-miRNA loop mutant library using a Surface Plasmon Resonance (SPR) was first carried out. Then, we conducted sequence analysis by ion PGM and one important motif for RND binding was obtained. The obtained RNA aptamers had strong bindings to RND according to SPR analysis of aptamer bindings to RND. Finally, Dicer-catalyzed processing was carried out and it was indicated that RND could inhibit the processing of RNA aptamers containing the motif.

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166 ncRNA functional profiling by gene deletion in yeast

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Sequencing approaches and high-resolution tiling arrays have revealed that eukaryotic genomes are extensively transcribed generating RNAs with no protein coding capacity. How many of these non-coding RNAs (ncRNAs) are functional and what is their function? *Saccharomyces cerevisiae* is a key model organism for investigating gene function. Protein coding gene deletion strains have provided a valuable resource for studying cellular processes. However, ncRNAs are absent from deletion strain collections, making it difficult to study the contribution of these RNAs in biological processes. Therefore, we have constructed over 500 molecular barcoded RNA gene deletion strains in diploid *S. cerevisiae*, including the annotated snRNAs, snoRNAs, tRNAs and recently identified stable unannotated transcripts (SUTs) and cryptic unstable transcripts (CUTs). The verified RNA deletion hemizygotes have been sporulated to generate haploid deletion strains, and then haploids mated to generate homozygous diploid deletion strains. Overall we have produced approximately 2000 ncRNA deletion strains. Interestingly, the majority of ncRNAs deleted were non-essential under nutrient rich conditions indicating that many ncRNAs may only be required under specific conditions. We have initially used the diploid hemizygote ncRNA deletion strain collection for ncRNA fitness profiling to explore the role of individual ncRNAs under different conditions. We identified ncRNA genes that show haploinsufficiency phenotypes or haploproficiency phenotypes in 9 different growth conditions providing important functional information for many of the ncRNAs.

One of the essential ncRNAs, RUF20 (SUT527), overlaps the 3' UTR of the essential gene SEC4, a GTPase required for vesicle-mediated exocytic secretion and autophagy. RUF20 essentiality is derived from its overlap with the SEC4 3' UTR. The SEC4 3' UTR is required for localization of SEC4 mRNA. We found that SEC4 mRNA expression was decreased and SEC4 3' UTR formation was affected when RUF20 expression was suppressed. Fluorescent in situ hybridization (FISH) revealed that RUF20 displays a similar localization to SEC4 mRNA and there is a mislocalization of SEC4 mRNA when RUF20 RNA is not transcribed. We have therefore identified a molecular function for the ncRNA RUF20 and suggest that the physical interaction between RUF20 and the 3' UTR of SEC4 influences SEC4 3' end formation and mRNA localization.

167 Role of the Goldilocks sRNA in Bacterial Gene Regulation

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Small non-coding RNAs (sRNAs) are critical for proper development and gene regulation, with many of these sRNAs playing an essential role in the cellular stress response. While their roles have been extensively studied in other systems, less is known about sRNA regulation in bacteria. One sRNA found in *Escherichia coli (E. coli)* and the closely related *Salmonella typhimurium*, Goldilocks (C.0293), is only expressed when cells are grown on minimal media. However, the specific function of this sRNA remains unknown. Affinity studies show that heat unstable nucleoid protein (HU) interacts with Goldilocks, and we hypothesize that this binding could activate HU to repress the gal operon in times of limited nutrient availability. To further study its function in *E. coli, in vivo* homologous recombination via the Red/ET recombination system was used to delete the Goldilocks gene. Preliminary studies suggest that the deletion of Goldilocks was successful and that it is essential to cell survival under limited nutrient conditions. The next step will be to screen growth of this deletion strain under a wide range of conditions.

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168 Inducible and modular lncRNA expression vectors.

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Long noncoding RNAs (lncRNAs) are a broad and new class of transcripts that do not encode proteins and potentially function in regulation of diverse pathways. Characterization of lncRNA function is challenging for many reasons, including their length, lack of conservation, overlap with coding transcripts, and relatively low abundance. At present, no commercial vectors are specifically designed for lncRNA expression in mammalian cells, limiting certain biochemical and cellular studies.

The current practice of using Pol II-driven protein expression vectors, designed for extremely high levels of coding mRNA transcription, to express lncRNAs may cause unwanted artifacts in expression and results analysis. To facilitate lncRNA investigation, we are developing lncRNA expression vectors that utilize tetracycline-inducible Pol III promoters and termination signals. Inducible and tunable expression is critical for controlling lncRNA expression levels, which are typically low. lncRNAs can be fused to boxB hairpins for biochemical isolation and fluorescent RNA aptamers for live-cell imaging. These vectors can also be linearized for in vitro transcription of the cloned lncRNA. Expression construct elements are designed to be easily removed or inserted using a custom multi-cloning site for modular and flexible cloning. Our intention is to create all-in-one cloning vectors to simplify and enable biochemical and cellular lncRNA research.

169 Tissue-specific induction of circular RNA during human fetal development revealed by statistically based splicing detection

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The pervasive expression of circular RNA from protein and non-coding loci is a recently discovered feature of gene expression programs in highly diverged eukaryotes, but the function of most circular RNAs are still unknown. We developed a novel algorithm that allowed us to discover striking general induction of circular RNAs in many tissues, including the heart and lung, during human fetal development. Beyond this global trend, specific circular RNAs are tissue specifically induced during fetal development, including a circular isoform of NCX1 in the developing fetal heart that, by 20 weeks, is more highly expressed than either the linear isoform or beta-actin. Regions of the human fetal brain, such as the frontal cortex have marked enrichment for genes where the circular isoform is dominant. In addition, while the vast majority of circular RNA production occurs at canonical U2 (major spliceosome) splice sites, we find the first examples of developmentally induced circular RNAs processed by the U12 (minor) spliceosome, and an enriched propensity of U12 donors to splice into circular RNA at un-annotated, rather than annotated, exons. Together, these results suggest a potentially significant role or circular RNA in human development.

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170 Structural Studies of Intact Long Noncoding RNAs in Plants and Mammals

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Long non-coding RNAs (lncRNAs) have emerged as key players in development, cancer and plant biology. Few structural studies of lncRNAs have been performed to date [Puerta-Fernandez, PNAS, 2006; Novikova, NAR, 2012; Wan, Mol. Cell 2012; Ilik, Mol. Cell 2013; Davidovich, NSMB, 2013]. Fundamental questions include: (1) do lncRNAs have a well-defined structure?, and (2) are lncRNAs organized into modular sub-domains? A third issue that remains poorly understood is the extent of conservation of lncRNAs, which is difficult to ascertain without knowledge of secondary structure. We developed a new experimental strategy called Shotgun Secondary Structure (3S) determination [Novikova, NAR, 2012; Novikova, Methods, 2013]. 3S helps eliminate large numbers of possible secondary folds corresponding to a single chemical probing profile, allowing us to produce the secondary fold of a lncRNA with little need for computational predictions. The technique enabled us to produce the first experimentally derived secondary structure of an intact mammalian lncRNA, the steroid receptor RNA activator, revealing it to be modularly organized into 4 sub-domains with many helices, internal loops and junctions [Novikova, NAR, 2012]. We have also applied 3S to the Coolair, Braveheart and Gas5 lncRNA systems. While Coolair and Braveheart are structured, Gas5 is a chain of stem loops separated by unstructured regions. We use the experimentally derived structures to find a given lncRNA in other species. New data on in vivo validation studies will also be described.

171 Nuclear circular RNAs and exon-intron circular RNAs in mammalian cells

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Circular RNAs (circRNAs) are a special species of transcripts in metazoans. Previous work has revealed the presence of a large number of circRNAs. However, circRNAs in the cell nucleus and possible existence of circRNA subtypes as well as their features remain to be investigated. We identified ~1300 human circRNAs in the nucleus (nuclear circRNAs). With a new computational pipeline, we annotated ~1700 circular RNAs with retained intron (Exon-Intron circRNAs, EIciRNAs), which were a novel subclass of circRNAs with higher possibility to reside in the nucleus. EIciRNAs as well as the nuclear circRNAs were prone to own longer flanking upstream and downstream introns, which included more ALU elements. Parent genes of both nuclear circRNAs and EIciRNAs tended to have more exons. Most of human and mouse EIciRNAs were derived from orthologous genes. EIciRNAs were more cell-type-specific than the nuclear circRNAs. Interestingly, EIciRNAs were generated from genes with higher expression levels. mRNAs of these genes rather than the other genes showed significant decrease upon knockdown of U1 snRNP, indicating a special association between EIciRNAs and U1 snRNP. Furthermore, analysis of GO pathway revealed that parent genes of EIciRNAs were enriched for functions associated with the nuclear part and the intracellular part.

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172 miRNA and mRNA 3'UTR processing variations in normal and hypertrophic hearts

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Introduction: microRNAs (miRNAs) and their mRNA 3' untranslated region (3'UTR) targets can both be expressed as multiple processing variants. Each variant could have different targeting properties, thus affecting miRNA-mediated regulation in the cell. We aim to comprehensively uncover these processing variants in the heart and evaluate their changes during cardiac hypertrophy. **Methods:** Transverse Aortic Constriction (TAC) was performed to induce cardiac hypertrophy in C57BL/6 mice. RNAs were extracted from the cardiomyocytes of sham-treated, pre-hypertrophic (2 days post-TAC), and hypertrophic (7 days post-TAC) mice, then subjected to small RNA- and polyA-tail (PAT)- sequencing. **Results:** The two sequencing datasets were first mined for differential miRNA and mRNA expression. 19 miRNAs and 618 mRNAs were found to be differentially expressed during hypertrophy. While gene ontology analyses of differentially expressed mRNAs were consistent with previous findings, we newly identified 430 mRNAs as differentially expressed, pinpointing changes during hypertrophy in finer detail. In assessing the data for the expression of processing variants, we found that there is a widespread occurrence of miRNA isomiRs in the cardiomyocyte, however their relative proportions remained largely unchanged during hypertrophy. Our mRNA 3'end-sequencing data identified novel 3'UTRs for 7,348 genes. Notably, there was a significant global shift towards shorter 3'UTR variants as hypertrophy develops. We shortlisted 583 genes with a statistically significant change in the proportion of its 3'UTR variants during hypertrophy, 7 of which we have validated through qPCR. Sucrose density gradient centrifugation revealed that different 3'UTRs of an mRNA could differ in their polysome associations. miRNA target prediction analysis from our sequencing datasets identified 104,001 miRNA-mRNA interactions in the cardiomyocyte. Differential analyses of these interactions suggested that the connectivity between miRNA and their 3'UTR targets are modulated during hypertrophy. **Conclusion:** We present an in-depth study of miRNA and mRNA processing variants in cardiomyocytes. Collectively, our data adds richer textures to a systems-level understanding of miRNA-mediated regulation during normal and hypertrophic states of the heart, which may aid in the development of miRNA-based therapy for cardiac disease.

173 Circular intronic RNA in the cytoplasm of *Xenopus* oocytes

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Because of their large size *Xenopus* oocytes provide a unique experimental system for the analysis of purified nuclear and cytoplasmic RNA populations. In an earlier study our laboratory found a population of stable intronic sequences in the nucleus of the developing oocyte. To our surprise, we found an even more abundant population of intronic sequences in the cytoplasm, derived from at least 9,000 specific introns. All of the cytoplasmic intronic sequences are resistant to RNase R, suggesting that they exist as circles (lariats without tails). The presence of inverted reads within our RNAseq data confirmed this conclusion. Because these circular intronic RNAs (ciRNAs) accumulate in the oocyte cytoplasm and persist during early embryogenesis, they could be important maternal factors for the early embryo.

To further study cytoplasmic ciRNAs we developed a system to express ectopic ciRNAs in living *Xenopus* oocytes independently of their endogenous cognate mRNA. We inject plasmid DNA directly into the nucleus and monitor the RNA transcribed from the plasmid, including ciRNAs. Included in the construct is a GFP reporter gene, which allows us to select successfully-injected oocytes by their green fluorescence. ciRNA itself is transcribed either from mCherry split by an intron coding for the ciRNA or from a portion of the host gene itself. Now that we have demonstrated the feasibility of the system, we will assay potential stability signals within the introns themselves. We have also engineered a ciRNA construct that includes MS2 hairpins, which will permit pulldown experiments to assess proteins associated with the ciRNA. Ultimately we hope to examine possible roles of cytoplasmic ciRNAs in translation control or mRNA stabilization.

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174 The biochemical RNA landscape of a cell revealed by Grad-seq

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High-throughput sequencing can rapidly profile the expression of theoretically all RNA molecules in any given organism but the primary sequence of these transcripts is a poor predictor of cellular function. This has been particularly evident for the regulatory small RNAs of bacteria which dramatically vary in length and sequence within and between organisms.

Here, we establish a new method (gradient profiling by sequencing; Grad-seq) to partition the full ensemble of cellular RNAs based on their biochemical behavior. Our approach enabled us to draw an RNA landscape of the model pathogen *Salmonella* Typhimurium, identifying clusters functionally related noncoding RNAs irrespective of their primary sequence. The map revealed a previously unnoticed class of transcripts that commonly interact with the osmoregulatory protein ProQ in *Salmonella enterica*. We show that ProQ is a conserved abundant global RNA-binding protein with a wide range of targets, including a new class of ProQ-associated small RNAs that are highly structured, and mRNAs from many cellular pathways. By its ability to describe a functional RNA landscape based on expressed cellular transcripts irrespective of their primary sequence, our generic gradient profiling approach promises to aid the discovery of major functional RNA classes and RNA-binding proteins in many organisms.

175 Intronic repeats facilitate the production of circular RNAs with a variety of cellular functions

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Although it was long assumed that eukaryotic pre-mRNAs are almost always spliced to generate a linear mRNA, it is now clear that thousands of protein-coding genes are non-canonically spliced to produce circular RNAs. Here, we provide novel insights into how circular RNAs are generated as well as how they function in cells. We and others recently showed that intronic repetitive elements, including sequences derived from transposons, are critical regulators of circular RNA biogenesis in human cells. When complementary sequences from two different introns base pair to one another, the splice sites from the intervening exon(s) are brought into close proximity, facilitating backsplicing. Whether this base pairing-based mechanism extends to many other eukaryotes is, however, unclear. For example, the introns flanking most *Drosophila* circular RNAs have been reported to lack complementary repeats. Nevertheless, we have now identified and characterized fly circular RNAs that are flanked by complementary sequences. Analogous to what we observed in humans, these repeats are necessary for the biogenesis of the circles in flies. Furthermore, the fly repeats are able to support circular RNA production in human cells, indicating that intronic repeats are generally used across eukaryotes to facilitate circular RNA production. Once generated, the functions for the vast majority of circular RNAs are unknown. As there is currently a lack of tools to modulate circular RNA levels, we have generated plasmids that efficiently over-express any desired circular RNA *in vivo*. Importantly, these plasmids produce a minimal amount of the associated linear RNA. Using these reagents, we have demonstrated that designer circles that contain an open reading frame and an IRES can be efficiently translated in cells. Likewise, designer circles that contain microRNA binding sites can function as efficient sponges *in vivo*. In total, our results reveal that intronic repeats facilitate the generation of circular RNAs in numerous species and that the mature circles can serve a variety of cellular functions.

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176 The Static Dynamics of Gene Regulation by the miR-10 Family of microRNAs

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MicroRNAs (miRNAs) are short non-coding RNAs that regulate gene output by targeting degenerate elements in 3'untranslated regions (3'UTRs) of mRNAs. miRNAs are often deeply conserved, but have undergone drastic expansions in the genomes of higher metazoans, resulting in families of miRNAs whose members typically vary by single nucleotide differences. The evolutionary significance of maintaining multiple copies of highly similar miRNAs is not understood. Each miRNA can regulate hundreds of target genes, yet it is not clear what functional differences exist between miRNA family members.

To address these questions we focus on four intertwined human miRNAs: *miR-10a*, *miR-10b*, *miR-99a*, and *let-7c*. The miR-10 family includes *miR-10a* and *miR-10b*, which differ by a single central nucleotide, as well as *miR-99a*, which has a single nucleotide deletion in the seed region. In the genome, *miR-99a* is part of a cluster with *let-7c*, while *miR-10a/miR-10b* exist within two Hox clusters.

To understand the functional differences of each miRNA, we developed a novel high-throughput assay called Luminescent Identification of Functional Elements in 3'UTRs (3'LIFE), and screened for targets of these four miRNAs amongst a panel of several hundred 3'UTRs. We identify a large number of novel target genes, with 27% directed by non-canonical interactions.

miR-10a and *miR-10b* targets are not identical; while they share largely overlapping gene sets enriched for multiple Hox genes, they also contain unique targets, suggesting functional divergence. *miR-99a* instead targets a distinct set of genes, but unexpectedly shares a significant portion of targets with *miR-10a/miR-10b*. In addition, while *let-7c* and *miR-99a* targets are completely different from each other, target analysis suggests novel cooperative functional roles.

In the case of all four miRNAs, we found consistent roles as tumor suppressors or oncogenes. Unexpectedly, each miRNA preferentially targets multiple genes within regulatory networks, such as RAS and retinoic acid signaling. We explore the expression of these miRNAs across evolutionary time in human tissue culture, *in situ* hybridization in mouse embryos, and in fluorescent reporters in *C. elegans*.

Our findings suggest that miRNAs are relatively static in their sequence and expression patterns, but are capable of readily evolving novel regulatory functions.

177 Hfq assists small RNAs in rearranging the structure of the coding region of *ompD* mRNA

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The Hfq protein is essential for the regulation of translation by bacterial small noncoding RNAs (sRNAs), which are important for the adaptation to changes in environmental conditions, and can also affect the virulence of pathogenic species. This Sm-like, ring-shaped protein facilitates the pairing of sRNAs to their mRNA targets. However, it has also been shown to be recruited by an sRNA to the regulated mRNA, or to compete with an sRNA for binding to mRNA. The detailed molecular mechanism of the Hfq contribution has been only explained for the pairing of sRNA DsrA with the 5′ untranslated region of E.coli rpoS mRNA. However, recent Hfq profiling data suggested that the coding sequence of mRNAs could also be an important target for this regulation. Indeed, the Salmonella ompD mRNA is bound in the coding sequence by four sRNAs, which repress its translation or accelerate its decay. Although Hfq binds to ompD mRNA and affects its expression, it is not clear how it contributes to sRNA interactions with the coding sequence of ompD, and whether it uses a similar mechanism as described for DsrA-rpoS pairing.

Here, the kinetics of association of RybB and MicC sRNAs to the 5′-terminal ompD mRNA fragment was monitored using a gelshift assay. The results showed that Hfq markedly increased the rates of association of both sRNAs to this mRNA. Next, the probing with structure-specific RNases was used to describe the secondary structure of ompD mRNA leader sequence. It was also used to probe how the binding of Hfq and sRNAs affects the structure of this mRNA fragment. The results suggest that while Hfq promotes the pairing of both sRNAs to ompD mRNA the binding of these sRNAs determines the specific local changes in the ompD mRNA structure.

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178 Expression landscapes of RNA-binding proteins and lncRNAs in normal cervix and cervical cancer

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Cervical cancer is caused by persistent infection and genomic integration of high-risk human papillomaviruses and is the second most common cancer among women worldwide. Approximately 500,000 incident cases of cervical cancer and ~320,000 cervical cancer deaths are estimated each year and more than 80% of the cases arise in developing countries. Using RNA-sequencing (RNA-Seq) approach, we examined seven normal cervical tissues and seven cervical cancer tissues for their expression landscapes of ~19,000 coding and 113,513 noncoding RNAs. We identified 614 differentially expressed coding transcripts enriched in cancer related pathways and 95 of them encoding RNA-binding proteins (RBPs) from the analyzed 1502 human RBPs. Moreover, we identified 34 differentially, abundantly expressed lncRNAs from normal cervix to cervical cancer. Further validation of the altered expression of 26 candidates, including 8 RBP genes by using TaqMan real-time PCR in a cohort of 47 human cervical tissue samples, including 24 normal cervical tissues and 23 cervical cancer tissues, showed that they are broadly involved in cervical carcinogenesis, many of which have not been reported. By using human vaginal keratinocyte-derived raft culture tissues with or without HPV16 and HPV18 infection, we further corroborated that these novel candidates, including FAM83A, SEMA3F, CLDN10, ASRGL1, and also two RBP genes RNASEH2A and NOVA1, are regulated by HPV infection. Finally, we found lnc-FANCI-2 was increasingly expressed along with cervical lesion progression from CIN to cancer, when compared to the normal tissues. In contrast, lnc-GLB1L2-1 was gradually decreased along with the lesion progression, when compared to the normal tissues. In conclusions, our study provides the first comprehensive expression atlas of RBPs and lncRNAs in normal cervix and cervical cancer, which enables us to develop potential biomarkers and intervention strategies for better diagnosis and treatment of patients with cervical cancer.

179 MicroRNA transferase function of AUF1 p37

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Eukaryotic gene expression is tightly regulated post-transcriptionally by RNA-binding proteins (RBPs) and microRNAs. Silencing the RBP AU-rich-binding factor (AUF)1 reduced the interaction of microRNAs with Argonaute 2 (AGO2), the catalytic component of the RNA-induced silencing complex (RISC). Analysis of this effect revealed that the AUF1 isoform p37 displayed high affinity for the microRNA let-7b, promoted the interaction of let-7b with AGO2,and enhanced AGO2-let-7-mediated mRNA decay. Our findings uncover a novel mechanism whereby microRNA transfer from AUF1 p37 to AGO2 facilitates microRNA-elicited gene silencing.

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180 An Overarching Role for U1 snRNP Telescripting in mRNA Regulation

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Our recent observation that most RNA polymerase II nascent transcripts in animal cells fail to produce full gene-length transcripts when U1 snRNP (U1) is inactivated revealed a new dimension in gene expression regulation. This essential U1 protective activity, which allows transcription to go farther (telescripting), suppresses premature cleavage and polyadenylation (PCPA) from cryptic signals (PASs) in introns. It is separate from U1's splicing function; both depend on U1's base pairing through its 5' sequence, but 5' modified U1 variants that cannot function in splicing are still effective in telescripting. Our studies have further shown that partial U1 inactivation with an antisense oligonucleotide to its 5'-end dose dependently shortens mRNAs. Widespread mRNA shortening of 3' untranslated regions (3'UTRs) occurs in cancer, proliferating cells and activated immune cells and neurons. 3'UTRs have many mRNA regulatory elements, including miRNAs targets that generally repress translation, and their removal contributes to oncogenicity. We investigated the potential role of U1 telescripting in these phenomena. We found that moderate U1 decrease shortened 3'UTRs of >1,000 genes, enhancing cell migration and invasiveness to 500% in HeLa cancer cells while its over-expression lengthened 3'UTRs of >2,000 genes that are already shortened in these cells and attenuated the same oncogenicity indicators. The strong correlation between U1's bi-directional effects on nascent transcripts' length and cell phenotype suggests that mRNA shortening in cancer can be explained by a deficit in U1 telescripting. These findings highlight U1 telescripting as an overarching mRNA regulation mechanism, revealing its pivotal contribution to cell aggressiveness and other activated phenotypes, and offering U1 modulation as a new investigational tool to modify them.

181 FOX-2 binding to pre-miRNA-20b influences strand bias

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An increasing number of RNA binding proteins (RBPs) known for roles in pre-mRNA processing are being found to act as auxiliary factors during miRNA biogenesis. Here we show that FOX-2 protein, a well-studied alternative splicing factor, specifically binds to GCAUG motifs present on miRNA hairpins *in vitro and in vivo*. Amongst others, pre-miR-20b was bound by FOX-2, an interaction mediated by a GCAUG motif present in the terminal loop. Using surface plasmon resonance (SPR) we could show that a recombinant FOX-RRM domain bound to pre-miRNA-20b with moderate affinity (3.6 μM). Mutation of the second guanidine residue (G_5), known to be essential for RRM-RNA interactions, completely abrogated binding. Utilizing different techniques we established that overexpression of both pre-miRNA-20b and miR-20b 5p, but not miR-20b 3p suppressed FOX-2 protein levels. FOX-2 silencing was lost upon mutation of the Fox-binding element (FBE) in the loop of pre-miRNA-20b. Northern blots and small RNA deep sequencing experiments showed that FOX-2 influenced the balance of miR-20b 5p versus miR-20b 3p strands. These data suggest a novel connection of FOX-2 to microRNA biogenesis by influencing strand bias of miR-20b.

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182 tRF5-Glu Detection and its Regulation of BCAR3 in Ovarian Cancer Cells

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Deep sequencing studies are revealing a never before known complexity of processed noncoding RNAs in human samples, including tRNA fragments (tRFs). tRFs belong to a class of RNA fragments known to accumulate during several types of cellular stress. Our lab collected total RNA from the urine of several ovarian cancer patients and detected high levels of multiple tRFs. One specific tRF, tRF5-Glu, was detected at higher levels within ovarian cancer patient samples as compared with non-ovarian cancer control samples. Ovarian cancer is difficult to detect, diagnose, and treat due to the inaccessible nature of the ovaries. Methods for early diagnosis of ovarian cancer and for distinguishing subsets of patients based on therapeutic response are needed to enhance treatment of this disease. We hypothesized that by determining the expression and function of tRF5-Glu in ovarian cancer cells that these data might provide new understanding of the ovarian cancer disease process and eventually a new avenue for therapeutic intervention. We found that tRF5-Glu functions like a microRNA by targeting the mRNA of the Breast Cancer Anti-estrogen Resistance 3 gene, BCAR3, and it does so in an estrogen-dependent manner. BCAR3 is known to regulate cancer cell migration through its protein interaction with p130cas and contributes to anti-estrogen resistance in breast cancer cells and may act in a similar manner in ovarian cancer cells. Using tRF5-Glu mimics and inhibitors we have studied the regulation of BCAR3 protein expression in multiple ovarian cancer cell lines with and without estrogen receptor alpha (ERα) expression and in the presence and absence of estrogen. We have found that BCAR3 regulation by tRF5-Glu is dependent on a functional ERα and that this regulation may be inhibited in the presence of a selective ERα down regulator, ICI.

183 Fluorescence Bimolecular Complementation enables Facile detection of Ribosome Assembly Defects in *Escherichia coli*

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In the last decade, crystal structures of ribosome rationalized decades of research to understand the mechanism of protein synthesis. However, given the pivotal role played by ribosome, much remains to be understood how this massive macromolecular machine is assembled with such exquisite precision. The maturation of ribosome from the constituent RNA and protein complements involve concerted interplay of several factors. Indeed, these factors seem to be responsible for the acceleration of otherwise non-spontaneous assembly under physiological conditions inside the cell. Identification and characterization of candidate factors involved in ribosome assembly is fraught with bottlenecks due to lack of facile technology. Hitherto, the involvement of assembly factors in ribosome assembly is validated whether their absence produces an altered ribosome profile and cold sensitive growth phenotype. The loss of assembly factors ensues premature subunits that are inept to associate to form functional ribosome competent for protein synthesis and this state is captured as altered ribosome profile in the density gradient fractionation. This traditional approach is cumbersome and not scalable for high-throughput screening to identify potential assembly factors. It is this bottleneck that we set out to overcome by employing bimolecular fluorescence complementation as a tool to detect the involvement of candidate factors in ribosome assembly. The fluorescent Venus protein is split into two non-fluorescent parts - the N and C-terminal fragments - and these are fused with the ribosomal proteins (r-proteins) S13 and L5, respectively, in *Escherichia coli*. When the r-proteins assemble onto the ribosome, this enables the two fragments to come in proximity to each other and this facilitates the association of the complementary parts of the Venus such that it is now rendered fluorescent. We have validated this concept by deleting the known assembly factors, RsgA and SrmB that participate in 30S and 50S ribosome assembly, respectively. The absence of these proteins abrogates the otherwise proficient complementation of Venus protein fragments thus capturing the existence of assembly defect. Our approach thus offers a facile methodology for large scale genomic screening for identifying potential assembly factors involved in ribosome assembly.

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184 A point mutation in the *Saccharomyces cerevisiae* U3 snoRNP protein Rrp9p identifies protein Rrp36p as its direct partner in pre-ribosomal RNA processing

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U3 snoRNP plays a key role in early steps of pre-rRNA processing. This C/D box snoRNP contains the C/D box proteins: Snu13p, Nop1p, Nop56p and Nop58p and a specific Rrp9p protein. Rrp9p is required for pre-rRNA maturation and cell growth. However, its role in the macro-complex (SSU-processome) carrying the catalytic activity for pre-RNA cleavage is rather elusive. The large C-terminal domain of Rrp9p folds into a helix-propeller structure. To get insight into the role of Rrp9p, we used this 3D structure and guessed that amino acids at the surface of the propeller, which are not conserved in propeller of proteins involved in other biological activities, but are conserved in the propeller of vertebrate's Rrp9p counterparts, could be needed for pre-rRNA processing. They were mutagenized and the effects on cell growth were tested. Through this approach, we found that R289A substitution in Rrp9p affects cell growth, especially at 20°C. The R289A Rrp9p protein is stable and incorporated in U3 snoRNPs. The level and composition of U3 snoRNP is preserved. However, a strong defect of 18S rRNA production is observed with the accumulation of several maturation intermediates: 35S, 23S, 20S and the appearance of a new 22S intermediate cleaved at the A0 and A3 sites, meaning that the kinetics of cleavages at sites A1 and A2 is strongly slowed down. Fractionation of ribosomes on sucrose gradients revealed the appearance of a 60S peak. We hypothesized that the R289A mutation might affect the efficiency of interaction of Rrp9p with other proteins of the SSU-processome. By two-hybrid assays, we identified a network of protein-protein interactions within the SSU-processome that includes Rrp9p. We showed that the R289A mutation affects a yet not identified direct interaction between Rrp9p and Rrp36p. Destabilization of this interaction probably explains the pre-rRNA maturation defect. We are currently studying the consequence of this destabilization on the network of protein-protein interactions that we identified. Based on our earlier studies on the intermolecular helix formed between U3 snoRNA and the pre-rRNA, we also investigate possible connections between defects in intermolecular helix formation and defects in Rrp9p-Rrp36p interaction.

185 ATP Hydrolysis by the DEAD-box protein Rok1 Releases the Assembly Factor Rrp5 from pre-40S Ribosomes.

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DEAD-box proteins are ubiquitous regulators of RNA biology. While commonly dubbed "helicases", their activities also include duplex annealing and RNA-protein complex remodeling. Rok1, an essential DEAD-box protein, and its co-factor Rrp5 are required for ribosome assembly. Here we present structural and biochemical analyses of Rrp5•Rok1 in the presence and absence of RNA and nucleotides, which indicate that structural changes in Rrp5•Rok1 lead to tight RNA binding of the complex in the presence of ATP but not ADP. Furthermore, in vivo analyses show that in ATPase-deficient mutants of Rok1, pre-40S assembly intermediates are enriched in Rrp5.Together, these data show that ATP hydrolysis by Rok1 releases Rrp5 from 40S subunits. More broadly, the data show that ATPase-dependent structural switches in DEAD-box proteins can be used to release binding partners from their RNA substrates without motor activity by the DEAD-box protein. We are currently working to obtain a structure of Rrp5•Rok1 on ribosomes.

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186 Multiple Functions of the Nop7-Subcomplex in the Assembly of the 60S subunit of Yeast Ribosomes

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Eukaryotic ribosome assembly requires the concerted action of >200 trans-acting proteins called 'assembly factors'. The evolutionarily conserved Nop7-subcomplex is a heterotrimer composed of three assembly factors – Nop7, Erb1, and Ytm1. In the absence of the Nop7-subcomplex, assembly of the large 60S subunit is abolished at an early step. Multiple ribosomal proteins and assembly factors fail to stably associate with pre-ribosomes and these particles are eventually turned over (Sahasranaman *et al.* (2011)). UV-crosslink sites of Erb1 and Nop7 on pre-rRNA indicate that they play a role in establishing tertiary structure of the large subunit (Grannemann *et al.* (2011)). Taken together, these results imply that the Nop7-subcomplex is required to form stable pre-ribosomes during early steps of 60S subunit assembly. However, additional roles of the Nop7-subcomplex proteins cannot be understood using depletion mutants. To investigate more specific roles of the Nop7-subcomplex, we constructed mutations in the essential N-terminal half of Erb1 that mediates Nop7-subcomplex formation by its interaction with Nop7 and Ytm1 (Tang *et al.*(2008)). Eight internal deletions in the N-terminal half of Erb1 affect pre-rRNA processing and pre-ribosome composition differently, allowing us to delineate roles of different regions of Erb1. The ribosome assembly defects in these mutants were characterized using affinity purification of pre-ribosomes, chemical probing of pre-rRNA structure, and protein-protein interaction assays. We identified two *erb1* mutants affecting 60S subunit assembly at a later step than that affected upon its depletion. Our results agree with the prediction that the Nop7-subcomplex is essential to stabilize the pre-ribosomes during early 60S subunit assembly. During later events, the Nop7-subcomplex influences the recruitment of specific assembly factors, potentially by affecting pre-rRNA structure.

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187 Eukaryotic rRNA Expansion Segments Function in Ribosome Biogenesis

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The secondary structure of ribosomal RNA (rRNA) is largely conserved across all kingdoms of life. However, eukaryotes have evolved extra blocks of sequences relative to that of prokaryotic rRNA called 'expansion segments' (ES). These expansion segments have also been referred to as variable regions, owing to their variability in length and sequences across organisms and sometimes, even within an organism. This variability seems to preclude important roles for expansion segments in the function of the ribosome.

We sought to systematically investigate the potential functions played by the 25S rRNA expansion segments in Saccharomyces cerevisiae ribosome biogenesis. Although studies have identified cellular functions for a few expansion segments before, a thorough characterization of the potential of roles of these expansion segments remains to be done, possibly because of the limitations in the availability of robust systems to study rRNA mutants. We used a temperature sensitive PolI mutant yeast strain in which transcription of rDNA is driven from a plasmid-borne rDNA copy with a *GAL* promoter in order to study the roles of expansion segments.

Using this rDNA mutagenesis system, we deleted each of the eukaryote-specific expansion segments in yeast 25S rRNA. The phenotype of the mutants was first assayed by studying their growth. Following this, northern blotting, primer extension assays and affinity purifications were used to zoom in on the precise ribosome biogenesis phenotype of these mutants.

This study is the first of its kind to systematically identify the precise functions of eukaryote-specific expansion segments by showing that they play roles in specific steps of ribosome biogenesis. The catalog of phenotypes we identified helps us build possible mechanistic models for the roles of expansion segments in ribosome biogenesis. Going forward, this study will help us unravel the yet unexplored functions of these eukaryote-specific expansion segments and pave the way for a deeper understanding of the mechanisms of ribosome biogenesis in general.

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188 hUTP24 is essential for processing of the human ribosomal RNA precursor at site A_1, but not at site A_0

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Production of ribosomes relies on more than 200 accessory factors, securing the proper sequence of steps leading to the faultless assembly of these ribonucleoprotein machineries. Among trans-acting factors are numerous enzymes, including ribonucleases responsible for processing of the large rRNA precursor synthesized by RNA polymerase I, encompassing sequences corresponding to mature 18S, 5.8S and 25/28S ribosomal RNAs. In humans, the identity of most enzymes performing individual processing steps, including endoribonucleases cleaving pre-rRNA at specific sites within regions flanking and separating mature rRNAs, remains unknown. Here, we investigated the role of hUTP24 protein in ribosomal RNA maturation in human cells. hUTP24 is a human homolog of the *Saccharomyces cerevisiae* putative PIN domain-containing endoribonuclease Utp24 (yUtp24), which was suggested to participate in the U3 snoRNA-dependent processing of yeast pre-rRNA at sites A_0, A_1 and A_2. We demonstrate that hUTP24 interacts to some extent with proteins homologous to the components of the yeast small subunit (SSU) processome. Moreover, mutation in the putative catalytic site of hUTP24 results in slowed growth of the cells and reduced metabolic activity, associated with a defect in biogenesis of 40S ribosomal subunit leading to decreased amounts of 18S rRNA, which is a consequence of aberrant pre-rRNA processing at the 5'-end of 18S rRNA segment (site A_1). Interestingly, in contrast to yeast, site A_0 located upstream of A_1 is efficiently processed upon UTP24 dysfunction. Finally, hUTP24 inactivation leads to an aberrant processing of 18S rRNA 2 nucleotides downstream of the normal A_1 cleavage site.

189 A dead-end fold? ITS2 secondary structure models reassessed.

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For the formation of ribosomal subunits a long RNA Polymerase I transcript gets, to a large extent co-transcriptionally, assembled with r-proteins, processed and modified. During this process many refolding events in the pre-rRNA will occur while dynamic interactions with trans acting factors are tightly co-ordinated to support maturation of the pre-rRNPs by step-wise removal of external (ETS) and internal (ITS) transcribed spacer regions from the pre-rRNA. During the formation of the 60S subunit, the spacer region separating the mature 5.8S and 25S rRNA sequences, ITS2, will be cleaved internally and then removed by 5' and 3' exo-nucleases. In *Saccharomyces cerevisiae*, the presence of this spacer is required for proper formation of the large ribosomal subunit and even small mutations in ITS2 can slow down or interfere with its removal and 60S assembly. Based on mutational analysis and phylogenetic comparison, structural requirements within the spacer have been proposed that guide its processing.

We will present an extensive phylogenetic comparison of ITS2 sequences to delineate shared structural elements and discuss how these alignments relate to current models, available structural probing data and published mutational analysis. Taken all data together it can not be excluded that some of these models might represent dead-end precursor molecules that do not support the formation of mature particles. To investigate the dynamics of ITS2 folding during 60S assembly we are currently using the ChemModSeq method, recently developed in our lab (Hector *et al*., 2014; *Nucleic Acids Res*. **42**: 12138-12154), that allows rapid and quantitative measurements of RNA structural rearrangements during the assembly of ribosomal intermediates.

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190 Utp14 is an activator of Dhr1 helicase function *in vivo* during ribosome biogenesis in yeast

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In eukaryotic ribosome biogenesis, U3 snoRNA base-pairs with the pre-rRNA to promote its processing. However, U3 must be removed to allow folding of the central pseudoknot, a key feature of the small subunit. Previously, we showed that the DEAH/RHA RNA helicase Dhr1 dislodges U3 from the pre-rRNA (1) and that mutations in both Dhr1 and Utp14 suppress the absence of the rRNA methyltransferase Bud23 *bud23*Δ. Here, we report that Utp14, an essential protein of the pre-ribosome, interacts with Dhr1 and regulates its function *in vivo*. Mutations in Utp14 that suppress *bud23*Δ map to a discrete sequence that is required for full interaction with Dhr1. Mutations in this region display a gradient of loss of interaction with Dhr1 that correlates with loss of function of Utp14. Surprisingly, the loss of function of Utp14 inversely correlates with accumulation of Dhr1 and U3 in a pre-40S particle. This co-sedimentation of Dhr1 with U3 is reminiscent of the accumulation of helicase inactive Dhr1 in a pre-40S particle containing U3. The similarity in the phenotype of *utp14* and *dhr1* mutants led us to propose that Utp14 activates the helicase activity of Dhr1 *in vivo*. This prediction was borne out by *in vitro* studies described in the abstract by Liu et al. Importantly, the degree of stimulation *in vivo* correlated with Utp14 function and its ability to interact with Dhr1. To test if Utp14 is also required for recruiting Dhr1 to the pre-ribosome we show that in the absence of either Utp14 or Bud23 Dhr1 can stably bind to the pre-ribosome. In contrast, Dhr1 binding is substantially reduced when both Utp14 and Bud23 are depleted. Thus, Utp14 and Bud23 together are required for efficient recruitment and activation of Dhr1. Because Utp14 mutations that suppress *bud23*Δ show reduced activation of Dhr1 and increase its presence in the pre-ribosome, we propose that Bud23 is required for productive Dhr1 activity. Thus, Bud23 may serve as a sensor of the status of ribosome assembly, controlling the productive release of U3 by Dhr1.

191 An epigenetic switch downstream of miRNA loss results in irreversible activation of let-7 oncofetal targets

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MicroRNAs (miRNAs) are ~22nt RNAs that post-transcriptionally repress their target mRNAs. miRNA activity typically confers approximately 2-3 fold repression on a targeted gene, consistent with a role for miRNAs as tuners of gene expression. Despite this modest effect however, miRNAs are critical for many biological processes.

By regulating central transcription factors and their downstream effectors, miRNAs increase their regulatory impact. Thereby, miRNAs are integrated into transcriptional networks that reinforce gene expression programs. Hence, there is a need for comprehensive description of the involvement of miRNAs in transcriptional networks in various cellular states.

In investigating the transcriptional programs regulated by miRNAs in somatic cells, we observed that Dicer deletion results in up-regulation of a let-7 targeted embryonic gene network. For a subset of let-7 oncofetal targets (characterized by high expression in embryos, inactivation in most adult tissues and reactivation in tumors), the magnitude of up-regulation is 50-100 fold, much greater than that typically reported for miRNA-mRNA interactions. This suggests effects additional to loss of post-transcriptional repression by miRNAs.

Subsequent ChIP-Seq analysis on H3K4me3 and H3K36me3 chromatin marks indicated transcriptional activation of these let-7 targets upon Dicer loss. To test if repression of these targets could be restored by re-expression of miRNAs, we stably expressed wild-type Dicer in Dicer KO cells. By global miRNA profiling and reporter assays, we observed complete restoration of the expression and post-transcriptional activity of let-7 and other miRNAs. However, the transcriptionally activated let-7 oncofetal targets fail to revert to their original wild-type expression levels. Computational analysis indicated that these genes are a subset of a larger, irreversible gene set activated by Dicer loss. This gene set is enriched for inflammatory genes regulated by NFKB. Thus, in somatic cells, miRNAs suppress an embryonic transcriptional network that is irreversibly activated upon Dicer loss.

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192 Structural Insights into the Binding and Inhibition of Lysine Specific Demethylase-1 by RNA

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While noncoding RNAs are emerging as key epigenetic regulators, the structural mechanisms of action remain largely undefined. One pathway of RNA-mediated regulation is the binding of transcripts to chromatin modifiers such as lysine specific demethylase 1 (LSD1). LSD1 catalyzes the demethylation of histone 3 at lysine 4 (H3K4) resulting in gene silencing. The well-characterized lncRNAs HOTAIR and TERRA are thought to act as scaffolds for LSD1 and other proteins. We have used X-ray crystallography, NMR, and EMSAs to interrogate proposed RNA binding domains of LSD1. These experiments produced the first known crystal structure of a complex between RNA and a chromatin remodeling enzyme and confirm previous reports that LSD1 preferentially binds G-quadruplex containing RNAs. In combination with activity assays, these studies suggest that RNA can bind both the previously uncharacterized N-terminal region of LSD1 and the amine oxidase domain to allosterically inhibit demethylation.

193 Novel computational metrics and approaches for evaluating population-wide differences in alternative splicing at the single-cell level

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The recent availability of technologies that enable the large-scale isolation of single cells and generation of whole transcriptome sequencing data has led to the development of computational methods to evaluate gene expression at the single cell level. This has enabled exciting studies of cellular heterogeneity and gene regulatory networks. However, there is a dearth of computational approaches to analyze alternative splicing at the single cell level. We have recently generated deep (>30 million reads per cell) RNA-seq data for hundreds of single cells during stem cell differentiation towards mature motor neurons. Here we present a computational, open-source, framework that enables the analysis of alternative splicing at the single-cell level, using Bayesian methods to assign splicing "modalities" and non-negative matrix factorization (NMF) to reveal how "modes" change across our profiled single cells. We identify hundreds of "bimodal" alternative splicing events that have, surprisingly, distinct sequence and transcript properties that distinguish them from single "modal" events, revealing the power of these technologies and analytical tools in revealing the biological importance of alternative splicing at the single cell level.

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194 Detecting riboSNitches with RNA folding algorithms: a genome-wide benchmark.

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A riboSNitch is an RNA element that changes secondary structure due to a single nucleotide variant. Although the phenotypic consequences of most riboSNitches are likely small, a few known riboSNitches have implications in diseases like Hyperferritinemia Cataract Syndrome and Retinoblastoma. RiboSNitches are likely important in contributing to allele-specific effects in post-transcriptional regulation, and the combination of many different riboSNitches present in any individual genome represents a level of phenotypic complexity on par with individual expression patterns. Thus, understanding the role that riboSNitches play in phenotypic outcomes is an important stepping-stone in the study of personal genomics. A recent parallel analysis of RNA structure (PARS) study probed transcriptome secondary structure in a family trio, and was the first study to identify riboSNitches genome wide. Out of over 12,000 transcribed loci differing in genotype between trio members, nearly 2,000 of these displayed significant structure differences between the individuals, identifying them as riboSNitches. We used this collection of riboSNitches and non-riboSNitches (transcripts with no structure change between trio members) to evaluate the riboSNitch prediction performance of eleven RNA folding algorithms. We found that the algorithms specifically tailored for riboSNitch prediction, which includes remuRNA, rnaSNP and SNPfold, performed better as a whole than more general RNA folding algorithms. We also found that predictions based on base pairing probabilities derived from the Boltzmann ensemble of structures were more accurate than predictions based on minimum free energy (MFE) representations of RNA structures, suggesting that MFE structures are too reductive for the purpose of structure comparison. Across all algorithms, prediction accuracies improved with increasing riboSNitch validation, indicating that low prediction performance is due in part to false positives in the data set or that some riboSNitches result from cellular conditions not replicable *in silico*. Accuracies significantly improved when performance was evaluated solely on the most confident predictions. This means that predicted riboSNitches with the largest structure changes are the most likely to be observed experimentally. In sum, our benchmark reveals several best practices for the prediction of riboSNitches, instrumental to the interpretation of a growing body of research on the functional impact of transcript secondary structure.

195 Spliceman: An Online Tool for Predicting and Visualizing the Effects of Genetic Variants on Splicing

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The mechanism by which single-nucleotide mutations cause splicing defects is poorly understood. These variants are mostly associated with disruption or creation of regulatory elements that are important in the substrate recognition by the splicing machinery. A splicing element's function often depends on its location. Natural selection has resulted in the accumulation of functional cis-elements at optimal positions relative to active splice sites. Conversely, the positional distribution of a motif of a specified number of nucleotides around annotated splice sites is a readout of that element's function. Spliceman server was launched in 2012 and exploits the relationship between location and function of splicing elements to predict how likely point mutations are to disrupt splicing.

We present a transformed version of the basic online tool that will be usable to scientists and students worldwide. We have implemented industry-standard input options (i.e., Spliceman now accepts Variant Call Format (VCF) files) to increase the througput of mutations that the web server can process. The output includes a usable prediction (derived from ROC curve analysis) in the form of $\log_{10}$(p-value) of a variant disrupting splicing. Furthermore, with the increasing number of identified and characterized proteins that bind specific exonic/intronic enhancers and silencer elements, it is now possible to provide the mechanistic prediction of which RNA-binding proteins (RBPs) are disrupted by the single-nucleotide mutation. Based on highest allelic binding difference to known RBP recognition motifs, Spliceman reports top 5 RBPs binding sites that are destroyed by each variant of interest. Additionally, we also provide a tool for visualizing the locations - within exons and introns - of sequence variants to be analyzed and the predicted effects on splicing of the pre-mRNA transcript in a clean and easily readable table view.

Finally, in addition to in silico analysis, Spliceman now offers users the opportunity to recommend variants for submission to our high-throughput in vitro and in vivo splicing assays. Results from these types of studies will allow us to utilize the excess capacity of our assays, help web users recruit experimental collaborators, and continuously update the prediction tool for identifying mutations that cause splicing defects.

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196 Rational engineering of the Neurospora VS ribozyme: substitutions of the I/V kissing-loop interaction with known kissing-loop interactions.

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The Neurospora VS ribozyme is a catalytic RNA that specifically recognizes and cleaves a stem-loop substrate. Substrate recognition is achieved through formation of a highly stable kissing-loop interaction between the stem-loop I (SLI) substrate and stem-loop V (SLV) of the trans VS ribozyme. In previous engineering studies, we investigated the possibility of engineering VS ribozyme variants to cleave isolated SLI substrates in which the length of stem Ib is either increased or decreased. Our results reveal that trans VS ribozyme variants with helix-length modifications in stem V can precisely and efficiently cleave a range of substrates that contain 3-6 base pairs in stem Ib. In the present study, we investigate the possibility of substituting the I/V kissing-loop interactions with known kissing-loop interactions. We first identified alternative kissing-loop interactions that are structurally similar to the I/V kissing-loop interaction of the VS ribozyme by performing a symbolic search of the PDB using WebFR3D. Next, we experimentally tested the ability of variant VS ribozymes to cleave variant SLI substrates that would allow for formation of an alternative I/V kissing-loop interaction. Interestingly, preliminary results with an alternative kissing-loop interaction indicate that this interaction is compatible with the cleavage reaction, but with a lower kcat/KM than observed with the wild-type VS ribozyme system. Molecular dynamic simulations are now being performed to help rationalize these kinetic results. In summary, our studies clearly establish that the sequence of the trans VS ribozyme can be adapted to cleave other folded RNA substrates.

197 Predicted conserved RNA structures are under selection, near cis-regulatory regions and widespread in the human genome

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We present a computational genome-wide screen for structured RNAs based on structural re-alignments of 17 vertebrates, identifying ~520k regions of conserved RNA structure (CRS). The screened regions correspond to ~50% of the human genome. CRSs cover 2.6% thereof and overlap a range of known sequences, from Rfam to long non-coding RNAs, and with most of the CRS in intronic or intergenic regions. By comparison to publicly available RNAseq data, ~25% of our candidates overlap with transcribed regions requiring an abundance of at least 3 reads. In complement to this we design CaptureSeq, confirming known expression of ~2400 CRS while revealing ~200 new cases of expression in human fetal brain. qRT-PCR furthermore revealed expression in 5 of 7 examined tissues in human and mouse. Interestingly, approximately one third of the CRS regions co-localize with cis-regulatory regions. We estimate that most CRSs are under purifying selection, many have low sequence identity (<50%) and some CRSs are estimated to be positively selected from a primary sequence perspective. Comparing the CRSs to causal autoimmune disease variants, we predict several SNPs to cause changes in the RNA secondary structure. To summarize, experimental analyses by CaptureSeq, qRT-PCR and RNA structure probing support expression as well as predicted structures of CRSs, even in cases with low (<50%) sequence identity between human and mouse examples.

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198 Whole genome computational analysis of tRNA-derived small RNAs in Arabidopsis thaliana RNA biogenesis mutants

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Post-transcriptional RNA fragmentation into smaller entities is a widespread and conserved mechanism leading to formation of small regulatory RNAs. In recent years, application of the modern high-throughput sequencing technologies revealed a novel, rapidly growing class of stable non-coding RNA molecules, derived from tRNA (tRFs). The RNAs belonging to this class are heterogeneous in size and their abundance was shown to change in response to environmental and developmental stimuli. Several studies have been initiated towards functional characterization of these molecules in a variety of model organisms. However, a global perspective of the biogenesis of this interesting group of ncRNAs is largely missing.

Using *Arabidopsis thaliana* as a model organism, we aim at identification and characterization of the components involved in tRNA and microRNA biogenesis pathways that may play a role in generation of tRFs. We are presenting our results of a global, high-throughput characterization of small RNA fragments originating from Arabidopsis tRNA and tRNA-like genomic sequences.

The RNA sequences used for this analysis were obtained using sRNA libraries from over 20 *A. thaliana* strains carrying mutations in genes associated with tRNA and microRNA biogenesis. In addition to our own experimental data, the set includes all high quality *Arabidopsis* short RNA sequences available from public sRNA-Seq databases. Thus, the entire dataset provides a broad perspective of tRNA-derived short RNAs in Arabidopsis for a wide cross-section of tissues, developmental stages, as well as biotic and abiotic stress conditions.

An advanced and user-friendly exploration of datasets and results of the analyses were implemented in a form of the "T-regs" web portal. The web site provides easy access to the tRFs data and allows for visualization of analysis results in a form of interconnected diagrams and expression profiles in addition to structural and alignment information.

The current state of the project and the most interesting examples of identified tRNA-derived fragments and their dependence on the tRNA and miRNA biogenesis pathway will be presented.

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199 R3D-2-MSA: The RNA 3D Structure-to-Multiple Sequence Alignment Server

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The RNA 3D Structure-to-Multiple Sequence Alignment web service (R3D-2-MSA) seamlessly links RNA three-dimensional (3D) structures to high quality RNA multiple sequence alignments (MSA) from diverse biological sources. R3D-2-MSA provides manual and programmatic access to curated, representative ribosomal RNA (rRNA) sequence alignments from bacterial, archaeal, eukaryal, and organellar ribosomes, using nucleotide numbers from selected atomic-resolution 3D structures and secondary structure (2D) models for selected organisms representing each domain. A web-based front end is available for manual entry and an API for programmatic access. Users can specify up to five ranges of nucleotides and fifty nucleotide positions per range. The R3D-2-MSA server maps these ranges to the appropriate columns of the corresponding multiple sequence alignment and returns data for display in a web browser, as JSON format for subsequent programmatic use, or as a download in FASTA, Clustal, Stockholm or tab-separated formats. The browser display provides a statistical summary of distinct sequences found and a complete list of taxonomic information, allowing one to explore the phylogenetic variation of the sequences. By linking 3D structures transparently to high-quality multiple sequence alignments, R3D-2-MSA accesses many more sequence variants for RNA structural motifs than are present in solved 3D structures, thus deepening our understanding of RNA evolution and facilitating RNA 3D structural modeling from sequence. The browser also displays the 3D structures of the nucleotides along with the sequence variation, so users can explore structure and sequence simultaneously. By resubmitting the output URL of previous queries users can quickly re-generate results. The service is freely available at http://rna.bgsu.edu/r3d-2-msa.

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200 Interfacial Motifs Improve Sequence-based Prediction of RNA-Protein Interaction Partners in Complexes and Networks

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The interactions between RNA and RNA-binding proteins regulate some of the most fundamental biological processes, disruptions of which are implicated in a wide range of diseases ranging from cancer to Alzheimer's disease. Most available methods for predicting RNA-protein interaction partners are hampered by relatively high false positive prediction rates. We have developed a new sequence-based machine learning method for predicting whether a given RNA-protein pair will interact. RPIMotif implements a Random Forest classifier and uses a feature vector that combines: i) conjoint triad representations of amino acids in protein sequences, ii) tetrad representations of ribonucleotides in RNA sequences, and iii) novel interfacial motifs extracted from known structures of RNA-protein complexes. RPIMotif has a higher true positive rate and a much lower false positive rate than our previously published RPISeq method (1). On an independent dataset of ~11,000 positive examples of experimentally validated RNA-protein interactions and ~1,000 negative examples generated by pairing non-RNA-binding proteins and ncRNAs, RPIMotif predicted partners with a true positive rate (TPR) of 0.96, false positive rate (FPR) of 0.27, and MCC of 0.63; RPISeq values were: TPR 0.97, FPR 0.63, MCC 0.40. Benchmarked performance comparisons of RPIMotif with other methods will be presented. Our results to date indicate that computational tools can be reliable enough to identify likely partners for proteins or RNAs of biomedical importance. Current work is directed at using RPIMotif to identify interaction partners in cellular signalling networks, e.g., long ncRNA-protein interaction networks implicated in cancer.

Reference:

1. Muppirala, U, Honavar, V, Dobbs, D. (2011) Predicting RNA-protein interactions using only sequence information. BMC Bioinformatics. Dec 22;12:489.

201 Spike-In RNA Variants (SIRVs): External transcript isoform controls in RNA-Seq

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RNA spike-in controls devised by the External RNA Controls Consortium (ERCC) are currently applied to assess sensitivity, input / output correlation, differential gene expression, etc. in RNA-Seq experiments. These control RNAs are monoexonic and do not represent transcript isoforms. However, the vast majority of genes in higher eukaryotes undergo alternative splicing, and transcript isoforms are present in concentrations spanning several orders of magnitude. To address this added complexity of transcriptomes, we have designed Spike-In RNA Variants (SIRVs) for the quantification of mRNA isoforms in Next Generation Sequencing (NGS). The initial consideration was to produce sets of transcripts which are variants of a given gene to provide for the training and evaluation of bioinformatics algorithms to accurately quantify, map and assemble isoforms. In detail, we have developed 7 transcript variant sets, based on human gene structures but with artificial sequences. For each of the genes, 6-18 transcript variants were derived either from known, annotated isoforms or additionally designed to comprehensively address alternative splicing, alternative transcription start and end sites, overlapping genes and antisense transcription. The SIRVs are designed to mimic human transcripts closely in terms of length (190-2500 nt) and GC content (30-51%), and the GT-AG exon-intron junction rule was observed. They do not show significant sequence similarities to any sequenced genome or transcriptome when searched against the NCBI database. Therefore, they can be spiked into total RNA from any sequenced organism - also alongside existing ERCC spike-in mixes - and are unambiguously identifiable in the resulting mRNA-Seq NGS data. In an initial experiment, the SIRVs were NGS-sequenced on their own or used as external standard by spiking reference RNAs. The ability of current and newly developed algorithms to identify these known input and to quantify transcript isoforms - also in dilutions - will be presented and discussed.

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202 Combining sequence and structure approaches to asses the landscape of SELEX derived aptamers to ribosomal protein S15

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The fitness landscape conceptually represents the extent to which observed molecules in nature overlap with the set of possible physical solutions to a biological problem. Understanding how peaks and valleys are distributed, irrespective of sequences observed in nature, can illuminate the selective pressures acting on the sequence and structure of a biological molecule. In this study, we use high-throughput sequencing (HTS) of SELEX derived populations to study the landscape of RNAs that can bind to the Geobacillus kaustophilus ribosomal protein S15. This protein is highly conserved among bacteria, yet there are at least four distinct mRNA structures that interact with S15 homologs to regulate gene expression. Given the diversity of natural structures, we wondered how many additional S15 interacting structures have yet to be found. Previous analyses of similar data focus on limiting the search space by choosing functional aptamers with few sequence or structure conformations, or identifying RNA binding motifs using approaches developed for discovering transcription factor binding sites. Unfortunately, sequence motifs have limited application when the protein may rely on a secondary structure motif, and structure based approaches are computationally slow. In order to characterize our HTS population, we leverage both sequence and structure approaches to address these short comings. We found the sequence pools have very high sequence diversity and only a small fraction of sequences are enriched. These enriched sequences form many tight but distinct clusters. We also compared these clusters using k-mer analysis, which revealed no common sequence motifs associated with binding. Using these clusters in secondary structure ensemble based approaches revealed that clusters fold into distinct, relatively different structures. Finally, some sequences identified using each method were experimentally tested for interaction with S15 to verify the efficacy of the analysis. Overall, our results indicate clustering by sequence does identify binders, but the high sequence diversity and high number of singleton clusters suggest under sampling relative to the total population diversity. Furthermore, k-mer analysis shows many different motifs may contribute to binding. The distinct structures found by our clusters suggests that many other RNA structures can bind S15.

203 Optimization of RNA Secondary Structure Prediction from Mapping Data in *Arabidopsis*

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RNA structure plays an important role in post-transcriptional regulation processes such as translation, RNA processing, and RNA stability. Yet, determining structure from sequence alone is a challenge that is currently being addressed through experimental and computational approaches. Recent advances in chemical modification strategies integrate both approaches by incorporating the chemical modification information into computational structure prediction, improving its predictive power. In chemical structure mapping, highly modified nucleotides correlate with a high probability of being unpaired. Rates of RNA modification are detected with reverse transcriptase (RT), which stalls and ceases synthesis at these modified sites. An untreated control experiment is also implemented to determine the natural rate of RT stalling in the absence of modification. Subsequently, RT stop events are counted via high-throughput sequencing, and the degree of each nucleotide's modification is calculated by combining counts from experiment and control.

Recently, several groups have demonstrated the power of this high-throughput strategy *in vitro*, *in vivo*, and at a transcriptome scale. Here, we present a number of analytical tools and approaches to data analysis and use them to investigate existing datasets of chemical modification experiments. We evaluate various analysis strategies and experimental choices with respect to their potential to maximize the structural information extracted from these datasets. We also highlight sources of error and uncertainty per the experimental design or constraints. Finally, we propose a refined model that has the potential to enhance the predictive power of these experimental approaches. Through these methods, we can better understand how to interpret data from chemical modification experiments and translate them into applicable structure predictions. Ultimately, improvements in these experimental methods and their analysis will drive a deeper understanding of RNA structure in biological functions and provide better strategies for the design of experimental RNA structure studies.

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204 lncRNA-RNA interactions across the human transcriptome

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Long non-coding RNAs (lncRNAs) are non-protein coding transcripts longer than 200 nucleotides. Although there is possibility that a fraction of lncRNAs are not functional and represent mere transcriptional noise, a growing body of evidence shows they are engaged in a plethora of molecular functions and have a considerable contribution to the observed diversification of eukaryotic transcriptomes and proteomes. Still, however, only ca. 1% of lncRNAs have well established functions and much remains to be done towards decipherment of their biological roles. One of the least studied aspect of lncRNAs biology is their engagement in gene expression regulation through RNA-RNA interactions. By hybridizing with mate RNA molecules, lncRNAs could potentially participate in modulation of pre-mRNA splicing, RNA editing, mRNA stability control, translation activation, or abrogation of miRNA-induced repression. Here, we present a similarity-search based method for identification of RNA-RNA interactions transcriptome-wide, which enabled us to find 18,871,097 lncRNA-RNA base-pairings across the human transcriptome. Further analyses show that the interactions could affect processing, stability and functions of 57,575 transcripts. An extensive use of RNA-Seq data provided support for approximately one third of the interactions, at least in terms of the two RNA components being co-expressed. We also created an online database to store the RNA-RNA interaction data. Altogether, our results suggest that lncRNA-RNA interactions are broadly used to regulate and diversify the transcriptome.

205 Autonomous Classification of RNA Structure Change

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Mutations in RNA will create a RiboSNitch, if important structural elements are disrupted. We are particularly interested in structural changes that will result in a functional consequence. Recent ultra-hightroughput techniques, such as SHAPE-MaP and PARS, enable the collection of structural RNA information on a genome-wide scale. With the ability to gather genome-wide structural information on RNA, it is important that to accurately classify these structural data in order to identify those structural changes that result in a phenotypic outcome. Furthermore, there are significant differences in signal to noise in transcriptome-wide data sets, such that false-discovery rates of riboSNitches can be significant. We therefore need an approach to determine whether a putative structure change is supported by the data. We set out to develop an automated approach to detect structure change in SHAPE data. We are using a training set of 3130 RNA mutations to detect riboSNitches. Comparison of our autonomous classification system against a crowd-sourced manual classification system for these putative riboSNitches will give insight into how well our autonomous system performs in comparison to the manual system. These data and analyses will also allow us to define the expected minimal change of a mutation in an RNA and thus identify interesting and therefore functional riboSNitches.

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206 Genome-wide RNA-Seq reveals links between the DEAD-box protein Dbp2 and cellular homeostasis through modulating RNA-DNA hybrids

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Cellular homeostasis requires a fine balance between energy uptake and consumption to maximize the cellular growth under different nutritional status. Our recent study in *Saccharomyces cerevisiae* found that the DEAD-box RNA helicase Dbp2 represses the induction of the *GAL* protein-coding genes upon carbon source switch by antagonizing the activating role of the *GAL* lncRNAs. This suggests a role for Dbp2 in gene regulatory programs that are responsive to cellular nutritional status. In addition, Dbp2 is associated with chromatin under typical growth conditions and either a carbon source switch or glucose deprivation results in rapid relocalization of Dbp2 to the cytoplasm, suggesting that Dbp2 integrates gene expression with nutritional status. To determine the entire complement of transcripts whose levels are affected by the absence of *DBP2*, RNA-Seq and strand-specific differential gene expression analysis were conducted in *dbp2*Δ and wild type strains. The results indicate that the expression levels of 30.27% of the protein-coding transcripts and 9.88% of the non-coding transcripts were affected in the absence of *DBP2*. GO annotation reveals overrepresentation of the ATP biosynthesis and carbohydrate transport factors, consistent with a role in energy homeostasis. Furthermore, we found that the target genes of *DBP2* tend to be enriched in RNA-DNA hybrids. We hypothesize that Dbp2 controls gene expression by regulating RNA-DNA hybrid formation, thereby enabling cells to rapidly adapt transcriptional programs in response to extracellular cues to maintain the homeostasis.

207 *RNA-Puzzles*: Critical assessment of RNA structure prediction

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RNA-Puzzles is a CASP-like collective blind experiment for the evaluation of RNA 3-dimensional structure prediction. The primary aims of *RNA-Puzzles* are to determine the capabilities and limitations of current methods of 3D RNA structure prediction based on sequence, to find whether and how progress has been made, and to illustrate whether there are specific bottlenecks that hold back the field.

Twelve puzzles have been set up and several assessments have been published. Groups of modelers around the world participate in this collective effort, the programs in prediction including RNAComposer, ModeRNA/SimRNA, Vfold, Rosetta, DMD, MC-Fold, 3dRNA, and AMBER refinement. Various criteria for structure assessment are included, for instance, RMSD, Deformation Profile, interaction network fidelity, atomic clashes and torsion angle deviations.

After the assessment, we find that some of the template-based prediction structures are predicted with high accuracy (e.g. Problem4) while large *de novo* prediction structures can also achieve a similar topology to the native structure with the help of contact constraints obtained from solution experiments, such as SHAPE, DMS, CMCT, and mutate-and-map.

According to the current puzzle assessments, some prediction methods can achieve a reasonable level of accuracy depending on the nature and size of the structure. Bottlenecks lie in: i) the accurate prediction of non-Watson-Crick base pairings and RNA modules; ii) achieving the global topology with correct structural geometries; iii) refining the predicted structures and removing deviations in bond lengths, bond angles and atomic clashes; iv) assessing the regions of RNA structures with mobility or least predictive power.

All submitted models and assessment results are available at http://ahsoka.u-strasbg.fr/rnapuzzles/.

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208 Dynamic analysis of Alternative PolyAdenylation from RNA-Seq (DaPars) Reveals landscape of 3' UTR usage across 7 tumor types

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The dynamic usage of mRNA 3′ untranslated region (3′UTR) resulting from alternative polyadenylation (APA) is emerging as a pervasive mechanism to regulate approximately 70% of human genes. The importance of APA in human diseases such as cancer is only beginning to be appreciated. Current APA profiling protocols use the partitioning and fragmentation of mRNA to enrich for polyA sites followed by high throughput sequencing (polyA-seq). These polyA-seq protocols, although powerful, have not been widely adopted. Therefore, global studies of APA in cancer are very limited. In contrast, whole transcriptome RNA-seq has been broadly employed in almost every large-scale genomics project, including The Cancer Genome Atlas (TCGA). We therefore developed a novel bioinformatics algorithm, termed Dynamic analysis of Alternative PolyAdenylation from RNA-Seq (DaPars), to directly infer dynamic APA events through standard RNA-seq. DaPars used a linear regression model to identify the exact location of the *de novo* APA site, and quantify the lengthening or shortening of 3′UTRs between different conditions.

When applied to 358 TCGA tumor/normal pairs across 7 tumor types, DaPars reveals 1,346 genes with recurrent and tumor-specific APAs. Most APA genes (91%) have shorter 3' UTRs in tumors that can avoid miRNA-mediated repression, including glutaminase (GLS), a key metabolic enzyme for tumor proliferation. Interestingly, selected APA events add strong prognostic power beyond common clinical and molecular variables, suggesting their potential as novel prognostic biomarkers. Finally, our results implicate CstF64, an essential polyadenylation factor, as a master regulator of 3' UTR shortening across multiple tumor types. Together, through the reanalysis of TCGA RNA-seq data using DaPars, our work demonstrates the importance of dynamic APA in cancer and expands our knowledge of the mechanisms and consequences of APA regulation during tumorigenesis.

209 STAR3D: a stack-based RNA 3D structural alignment tool

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The various roles of versatile non-coding RNAs typically require the attainment of complex high-order structures. Therefore, comparing the 3D structures of RNA molecules can yield in-depth understanding of their function conservation and evolutionary history. Recently, many powerful computational tools have been developed to align the RNA 3D structures. Although some rely on both backbone conformations and base-pairing interactions, none of them considers the entire hierarchical formation of the RNA secondary structure. One of the major reasons for this problem is that matching 2D structures to the 3D coordinates directly is particularly time-consuming. Here, we propose a novel RNA 3D structural alignment tool, STAR3D, to take into full account the stack relationship without the complicated comparison of the secondary structures. The 3D conserved stacks in the inputs are identified by joining small building components, and then combined into a tree-like consensus of the secondary structures. After that, the loop regions are compared one-to-one in accordance with their relative positions in the consensus tree. The experimental results show that the alignments generated by STAR3D are more accurate for both non-homologous and homologous RNAs than other state-of-the-art tools with shorter running time.

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210 Inference of recurrent RNA 3D motifs from sequence

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Predicting RNA 3D structure from sequence is a major challenge in molecular biophysics. An important sub-goal is to accurately infer recurrent 3D motifs from RNA internal and hairpin loop sequences, given a correct 2D structure. We have developed and validated new probabilistic models for 3D motif identification based on hybrid Stochastic Context-Free Grammars and Markov Random Fields (SCFG/MRF). The SCFG/MRF models are constructed using structural information from atomic-resolution RNA 3D structures. To parameterize each model, we use all instances of each motif found in the RNA 3D Motif Atlas and the associated annotations of pairwise nucleotide interactions. Knowledge of isostericity relations for non-Watson-Crick basepairs and their substitution patterns is used in scoring sequence variants. SCFG techniques account for nested pairs and insertions, while MRF ideas handle crossing interactions and base triples. Given the sequence of an internal or hairpin loop from a secondary structure as input, we align the sequence to each probabilistic model and use alignment score and edit distance to known sequence variants together to match to motif groups. The SCFG/MRF models are sufficiently distinct in sequence space that they usually match individual sequences from 3D motif instances to the correct model. We use test sets of randomly-generated sequences to set acceptance and rejections for each motif group and thus control the false positive rate. Validation was carried out using novel sequence variants from multiple sequence alignments. The JAR3D software for motif inference is available for download on Github and a web server is available for inference of single loops or all loops in a folded RNA. JAR3D is structured to automatically incorporate new motifs as they accumulate in the RNA 3D Motif Atlas when new structures are solved, and so its predictive power will grow over time.

211 JAR3D: A Website for Identifying RNA 3D Motifs in Secondary Structures

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A wide variety of functional ncRNAs are being sequenced and their secondary structures predicted. Far fewer ncRNAs have had their 3D structures determined, but those that have been solved show a rich variety of 3D motifs, especially in the "loop" regions between helices. Many of these motifs mediate important RNA-RNA or RNA-protein interactions. Recurrent internal loop and hairpin loop motifs are collected and clustered by the RNA 3D Motif Atlas which provides primary data for JAR3D, a new web service that connects RNA hairpin and internal loop sequences to known 3D instances of these motifs. JAR3D ("Java-based Alignment of RNA using 3D structure information") is freely available at http://rna.bgsu.edu/jar3d

Users of JAR3D can input one or more sequences of a given RNA hairpin or internal loop and JAR3D scores the sequence(s) against all motif groups, returning the top 10 matches, diagnostic information, and links to RNA 3D Motif Atlas entries. Exact sequence matches are noted, making JAR3D a useful way to look up motif groups for a known 3D sequence. Moreover, JAR3D can match novel sequences to 3D motif groups and also evaluates the quality of matches to avoid false positives. Matches are evaluated using a combination of edit distance (between the input sequence(s) and the sequences of known 3D instances) and alignment score (the score of the sequence(s) against probabilistic models for likely sequence variability, based on the 3D instances and our understanding of sequence variability in RNA basepairs and base-backbone interactions). Once a match is identified, JAR3D can align the input sequence(s) to the motif group and thereby to known 3D instances of the motif, allowing the user to evaluate the correspondence on a fine-grained level and take a further step toward 3D modeling of the particular sequence(s).

Additionally, to facilitate analysis of the loops in an entire RNA, users can input one or more aligned full RNA sequences (with length up to 500 nucleotides) along with a predicted secondary structure, and JAR3D will separate out the individual internal and hairpin loops and match them to motif groups.

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212 The Roles of Active Site Guanines in the Hairpin and glmS Ribozymes

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The small ribozymes often involve a specific active site guanine that plays a crucial catalytic role. We used molecular dynamics (MD) simulations and hybrid quantum chemical/molecular mechanical (QM/MM) calculations to probe the roles of the active site guanines in hairpin and *glmS* ribozymes. In both cases, the biochemical data have suggested a crucial catalytic role for G8 and G40 active site guanines in hairpin and *glmS* ribozyme, respectively. Our older MD simulations suggested that the deprotonated form these guanines are not tolerated by the active sites in both studied ribozymes.[1,2] However, our more recent simulations show that the observed expulsion of the deprotonated guanine form the active site of both ribozymes might be caused by not enough accurate description of non-canonical conformations of sugar-phosphate backbone and hydrogen bonding interactions in contemporary force fields.[3] In addition, the QM/MM calculations suggested that in both cases the deprotonated guanines are sufficiently reactive to overcome the thermodynamic penalty arising from their rare protonation states, and thus are able to act as a general base.[4,5,6]

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213 Investigation of the *glmS* Ribozyme: Role of the Active Site Guanosine and Exogenous Species in the Self-Cleavage Mechanism

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The *glmS* ribozyme is a small, self-cleaving RNA found in many gram-positive bacteria. Along with other small ribozymes, it cleaves through a general acid-general base mechanism wherein the 2'-hydroxyl at the cleavage site attacks the adjacent phosphate, resulting in a 2',3'-cyclic phosphate and a 5'-hydroxyl product. The *glmS* ribozyme requires the presence of a small molecule coenzyme, glucosamine-6-phosphate (GlcN6P), which appears to serve as the general acid, thus making the RNA a riboswitch-ribozyme. Key active site residues aid in the binding of GlcN6P, and one residue in particular, G33 in the *B. anthracis* construct, is well-positioned to act as the general base. However, studies have shown that G33 exhibits a pK_a shifted away from neutrality (≥10 compared to the pK_a of 9.2 for free guanine in solution) [1], which is thought to be detrimental for general acid-base catalysis. As demonstrated by recent work from our labs [2], this basic-shifted pK_a may actually aid the function of the general base, wherein a pK_a that more closely matches the high pK_a of the 2'-hydroxyl nucleophile is better suited to abstract its proton. Theoretical studies and pK_a calculations suggest that G33 may simply serve as a hydrogen bond acceptor to the 2'-hydroxyl nucleophile until attack on the adjacent phosphate begins, whereupon G33 accepts the proton from the 2'-hydroxyl, an event facilitated by the elevated pK_a of G33. We will also present our latest experimental results on the dependence of the reaction on exogenous species including metal ions.

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214 Engineered riboswitch that aminoacylates tRNA with unnatural amino acid

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Flexizymes are a group of artificial ribozymes that catalyze aminoacylation of tRNA using a chemically synthesized amino acid substrate. In nature, this is a reaction performed by protein aminoacyl-tRNA synthetases (ARS) [1]. tRNAs artificially charged with unnatural amino acids are especially useful in biological applications, as they can be used for genetic code reprograming and producing proteins or peptides with novel functionalities [2].

It has been shown that the activity of flexizymes is in general not restricted by the functional group of the amino acid substrate, thereby making these ribozymes highly promiscuous or "flexible" towards the amino acid substrates [1]. However, one drawback that limits the future of flexizyme-based genetic code reprograming is that flexizymes lack the ability to bind and charge a specific tRNA. Being able to specifically catalyze the aminoacylation reaction with one tRNA species, such as the orthogonal tRNA, would enable the ribozyme to reprogram the genetic code *in vitro* or *in vivo*. In addition, a ribozyme reminiscent of protein ARS with regard to tRNA binding would provide evidence in support of the RNA world hypothesis. In this project, we use a rational engineering approach to redesign the flexizyme so that it binds tRNA specifically. We propose that 1) similar to the natural aminoacyl tRNA synthetase, this new ribozyme would identify and bind the discriminator base as well as the anticodon of tRNA 2) the specificity of this new ribozyme can be allosterically reprogrammed by introducing mutations to its anticodon recognition motif (*i.e.*, the specifier).

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215 Studying RNA Backbone and Base Dynamics in the Kink-Turn Motif and Leader-Linker Interaction of the Glycine Riboswitch using Site-Directed Spin Labeling and Electron Paramagnetic Resonance Spectroscopy.

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Genetic regulation effectuated by mRNA riboswitches is associated with conformational changes and structural rearrangements in RNA secondary and tertiary structure. In the glycine riboswitch, two aptamer domains selectively recognize and bind the cognate ligand glycine and, through subsequent interaction with a downstream expression platform, induce regulation of genes associated with glycine metabolism. The recently described kink-turn motif and leader-linker interaction in the glycine riboswitch has been investigated using biochemical methods and was shown to play a functional role in the ligand binding process. Here we report results of site-directed spin labeling (SDSL) and continuous wave electron paramagnetic resonance spectroscopy (CW EPR) experiments whereby we characterized local RNA backbone and base dynamics of select sites within the kink turn motif and leader-linker interaction of the *Vibrio cholerae (VC)* glycine riboswitch in varying environments.

CW EPR, when used with SDSL, is an effective technique for investigating changes in site-specific dynamics within biological systems. Advancements in RNA solid state synthesis and RNA SDSL have allowed for nitroxide spin probes to be efficiently incorporated into various sites within RNA molecules. Changes in conformation and dynamics in the labeled regions directly influence CW EPR spectra and, consequently, RNA structural modulation can be monitored through this method. Here, we incorporate spin labels into the *VC* glycine riboswitch through the use of optimized ligation methodologies through which a relatively short (20 nt), synthetically modified and spin-labeled RNA fragment is joined to a larger in vitro transcribed fragment (212 nt). We performed CW EPR experiments at multiple frequencies and empirical analysis of spectral line shapes was used to describe changes in local riboswitch backbone and base dynamics. More specifically, variable temperature spectra for four riboswitch folding states in the absence or presence of salts and glycine ligand were collected to monitor changes in dynamics upon riboswitch folding. Our results provide detailed characterization of the leader-linker interaction and support current understanding of characteristic structural features of a kink-turn motif upon riboswitch RNA folding and ligand binding.

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216 Spin-Labeling Magnetic Resonance Studies of Conformational Dynamics and Flexibility of the 232 nt Glycine Riboswitch

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Site-directed spin-labeling (SDSL) coupled with electron paramagnetic resonance (EPR) has become a popular tool for characterizing conformational flexibility and dynamics in protein and lipid assemblies. Here, we show our achievements made in developing a suite of spin-labeling based magnetic resonance (MR) approaches for characterizing dynamics, conformational sampling, and local hydration environment of the glycine riboswitch. A series of singly and doubly spin-labeled 232 nt RNAs were prepared via splinted ligation of a shorter spin-labeled synthetic oligonucleotide with a larger fragment generated from in vitro transcription. For these investigations, spin labels have been incorporated at either modified backbone or base locations. Continuous wave (CW) EPR line shapes and distance distribution profiles from double electron-electron resonance (DEER) pulse EPR spectroscopy reveal changes in backbone dynamics and conformational sampling of the kink-turn motif under various environmental conditions such as changes in ionic strength or presence of magnesium and cognate ligand - glycine. The results are consistent with trends of biochemical studies and show the utility of DEER applications to characterize the conformational sampling, flexibility, and conformational changes of large RNAs.

Additionally, we have assembled a custom microwave transmitter that is interfaced with our existing EPR/NMR equipment for performing novel, low-field (0.35 T), Overhauser dynamic nuclear polarization (ODNP) enhanced NMR spectroscopy that is useful for interrogating the hydration landscape. The ODNP NMR technique allows one to probe changes in dynamic motions of H2O in the spin-labeled regions, thus making connections between changes in conformational sampling determined from line shape and distance measurements from EPR and local hydration dynamics under induced environmental conditions and during RNA folding. Taken together this work represents one of the first SDSL MR characterizations of large SL-riboswitch constructs prepared by splinted ligation methods; nicely laying the foundation for more in-depth investigations into the structure, dynamics, and folding of the glycine riboswitch to elucidate molecular mechanisms of regulation and function.

217 Twister ribozymes as highly versatile expression platforms for artificial riboswitches

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The recent description of a new class of small endonucleolytic ribozymes termed "Twister" opened new avenues into the field of RNA biology and synthetic biology. Here we present for the first time a series of approaches and designs for the employment of the twister ribozyme as an expression platform in the construction of artificial riboswitches that are able to control gene expression in *E. coli*. In our design the ribozyme motif sequesters the ribosome binding site allowing control of translation initiation. The twister ribozyme reveals to be an outstandingly flexible expression platform and enables the construction of many different one- and two-input regulators for controlling gene expression in a ligand-dependent manner.

We first generated one-input dependent riboswitches some of which outperform previous artificial genetic regulators, by attaching aptamer domains to two different sites of the catalytic domain. The fact that the twister ribozyme scaffold offers at least two independent sites for attaching ligand-sensing aptamer domains, opens the way to the development of Boolean logic operators at the post-transcriptional level by constructing compact two-input riboswitches that sense and respond to two small molecular signals at once. Using both a rational design and a screening strategies we were able to generate a broad range of binary Boolean logic gates such as AND, NAND, OR, NOR, ANDNOT and ORNOT operators.

Besides the important implications for synthetic biology, our results represent the first proof for the involvement of twister ribozymes in the control of gene expression in bacteria. The observed versatility in gene-regulatory setups hints at possible roles of naturally occurring ribozyme motifs.

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218 Bridging the Gap Between RNA Based Protocells and Multiple Turnover in RNA Ribozymes

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It is widely believed that RNA once acted as both the heredity material and cellular machinery of early life. While this hypothesis offers many attractive features that support RNA as being active in this role, there are still many challenges protocells would have had to overcome without being afforded the luxury of modern cellular machinery. One important function for the proliferation of life using a polymeric genetic storage material like nucleic acids involves the replication of that material. This becomes problematic when strand dissociation and exchange becomes rate limiting. In contemporary cells, protein helicases are used to separate double-stranded nucleic acids. Another important function includes concentrating otherwise dilute nucleic acids into a small enough volume where catalysis and reactions can occur. In areas such as thermal vents and rock surfaces, the effective volume can be vast, causing nucleic acid concentrations to be very low. With so many scenarios plausible for the emergence of life, all avenues toward addressing these two challenges of strand separation and co-localization of genetic material must be explored.

Herein we report that alkaline conditions drive product release during a two-piece hammerhead ribozyme reaction under single-turnover and multiple-turnover conditions. We chose the hammerhead ribozyme for study of RNA catalysis. This ribozyme is ideal for studying effects of pH and compartmentalization on catalysis because its kinetics have been well-characterized under a variety of conditions. We have mimicked intracellular compartmentalization and crowding by producing complex coacervates composed of anionic RNA nucleotides with cationic poly(allylamine). We characterize complex coacervates composed of mononucleotides and poly(allylamine) under varying pH and ionic strength, as well as the partitioning properties of RNA and magnesium into these systems. Excellent partitioning of RNA was observed under all conditions, while changes in Mg^{2+} partitioning and surface charge were seen as a function of ionic strength and pH, leading to a better understanding of environments where coacervation of nucleic acid material would have occurred. In the future, the combination of these two scenarios will allow for the production of a functional protocell able to undergo strand exchange and catalysis under multiple turnover conditions.

219 Antibiotic Drug Development Directed Against the T-Box Mechanism of Gram Positive Bacteria

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With the increasing emergence of multi-drug resistance bacteria, development of new antibiotics directed against novel targets is of critical importance. The T-box Specifier Loop of Gram positive bacteria represents one such novel target for drug discovery and design. Given the essential nature of T-box regulated operons we hypothesize that alterations of T-box function will result in bacterial cell death or growth arrest. We have used in silico analysis of the T-box Specifier Loop to identify small compounds likely to disrupt T-box function. These compounds were tested against a variety of Gram positive and negative bacteria with microdilution assays to determine antibacterial activity and spectrum of activity. From these assays we have identified a hit compound that displays activity with a variety of Gram positive bacteria only, suggesting target specificity. Mass spectrometry of hit compound and T-box Specifier Loop indicates target binding in vitro. Additionally, mammalian cell cytotoxicity assays conducted with this hit compound and human cell lines demonstrate a low amount of cytotoxic effects in tissue culture. Structure activity relationship assays are promising and have shed light on structural components necessary for antibacterial activity. Therefore, given the low mammalian cytotoxicity and effective antibacterial activity against Gram positive pathogens further exploration of the T-box Specifier Loop and small compounds that disrupt its function is warranted for antibiotic drug discovery.

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220 Structure and Dynamics of the Twister Ribozyme Suggest a New Mode for RNA Catalysis

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The twister ribozyme is a recently discovered self-cleaving nucleolytic ribozyme that has been estimated to have one of the fastest known catalytic rates for O2'-transphosphorylation. Despite experimental effort to characterize the ribozyme both structurally and biochemically, the specific roles of key conserved residues in the reaction remain elusive. We present a series of molecular dynamics simulations of the twister ribozyme at different stages along the reaction pathway in an effort to provide a model of the active site and a description of the residues directly involved in catalysis. Simulation results suggest that the extrusion of U6 from the active site to form a crystal packing contact is not representative of a catalytically active conformation. Relaxation in solution results in a stacking interaction with G45 that may be catalytically relevant. Analysis of a transition state mimic model provides evidence that extrusion of U6 disturbs the hydrogen bonding network that allows G45 to stabilize the negative charge on the scissile phosphate during the transition state. This hydrogen bonding between G45 and both of the non-bridging pro-R oxygen and the nucleophile, supports the hypothesis that this residue acts to promote "inline" conformations and facilitate activation of the nucleophile as a general base in the reaction. In the crystal stucture, a well conserved hydrogen bonding network constrains A7 to the syn conformation positioning the N3 amine within 4.5 Å of the O5' leaving group. Finally, we propose that the pKa of A7:N3 could be significantly shifted towards neutrality by the ribozyme environment, and thus be poised to act as the general acid in the reaction.

221 Revisiting the Catalytic Mechanism of Hammerhead Ribozyme

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The hammerhead Ribozyme (HHRz) catalyzes the site specific cleavage of a single phosphodiester bond within its RNA substrate. Originally discovered in plant viroids, thousands of HHRz-like sequences have been found in all domains of life. The cleavage mechanism of HHRz has been studied extensively but many questions still persist. In the crystal structures, G12 appears well positioned to serve as a general base and thereby activate the 2'-hydroxyl of C17 for an in-line nucleophilic attack on the scissile phosphate. The 2'-OH of G8 is proposed to be the general acid. However, neither G12 nor G8 are expected to be a particularly powerful general base or general acid. In addition, certain discrepancies exist between the crystal structure and the biochemical data. For example, the role of divalent metals is still controversial because the crystal structures do not show a divalent metal within the active site of the HHRz. However, biochemical data suggests that divalent metals greatly accelerate HHRz reaction rate and appear to interact with the pro-Rp oxygen of the scissile phosphate. We studied different HHRz mutants using both kinetics and X-ray crystallography to address these discrepancies. Our results suggest that HHRz may be using a metal mediated catalysis to cleave the phosphodiester bond. These studies provide new insights and new questions about the catalytic mechanism of HHRz.

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222 In vitro evolution of distinct self-cleaving ribozymes in diverse environments

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Folding and catalysis by biopolymers is exquisitely tuned to the specific chemical environments in which they evolve. Understanding evolution of biopolymer function, therefore, requires a determination of the impact of the local environment on the distribution of functional biopolymers in sequence space. *In vitro* evolution experiments have long been used to evaluate the potential roles of RNA in the origin and early evolution of life; however, the conditions under which these experiments have been conducted do not reflect our understanding of chemical environments on the early earth. To test the impact of environmental factors relevant to RNA's potential role in the earliest forms of life, we evolved populations of self-cleaving ribozymes in an anoxic atmosphere with varying pH in the presence of either Fe^{2+} or Mg^{2+}. Establishing the impact of Fe^{2+} and pH on the evolution of ribozymes is relevant to RNA's role in early life due to the abundance of soluble Fe^{2+}, and wide range of pH values for environments in which life may have first evolved. Populations evolved under different conditions are dominated by different RNA sequences and secondary structures, demonstrating global differences in the underlying fitness landscapes. Our comparison of RNA populations reveals that counterion identity and pH have a dramatic impact on the evolution of RNA catalysis, and therefore represent critical factors in establishing the potential role of RNA in origin and early evolution of life.

223 Exploiting the Ever Expanding Chemical Repertoire of RNA Catalysis:Ribozyme Catalyzed Nucleobase Modification

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Ribozyme 1.140 is an artificial ribozyme that came out of a selection for self-thiophosphorylation. To study the ribozyme-catalyzed product in more detail, we separated the catalytic and substrate functions into individual polynucleotide chains. Surprisingly, both RNA and DNA substrate strands were phosphorylated by the ribozyme, even though DNA lacks the 2'OH that serves as phosphoryl acceptor in all other known kinase ribozymes. These results along with additional experiments establish the nucleobase of G2 as the phosphoryl acceptor. We then mutated G2 with different nucleotide analogs where only 7-deaza-dG and 2-Aminopurine version of substrate showed evidence of thiophosphorylated product, while Inosine version of the substrate showed no evidence of thiophosphorylation, thus establishing that -N2 exocyclic amine is the likely site of modification. This is the first observation of ribozyme-catalyzed covalent modification of the nucleobase.

We engineered the ribozyme to target three functional RNA molecules: an ATP binding RNA Aptamer, a fluorescent "Mango" aptamer and a hammerhead ribozyme. We demonstrated that a functional ATP binding aptamer loses its ligand binding function upon ribozyme-catalyzed modification. We have shown that activity of the fluorescent Mango aptamer can be controlled by our trans acting ribozyme. Recent developments in Fluorescent RNA aptamers are promising for RNA based synthetic biology, thus regulation of the fluorescence might be of interest to the field. The target site for covalent modification in the hammerhead ribozyme (HHRz) lies outside the catalytic core but within a loop that

forms tertiary interactions required for low Mg^{2+} activity. Covalent modification by ribozyme 1.140 resulted in loss of activity in the HHRz below 1 mM Mg^{2+}, but the activity was fully restored by elevating the Mg^{2+} concentration to 20 mM. Thus, ribozyme-mediated covalent modification can not only be used to completely "turn off" functional RNAs but may also be used to fine-tune the activity of desired target RNAs. Our study shows that the chemistries catalyzed by in vitro selected ribozymes can potentially be exploited to build modulatable components for synthetic and natural biological circuits or therapeutics.

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224 Directed Evolution of Nucleoside Kinase Ribozymes that target a quasi-diffusible substrate.

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The RNA World hypothesis posits that RNA played an important role during the origin and evolution of life by acting as both genetic information carrier and catalyst. The existence of catalytic RNAs and regulatory RNAs strengthens this hypothesis and provides RNA tools for synthetic biology. As phosphoryl transfer is one of the most fundamental chemical reactions in modern biology, the study of RNA enzymes (Ribozymes) with kinase activity is of great interest.

In vitro evolution has yielded many kinase ribozymes and DNAzymes; however, previous strategies for *ribozyme* selections have had few or no constraints on phosphoryl acceptor sites, ligation-based strategy has been used previously to select *DNAzymes* that self-phosphorylate the 5'OH of the polynucleotide chain[1]. Many ribozymes that phosphorylate polynucleotide chains exist but phosphorylation of freely diffusible metabolites by ribozymes remains largely unexplored. To address this issue, our lab has developed a ligation-based strategy for the selection of nucleoside kinase ribozymes that specifically requires the evolving ribozymes to phosphorylate the 5'OH of a tethered mononucleoside. We rationally designed the initial library by seeding with ATP and GTP aptamers within a structural scaffold that is organized by a tetraloop-tetraloop receptor element to nucleate folding and to increase the frequency of active ribozymes. After 8 rounds of selection, we generated ribozymes that use ATP as the donor and phosphorylate the 5'OH of a Guanosine that is flexibly linked to the ribozymes via hexaethylene glycol (HEG). Our study will enable us to better understand the capabilities and limitations to catalyze chemical reactions in a multiple turnover fashion.

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225 Pushing molecular mimicry to the extreme: S15 and its regulatory mRNAs

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Bacteria have evolved an elegant negative feedback mechanism to regulate their ribosomal protein (r-protein) expression whereby a given r-protein binds a structured region in the 5'-UTR of its own transcript. In most cases this mRNA structure shares striking homology to the region of rRNA the r-protein binds and the mRNA mimics it to compete for r-protein binding. Ribosomal protein S15 appears to be an exception to this paradigm. The rRNA secondary structure S15 binds is widely distributed among bacterial phyla, consisting of a GU/GC motif ~1 helical turn from a GGC in a 3-helix junction. Despite this, four different natural mRNA regulatory structures exist in different bacterial phyla for S15. It remains unknown whether the predicted secondary structures of these mRNAs conceal mimicry to rRNA, whether there are mRNA-specific binding motifs, or whether there are phyla-specific binding profiles for these mRNA regulators. We used SELEX to identify specific RNA sequences or motifs that S15 recognizes with high affinity. Structure probing and mutagenesis experiments suggest one of these synthetic RNAs for S15 shares a GU/GC motif with rRNA, yet has a distinct overall secondary structure from all other RNAs. We explored the binding between an example of each mRNA and their S15 homologs using in vivo reporter assays as well as in vitro binding assays. Based on these studies we present a model postulating that the plastic nature of RNA enables the S15 mRNA regulators to fold into a variety of different secondary structures that hide at least one of the rRNA-binding motifs, making them all distinct yet partial mimics of rRNA.

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226 Defining Cooperative Interaction Networks in RNA

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In addition to the well-known role of RNA in protein coding, non-coding RNAs function in numerous essential biological processes, including membrane protein targeting, gene expression and regulation, and genome maintenance. Furthermore, recent years have seen an explosion in the discovery of new non-coding RNAs, often of unknown function. To accomplish their diverse functions, many non-coding RNAs adopt three-dimensional structures that bring relevant chemical groups together to allow specific recognition of binding partners. Although structural motifs and functional groups have been identified within many RNAs, the underlying physical properties and mechanisms that give rise to a functional RNA are largely unknown. To identify the connections between structural elements that facilitate active site interactions in a folded RNA and determine how their effects are communicated through the RNA structure, I am applying recent RNA array technology to develop a high-throughput method for measuring the thermodynamic and kinetic effects of single and combinatorial mutagenesis in the *Tetrahymena* group I intron. This method allows *in situ* transcription on an Illumina MiSeq platform and quantitatively monitors reaction progress using an optical readout. Initial experiments indicate that the kinetic behavior of the group I intron on the array mirrors that in solution for the wild-type sequence and several previously characterized sequence variants, strongly supporting the efficacy of this approach. These functional data will be used to determine the effects of mutations on individual reaction steps and develop physical models for the connectivity within the RNA, the predictive value of which will be tested by further functional studies and chemical probing techniques to elucidate the structural perturbations caused by mutagenesis. The combined structural and functional data for single and combinatorial group I intron variants will indicate the degree of connectivity or independence within an RNA, delineate cooperative interaction networks, and reveal the structural connections underlying observed functional effects.

227 Kinetic mechanism of glycine sensing by single T-box riboswitches

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T-box riboswitches critically control the expression of proteins involved in tRNA charging, amino acid biosynthesis and transport in Gram-positive bacteria by sensing the intracellular ratio of charged to uncharged tRNA. Unlike typical riboswitches that sense small metabolites, the T-box riboswitch is unique in its ability to recognize a complex macromolecular ligand, tRNA. Among the various T-box riboswitches, the *glyQS* RNA from *B. subtilis* has been biochemically well characterized due to its relatively limited size. It contains a long stem-I, a single stranded region with stem-III, followed by an expression platform that can form mutually exclusive anti-terminator (AT) or terminator hairpins. Stabilization of the AT hairpin by uncharged tRNA during transcription is thought to result in a full-length mRNA transcript, while the presence of an amino acid on the 3'-end sterically destabilizes a key tRNA interaction with the AT hairpin, resulting in premature transcription termination. Although a crystal structure of the stem-I:tRNA complex was recently solved, the conformational features and ligand sensing dynamics of the full T-box riboswitch, as well as the roles of conserved motifs in tRNA binding, are still poorly understood. Furthermore, how the presence of the small amino acid glycine on one end of such a large ligand affects riboswitching has remained unclear. Using single molecule fluorescence resonance energy transfer (smFRET), we first investigated the solution conformation of the full-length *glyQS* T-box riboswitch and several variants. We demonstrate that tRNA binding does not induce large-scale conformational changes in the riboswitch, suggesting a pre-organized conformation. In addition, we developed a single molecule coincidence analysis that directly assesses the tRNA binding and dissociation kinetics. We show that, contrary to previous ITC results obtained at much higher RNA concentrations, stem-I alone is insufficient to stably anchor tRNA, which can only be achieved in the presence of the AT hairpin. Finally, our results demonstrate that the conserved double T-loop motif only slightly accelerates tRNA association, whereas tRNA aminoacylation significantly destabilizes binding by greatly accelerating ligand dissociation. Based on our results, we propose a kinetic partitioning model for the molecular mechanism of co-transcriptional T-box riboswitching.

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228 Genome-Wide Discovery of Riboswitches by RNA Structure Profiling

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Riboswitches are RNA-based genetic switches that play an important role in bacterial metabolism and survival. Mostly found in the untranslated regions of mRNAs, riboswitches bind directly to metabolites to undergo conformational changes to alter gene expression outcomes by regulating transcription, translation and RNA decay. Existing approaches to identify riboswitches rely on comparative analysis to look for sequence and structure homologies to known riboswitches. Such approaches prevent us from recognizing alternate folds to previously studied ligands, and from finding new classes of riboswitches that bind to novel ligands. Therefore, to address the need for an unbiased approach for riboswitch discovery, we developed a new framework named PARCEL (for Parallel Analysis of RNA Conformations Exposed to Ligand binding), that incorporates high-throughput experimental detection of ligand-induced RNA structural changes to identify riboswitches genome-wide. We applied PARCEL to the transcriptomes of *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*, and identified 20 out of 24 existing TPP, FMN and SAM riboswitches, as well as a large number of new putative riboswitches in these three bacterial speces. Interestingly, new riboswitches take on different folds from the known consensus to bind to their ligands. Moreover, contrary to previous findings, they are found in both coding and non-coding regions of the transcriptome. They are also found in pathways outside of the metabolic and biosynthetic pathways of their ligands. These findings reveal that riboswitches are much more wide-spread than previously appreciated, and provide insights into the diverse properties and distribution of riboswitches. Extending PARCEL to other species, including eukaryotic organisms, will greatly expand our understanding of RNA-based gene regulation across kingdoms.

229 Uncovering new autocatalytic ribozymes for aminoacylation

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Ribozymes, also termed catalytic RNA molecules, have been extensively investigated by *in vitro* SELEX experiments, and characterized by kinetic assays. Ribozymes are involved in RNA cleavage, ligation, capping, polymerization, phosphorylation and acyl activation. RNA aminoacylation plays an important role in the evolution from the late RNA world to the modern DNA and protein world. Several ribozymes catalyzing amino acid transfer from various activating groups (such as AMP) have already been selected and characterized in the past two decades, documenting the possibility of transesterification in the absence of aminoacyl tRNA synthetase.

With a newly designed SELEX protocol minimizing the burden of constant tracks necessary for PCR amplification, we are interested in uncovering small ribozymes of the order of 20 nucleotides that could catalyze transesterification while using a terminal loop as the binding site for activated amino acids. The rational of this design lies in the possibility of observing an amino acid selectivity based on the nature of the loop, and thus implement some minimal coding rules for aminoacylation. Furthermore, stem-loop ribozymes constitute plausible tRNA candidates for an elementary translation.

We present the validation of an optimized experimental protocol that is significantly reducing the burden of constant tracks compared with existing protocols, while at the same time being very specific for the selection of 2' or 3' aminoacylated RNA. Our *in vitro* selection starts with a random RNA pool with as little as 10 conserved nucleotides, and a random track varying from about 10 to 30 nucleotides. Following our protocol, RT-PCR can easily re-generate a selected pool that can be used for the next round selection. We present results obtained after just a few cycles of selection.

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230 Ribose Dynamics of the Leadzyme

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The lead-dependent ribozyme or leadzyme is among the smallest of the known catalytic RNAs. Lead-dependent cleavage occurs between C6 and G7 within a six-nucleotide asymmetric internal loop. In structures of the leadzyme determined by NMR spectroscopy and X-ray crystallography the scissile phosphate is not positioned for in-line nucleophilic attack. This necessitates a conformational rearrangement of the active site to be consistent with the proposed transition state. We have previously probed the ribose structure and dynamics of the guanosine residues in the asymmetric loop using LNA (locked nucleic acid). We now report a study of the relationship between conformational dynamics and catalytic function at the cleavage-site residue C6. We have substituted C6 with a 2'-hydroxyl-bicyclo [3.1.0] hexane nucleotide, which is restricted to the C3'-endo ribose conformation but, unlike LNA, maintains the nucleophilic 2'-hydroxyl, and found a drastic attenuation of self-cleavage activity. In parallel, we are undertaking NMR spin relaxation experiments to examine ribose repuckering events at the C6 position using our previously-reported site-specific ^{13}C isotopic labeling scheme. These studies in totality will yield improved understanding of the relationship between relationship between RNA backbone dynamics and cleavage mechanism in the leadzyme.

231 Characterization and Optimization of the 10DM24 Deoxyribozyme as a General Tool for Fluorophore Labeling of Large, Unstructured RNA Transcript

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Fluorescent techniques can offer unique insights into the mechanisms and dynamic properties of biological systems. Preparing long, site-specifically modified fluorescent mRNAs by splinted ligation can be inefficient, time consuming, and costly. As an alternative to traditional labeling techniques, we are using the 10DM24 deoxyribozyme to prepare fluorescent pre-mRNAs for single-molecules studies. With the aid of an RNA cofactor, 10DM24 can covalently modify the 2' hydroxyl of a target adenosine in a larger RNA using GTP or fluorescent GTP derivatives. Notably, this method allows for incorporation of fluorophores suitable for single molecule fluorescence imaging such as Cy3 or Cy5. While 10DM24 is an effective tool for short RNAs (<100nts), we seek to expand 10DM24 for use with longer RNAs. We have shown that 10DM24 can also transfer fluorescent derivatives of GMP to specific sites within large, capped pre-mRNA splicing substrates. However, different RNA sequences within these substrates are modified to different extents and, in larger RNAs, metal-catalyzed hydrolytic degradation efficiently competes with the labeling reaction. Moreover, we have shown that these fluorescent, modified RNAs are efficient substrates for the yeast debranching enzyme, Dbr1. Based on these observations we seek to further our understanding of the mechanism of 10DM24 and to optimize the reaction conditions and chemistry to reduce RNA degradation and debranching. We have found that 10DM24 can be simplified by reducing the length of the P3 helix, while reaction efficiency at low metal ion conditions can be increased by addition of molecular crowding agents. These data lay the foundation for creation of a deoxyribozyme that is generally useful for site-specific labeling of long, unstructured RNAs for bulk and single molecule studies.

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232 Experimental and computational analyses of the transition state for non-enzymatic catalysis of RNA 2'-O-transphosphorylation by divalent metal ions

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The catalytic mechanisms of phosphoryl transfer enzymes commonly include one or more divalent metal ion cofactors that form multiple catalytic interactions with the reactive phosphoryl group. These interactions are often proposed to result in the stabilization of a more associative transition state (TS) for this addition-displacement reaction. However, the metal ion catalytic modes that operate in enzymatic and non-enzymatic transphosphorylation reactions have been difficult to resolve, and experimental information on how they affect transition state structure is limited. Here, we characterize the transition state for non-enzymatic RNA 2'-O-transphosphorylation catalyzed by Zn^{2+} ions using primary and secondary kinetic isotope effects (KIEs), and solvent kinetic isotope effect (SKIE) analyses together with quantum mechanical calculations. Catalysis by specific base and by Zn^{2+} both show SKIEs ($^{H}k/^{D}k$) of ~10 and have similar proton inventories consistent with there being no significant mechanistic differences due to involvement of acid/base catalytic modes. For the specific base reaction we observe normal (1.037 ± 0.004) 5'O leaving group and inverse (0.996 ± 0.002) 2'O nucleophile isotope effects and a non-bridging oxygen effect of near unity (0.996 ± 0.001) which indicates a late, anionic TS, as previously reported. Similar KIEs are observed for the Zn^{2+}-catalyzed reaction suggesting that there is no overall change in reaction mechanism. However, the 2'O and 5'O KIEs are both more inverse (0.986 ± 0.004 and 1.015 ± 0.002, respectively) compared to the specific base results, indicating stiffer bonding environments at both positions in the TS stabilized by Zn^{2+}. QM calculations support a model consistent with the experimental results, in which a more associative TS is stabilized by interactions with a Zn^{2+} ion coordinated to the 5'O and a second coordinated to a non-bridging oxygen that also interacts with the 2'O nucleophile via a coordinated water molecule. Thus, these results lend experimental and computational support for a long-proposed general principle regarding the effect of metal ions on the TS for phosphoryl transfer, and provide a framework for understanding how catalytic metal ion interactions influence TS bonding in biological catalysis.

233 Noncanonical Base-Pairing in Nonenzymatic Primer Extension

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Template-directed nucleic acid polymerization has been hypothesized to be a model for the synthesis of informational polymers prior to the emergence of enzymes.[1] Accurate template copying requires that chemically activated nucleotide monomers form Watson-Crick base pairs with the template strand. Here we explore the effect on nonenzymatic copying of RNA of the presence of modified nucleotides that form noncanonical base pairs with the template. Our on-going studies focus on the use of oxidatively modified nucleotides for RNA primer extension studies. A reasonably fast rate of primer extension with such monomers might lead to the generation of mutations during RNA replication; alternatively strong stalling of primer extension following the incorporation of a modified nucleotide could interfere with replication. From an evolutionary perspective, such phenomena could influence rates of sequence divergence and thus the discovery of novel functions, as well as providing enhanced structural functionality in early ribozymes.

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234 Altered (transition) states: mechanisms of solution and enzyme catalyzed RNA 2'-O-transphosphorylation

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Although there have been great strides in defining the mechanisms of RNA strand cleavage by 2'-*O*-transphosphorylation, a detailed understanding of molecular interactions in the transition state has remained elusive. Decades of detailed experimental and computational analyses reveal a complex free energy landscape that includes both stepwise and concerted mechanisms. In solution, the specific pathway followed depends on interactions with acid, base and metal ion catalysts. The sensitivity of this landscape to interactions with catalysts raises questions that are fundamentally important for understanding biological catalysis. In order to determine how catalytic modes such as acid/base and metal ion catalysis influence transition state structure and bonding in both solution reactions and enzyme active sites, we have applied kinetic isotope effects (KIEs) for the 2'-*O*-transphosphorylation of ^{18}O substituted dinucleotide RNA substrates. Primary ^{18}O KIEs and solvent D_2O isotope effects were measured to probe the mechanism of base-catalyzed 2'-*O*-transphosphorylation of RNA. The observed ^{18}O KIEs for the nucleophilic 2'-O and in the 5'-O leaving group are both large relative to reactions of phosphodiesters with good leaving groups, indicating that the reaction catalyzed by hydroxide has a transition state with advanced phosphorus-oxygen bond fission to the leaving group and phosphorus-nucleophile bond formation. A breakpoint in the pH vs. rate profile at pH 13 is attributed to the pK(a) of the 2'-OH nucleophile. A smaller nucleophile KIE is observed below the pK(a) that is interpreted as the combined effect of the equilibrium isotope effect on deprotonation the intrinsic KIE on bond formation. The primary ^{18}O KIE results and the lack of a kinetic solvent deuterium isotope effect together provide strong evidence for a late transition state and 2'-O nucleophile activation by specific base catalysis. In order to compare the solution catalyzed transition states between two mechanistic extremes, KIEs for acid catalysis were determined and revealed an altered transtition state. The ability to obtain a chemically detailed description of 2'-*O*-transphosphorylation transition states provides an opportunity to advance our understanding of biological catalysis significantly by determining how the catalytic modes and active site environments of phosphoryl transferases influence transition state structure.

235 A novel ligand that selectively targets CUG trinucleotide repeats

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Development of small molecules that can recognize specific RNA secondary and tertiary structures is currently important research topic for developing tools to modulate gene expression and therapeutic drugs. Expanded CUG trinucleotide repeats, known as toxic RNA capture the splicing factor MBNL 1 and are the causative of human disease named myotonic dystrophy type 1 (DM 1). The structural study of expanded CUG repeats showed that expanded CUG repeat could form imperfect hairpin structures containing a number of U-U mismatch as flanked by two C-G base pairs. While there were no drugs available for DM1 disease, one of important potential approaches to treating DM 1 disease is to develop small molecules targeting the CUG trinucleotide repeats, which sequester MBNL 1 from RNA.

Here, we described the rational molecular design, synthesis, and binding analysis of 2,9-diamino-1,10-phenanthroline (**DAP**) derivatives. The ligand **DAP** selectively bound to CUG trinucleotide repeats. The results of melting temperature (T_m) measurement showed that in presence of ligand **DAP** the highest ΔT_m of 7.8 °C was observed for $r(CUG)_9$ repeat, whereas the $r(CAG)_9$ and $r(CGG)_9$ repeats were not stabilized at all under the conditions. Surface Plasmon Resonance (SPR) experiments provided that **DAP** bound to $r(CUG)_9$ and $r(CCG)_9$ but not to $r(CAG)_9$ and $r(CGG)_9$. The binding stoichiometry and binding affinity of **DAP** to $r(CUG)_9$ was determined by isothermal titration calorimetry (ITC) measurement. The result of ITC measurement indicated that synthetic ligand **DAP** bound to $r(CUG)_9$ repeat with 4:1 stoichiometry with 2.1 μM binding affinity.

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236 Chemical synthesis and incorporation of m1G phosphoramidite into tRNALeu/UUR containing a site-specific m1G9 modification

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The m1G9 modification exists in human mitochondrial tRNALeu/UUR. This modification is enzymatically synthesized by the recently discovered MRPP1 enzyme, which is an unusual methyl transferase that must form a complex with a metabolic enzyme SDR5C (MRPP2) to have activity and is isolated from human cells in complex with SDR5C and with the mito Rnase P (MRPP3). Most importantly, the m1G9 modification is necessary for mitochondrial tRNA to fold into the correct tertiary structure. Without the modification, many mitochondrial tRNAs fold into an aberrant structure that cannot function in protein synthesis. In order to study the MRPP1-3 complex of tRNALeu/UUR and the tertiary structure of the tRNA, here we report the chemical synthesis of m1G phosphoramidite and its incorporation into mito tRNA that contains m1G9. (1). The m1G phosphoramidite was synthesized from guanosine in seven steps. (2). The modified RNA was then synthesized by solid-phase synthesis with double coupling to the m1G phosphoramidite. (3). An RNA 18 mer: 5'-GUU AAG AUm1G GCA GAG CCC-3' was then obtained and confirmed by MALDI-TOF MS. (4). Enzymatic ligation of this modified RNA 18 mer with an RNA fragment obtained by transcription to yield the full length RNA.

237 Redox Sensitive Flavin Aptamers

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Nucleotide cofactors used by modern biochemistry are sometimes considered to be remnants of catalytic elements from an RNA world. While it is well established that RNA can bind redox-active cofactors, there has been little direct attention paid to differential binding of oxidized and reduced forms of those cofactors. RNA enzymes to enhance redox potential of bound cofactors, although this strategy has not yet been exploited, it can potentially use differential cofactor binding as a function of redox state.

We used directed evolution to identify aptamers that differentially bind to FAD and its reduced form $FADH_2$. The selected aptamers bind immobilized FAD considerably better than immobilized $FADH_2$. We next used in-line probing to identify nucleotides that are either sensitized or protected from in-line attack by the 2'OH upon ligand binding in solution. For aptamer 12.29, FAD modulated in-line cleavage signal at several sites, but not by $FADH_2$Based on the predicted secondary structure of aptamer 12.29, we truncated the original 91-nt aptamer to a much smaller 51-nt version, which still retained FAD binding function. The truncated aptamer also binds to FAD but not $FADH_2$. We are also studying another promising candidate aptamer 12.8, which also shows FAD modulated in-line cleavage signals. Studying how RNA molecules can distinguish between redox states of cofactors will enable us to understand how prebiotic RNA enzymes may have used these cofactors for electron transport reactions.

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238 Tautomerism in RNA Biochemistry

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Tautomers are structural isomers that differ from one another based on the position of exchangeable proton(s). Nucleic acid bases have carbonyl and amino functional groups, which contain solvent-exchangeable hydrogen atoms that can participate in keto-enol and amino-imino types of tautomerism. Presence of multiple tautomers not only increases the structural and chemical diversity of nucleic acid bases but also enhances their ability to form pairing with multiple bases.

Structural diversity arising from tautomerism has been suggested to play a functional role in the nucleic acid biochemistry. In DNA, spontaneous formation of minor tautomers has been speculated to contribute to mutagenic mispairings during DNA replication, whereas in RNA, minor tautomeric forms have been proposed to enhance the structural and functional diversity of RNA enzymes and aptamers. This work focusses on the role of tautomerism in RNA biochemistry specifically focusing on the role of tautomerism in catalysis of small self-cleaving ribozymes such as GlmS, hammerhead, hairpin and Varkud, and in the recognition of ligand analogues by the purine and the TPP riboswitches.

Tautomerism of nucleic acid bases is also relevant to the origin of life question, and pertinent for understanding why Nature selected A, T, G, C and U as nucleic acid bases for encoding the genetic information of all life forms. The importance tautomerism to the RNA world hypothesis will also be discussed. Tautomeric nucleoside analogues also have therapeutic applications as antiviral drugs against RNA viruses because of their ability to induce lethal mutagenesis, increasing the viral mutation rates above the error catastrophe limit of a virus.

Finally, considering that the presence of multiple tautomers of nucleic acid bases is a rare occurrence, and that tautomers typically interconvert on a fast time scale, methods for studying rapid tautomerism in the context of nucleic acids under biologically relevant aqueous conditions will also be discussed.

239 *NCS2** links tRNA modification to pathogenic phenotypes in yeast

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Fungal pathogens pose a major threat to human health and biodiversity. However, little is known about the molecular mechanisms, which facilitate pathogenicity. One of the key prerequisites for the pathogenicity of yeast is the ability to grow at high temperatures.

Interestingly, *NCS2** - a single nucleotide polymorphism (SNP) in *NCS2* - allows *Saccharomyces cerevisiae* to grow at high temperatures and was identified in yeast isolated from a human patient. *NCS2* is a member of the *URM1* pathway, which is required for the sulfur modification of uridine (U_{34}) at the wobble position in the three eukaryotic cytoplasmic tRNAs, tE^{UUC}, tK^{UUU}, and tQ^{UUG}.

Importantly, thiolation deficient yeast is unable to grow at high temperature. Therefore, we tested whether increased tRNA thiolation can explain the phenotypes observed for *NCS2**. First, we established that *NCS2** confers resistance to stress conditions, which are linked to tRNA thiolation and that this effect depends on tRNA modification and not on other unknown functions of *NCS2**. Second, we observed that diploid strains (*NCS2/NCS2**) phenotypically behave as *NCS2** yeast under stress conditions, suggesting that *NCS2** is dominant over *NCS2*. Third, we established that the amino acid change introduced by the SNP is specific and cannot be replaced by a closely related amino acid. Fourth, we showed by yeast-two hybrid experiments that the interaction between Ncs2*p and Ncs6p is enhanced relative to Ncs2p. Finally, we investigated the role of tRNA thiolation in the clinical relevant yeast strain *Candida albicans*. If *NCS2* is knocked out in *C. albicans,* we observed distinct colony morphology at higher temperatures and a changed sensitivity to stress.

Currently, we are examining the *in vivo* significance of the pathogenic potential of *S. cerevisiae* in mice.

Taken together, our results suggest, that changes in the interaction of Ncs2*p and Ncs6p lead to enhanced tRNA thiolation thus contributing to the pathogenicity of yeast.

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240 Characterization of Archease-dependent and independent RtcBs

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RtcB is a noncanonical GTP and Mn(II)-dependent RNA ligase that is essential for tRNA maturation. Archease is a protein cofactor of RtcB that has been shown to accelerate RNA ligation and modify its NTP cofactor specificity. Through phylogenetic analysis, we have learned that the rtcB gene is widely distributed in all three domains of life; however, the archease gene is not widely found in bacteria. We are studying Archease-dependent and independent RtcBs from various bacteria to gain further insight into Archease function. Here we present kinetic and mechanistic data describing fundamental differences between Archease-dependent and independent RtcBs. Furthermore, we have shown that Archease is functionally interchangeable between Archease-dependent RtcBs, even across domains of life. However, Archease fails to activate RtcB from organisms which don't encode Archease. Our work adds to the understanding of how Archease affects the ability of RtcB to ligate RNA which is important for gaining insight into the process of tRNA maturation.

241 The role of RNA modifications in zebrafish development

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Chemical modifications of nucleosides are found in all classes of RNA. In particular non-coding RNAs like rRNAs and tRNAs contain a plethora of modifications, which are often conserved in all domains of life. However, most research on RNA modifications is performed in unicellular organisms. Interestingly, modification mutants in yeast or bacteria, show growth phenotypes mainly under stress. However, similar mutations in higher eukaryotes can lead to severe phenotypes and embryonic lethality. We sought to systematically characterize the developmental role of RNA modifications in zebrafish as a vertebrate model. To this end, we first established the presence of modified nucleosides in tRNA isolated from zebrafish by thin-layer chromatography and mass spectrometry. Second, we performed a comprehensive analysis of the expression pattern of genes required for RNA modification during zebrafish development. Using whole mount *in situ* hybridization, we found that expression patterns of modification genes largely coincide with tissues that appear to have high levels of protein translation, like the brain or the pectoral fin bud. However, spatio-temporal localization pattern of members of the same pathway do not always overlap. Currently, we are expanding our analysis to all putative RNA modifying genes. Finally, to functionally characterize selected RNA modification genes, we are generating knockout zebrafish lines using CRISPR-Cas9 technology. Our analysis will help us understand how loss of RNA modifications causes phenotypes in higher eukaryotes and provide insights into their developmental regulation.

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242 CRISPR/cas9 targeting of HIV-1

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CRISPR/Cas9 is a gene editing technology notable for ease of targeting design. We have designed CRISPR chimeric guide RNAs against the HIV-1 genome including the regions containing tat, TAR, and the U3 region of the long terminal repeat (LTR). Cas9 protein induced double stranded breaks in target sequences via the guide RNA provides a potential way to reduce or eliminate HIV-1 gene expression and integrated HIV-1 genome by gene disruption or elimination, since the LTR and TAR occur on the 5' and 3' regions of the HIV genome. Here, we describe how we have targeted these sequences in both latently and actively infected PBMCs, in addition to a mCherry reporter CD4+ CEM cell line, providing evidence that HIV-specific CRISPR-mediated gene editing reduces levels of p24 produced by cells infected with different subtypes of HIV-1. This data suggests that CRISPR may be used to reduce and eliminate HIV-1 infection in actively infected and latent cell populations.

243 Structure / Function Relationship of the Archaeal Box C/D RNP Proteins

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One of the most common post-transcriptional modifications of RNA is 2'-O-methylation. Box C/D ribonucleoprotein (RNP) complexes are responsible for many such modifications in Eukaryotic and Archaeal organisms. Each Archaeal Box C/D RNP is made up of a ‘guide' sRNA and three core proteins. The guide RNA has conserved motifs (C, D, C', D') and specific secondary structures. The proteins, Nop5, Fibrillarin, and L7Ae, interact with these structures and bring in the target RNA to be modified. Study of the nature of the interactions within this complex is important to our understanding of its function. Here, we are determining the crucial amino acids of the proteins within this system by in-vitro and in-vivo methods.

Methanocaldococcus jannaschii is being used to test in-vitro 2'-O-methyation at C34 of elongator tRNA-Met and U39 of tRNA-Trp. *Haloferax volcanii* is being used to test in-vivo 2'-O-methylation at C34 of elongator tRNA-Met and G1934 of ribosomal RNA. Select mutants of Nop5 or Fibrillarin were introduced into strains lacking the native protein and their ability to produce 2'-O-methylations was determined by limited dNTP-mediated reverse transcription. Mutants were selected based on homology modelling and literature review. Some preserved the methylation, while others showed no modification.

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244 Adenosine-to-Inosine RNA editing patterns in *C. elegans*

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Adenosine deaminases that act on RNA (ADARs) are enzymes that edit RNA by converting adenosines into inosines. Inosine is recognized as guanosine by translation machinery, splicing factors, and other RNA binding molecules. Thus, these changes can alter the coding potential of the mRNA, RNA splicing and miRNA/siRNA binding depending upon where in the mRNA the editing occurred. In mammals, RNA editing is essential for normal development and proper neuronal function. Consistent with this, aberrant RNA editing levels have been observed in human patients suffering from neuropathological diseases as well as cancer. Interestingly, many of these studies have also revealed that RNA editing levels do not correlate with ADAR expression, suggesting there are cellular mechanisms that regulate RNA editing. Our goal is to use both *in vitro* biochemistry, as well as genomics and molecular biology to elucidate the mechanisms that regulate ADAR activity. As *C. elegans* express only one ADAR enzyme, ADR-2, these organisms are ideal to monitor the mechanisms that regulate RNA editing. *C. elegans* ADR-2 will be tagged with FLAG using CRISPR technology. This will allow immunoprecipitation of ADR-2 permitting analysis of ADR-2 associated proteins and mRNA.

245 Pus10 is involved in production of pseudouridine 54 of specific mammalian tRNAs

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The TψC loop of tRNA generally contains T (ribothymidine or 5-methyluridine) at position 54 in most Bacteria and Eukarya, and Ψ (pseudouridine) and C at positions 55 and 56, respectively in most organisms. TrmA and TruB homologs produce T54 and Ψ55, respectively, in Bacteria and Eukarya. Archaeal tRNAs commonly have Ψ54 (or m^1Ψ54) instead of T54 and Pus10 is responsible for both Ψ54 and Ψ55 in these tRNAs.

The pus10 gene is present in nearly all Archaea and most higher eukaryotes, but not in Bacteria and yeast. This coincides with the presence of Ψ54 in archaeal tRNAs and some tRNAs (Gln, Trp, Pro, etc.) of animals, and its absence in the tRNAs of Bacteria and yeast. Certain tRNAs that function as primers for replication of retroviruses also contain Ψ54. The enzyme for tRNA Ψ54 synthase activity in eukaryotes has not yet been identified. We show here that HeLa cell extracts contain Ψ54 synthase activity that is specific for some tRNA transcripts but not for others. This activity is reduced in the extracts of Pus10-knock down cells with a concomitant increase in tRNA T54 methyltransferase activity, suggesting that these two activities compete for modifying the same U54 of the tRNA.

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246 Orchestrated positioning of post-transcriptional modifications at the branch point recognition region of U2 snRNA

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Spliceosomal U snRNAs are heavily modified post-transcriptionally with functionally important modifications. Yet, little is known how the modification pattern is established. Do modifications occur at random, or is there a series of sequential events that occur in a hierarchical order? Using an *in vivo* yeast cell system, we tested human, *Xenopus*, and *Drosophila* guide RNAs for modification of U2 snRNA at the branch point recognition region. We also tested a newly identified *Schizosaccharomyces pombe* box H/ACA guide RNA for U2-43/44 pseudouridylation. Our data suggest the existence of crosstalk between different modified positions in U2 snRNA. For instance, the modification within dinucleotide Ψ44Ψ45 in *Drosophila* U2 snRNA (or equivalent Ψ43Ψ44 in vertebrate U2) must occur stepwise: specifically, guide RNA-dependent pseudouridylation of position 45 (position 44 in vertebrates) first and RNA-independent Pus1p-catalyzed pseudouridylation of position 44 (position 43 in vertebrates) next. In fact, when tested in yeast, a preexisting Ψ44 inhibited formation of Ψ45 mediated by a guide RNA-dependent mechanism. The same was true for C_m41Ψ42 (C_m40Ψ41 in vertebrates): 2'-O-methylation of position 41 interfered with formation of Ψ42. We discuss possible mechanisms that cells utilize to reach the required post-transcriptional modification patterns; these might involve alterations in canonical guide RNA structure and/or RNA-independent modification at certain positions.

247 Enteroviruses Direct Cleavage of Human Proteins Involved in RNA Modification

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Enteroviruses such as poliovirus are lytic viruses with a positive-stranded RNA genome. Their replication results in rapid and major changes in host cell physiology. We conducted a proteomics screen of phosphorylation, ubiquitination, and protein abundance landscapes in polio-infected cells, and discovered that several host proteins are cleaved in the course of infection. Among the impacted proteins, several are known to take part in RNA modification (IKAP) and in reading modified bases (YTHDF family members). We propose that RNA modifications such as N6-methyladenosine participate in previously unsuspected antiviral pathways.

248 Mechanism of a multifunctional tRNA methyltransferase family

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The tRNA methyltransferase (Trm10) methylates N1 of guanosine residues at the 9th position (G9) of multiple tRNAs using S-adenosyl methionine (SAM) as the methyl group donor. Trm10 was originally discovered in Saccharomyces cerevisiae but is nearly ubiquitous in Archaea and Eukarya, and familial mutations in a human homolog of Trm10 (TRMT10A) are associated with numerous abnormalities, including defects in glucose metabolism and neurological dysfunction. Despite classification of Trm10 enzymes as members of the SpoU-TrmD (SPOUT) family of methyltransferases, the molecular mechanism of these enzymes is not completely understood, and cannot be inferred directly due to limited sequence similarity to other more well-studied family members. Moreover, biochemical analysis of Trm10 homologs identified in human mitochondria and some Archaea revealed the surprising ability of some Trm10 family members to catalyze m1A9 methylation, either in addition to, or instead of the prototypical m1G9 activity. This flexibility in terms of substrate nucleotide is particularly remarkable in light of the different pKa values expected for the N1 atom in G9 vs. A9-containing tRNA substrates. For all of these reasons, we hypothesize that Trm10 enzymes exhibit an atypical mechanism of N1 methylation, and aimed to determine the molecular features of catalysis utilized by this unusual and important enzyme family.

Here we use site-directed mutagenesis to alter conserved residues implicated in Trm10 catalytic activity based on their positions in a recently solved crystal structure and utilize single-turnover kinetic assays to evaluate activities of the resulting proteins. Although the SAM-binding site predicted by the crystal structure is supported by this analysis, these data argue against an important role for a previously proposed general base in the reaction. Analysis of additional Trm10 variants is underway, with the ultimate goal of elucidating the complete catalytic mechanisms exhibited by members of this methyltransferase enzyme family.

249 N^6-methyladenosine-dependent nucleocytoplasmic translocation of HNRNPG protein regulates gene expression

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N^6-methyladenosine (m^6A) is the most abundant and dynamic internal modification in eukaryotic mRNA and long non-coding RNA. m^6A is indispensable for cell viability and development, yet its functional mechanisms are still poorly understood. Here, we show that the m^6A methylation at a specific hairpin in the abundant, nuclear-localized *MALAT1* (metastasis associated lung adenocarcinoma transcript) lncRNA enhanced the interaction of this hairpin with heterogeneous nuclear ribonucleoprotein G (HNRNPG). HNRNPG is one RNA-binding protein shuttling between nucleus and cytoplasm, and regulates RNA transcript processing steps. Further studies showed that m^6A was enriched within HNRNPG binding sites. Through combining HNRNPG PAR-CLIP and MeRIP-seq, we mapped transcriptome-wide m^6A sites within HNRNPG binding sites. Interestingly, transcriptome-wide loss of m^6A modifications by knocking down m^6A-methyltransferases (METTL3 or METTL14) significantly increased the translocation of the HNRNPG protein from nucleus to cytoplasm, and increased the number and size of cytoplasmic granules containing the HNRNPG protein. We also found that HNRNPG, METTL3, METTL14 co-regulated the expression and splicing of a significant number of genes, suggesting the physiological relevance of m^6A within HNRNPG binding sites. Taken together, our working model suggests that m^6A methylation of nuclear-localized lncRNAs (such as *MALAT1*) retains HNRNPG protein in the nucleus for mRNA processing, and altered m^6A levels affect the nucleocytoplasmic translocation of the HNRNPG protein, resulting in the change of gene expression and alternative splicing. These findings illustrate the m^6A-mediated location and function of RNA-binding proteins in the cell.

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250 Target recognition, regulation and function of Pus1-dependent mRNA pseudouridylation

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Pseudouridine, the 5-ribosyl isomer of uridine, heavily decorates structural RNAs throughout all domains of life. Recently, our lab has discovered pseudouridines in mRNAs via transcriptome-wide pseudouridine profiling (Pseudo-seq) [1]. mRNA pseudouridylation is widespread in yeast and human cells, and most mRNA sites appear to be regulated; however, little is known about the mechanisms responsible for selective, regulated modification of specific sequences. Here we have investigated the basis for mRNA targeting by the conserved pseudouridine synthase Pus1. Pus1 has more than 50 mRNA targets whose modification status varies with growth conditions in ways that are not explained by changes in substrate or enzyme abundance. To gain insight into the mechanism of regulated substrate recognition by Pus1, we have reconstituted Pus1-dependent modification of synthetic mRNAs in vitro. By computational prediction, mutational analysis and high-throughput RNA structure probing in vitro, we have identified a stem loop structure that is necessary and sufficient for Pus1-dependent pseudouridylation of many mRNAs. These structures are evolutionarily conserved in some substrates implying biological significance. We further show that overexpression of wildtype - but not catalytically inactive - Pus1 reduces growth rate and causes aberrant mRNA modification patterns, consistent with physiological functions for some mRNA pseudouridines. Work is ongoing to determine whether and how mRNA structural dynamics regulate mRNA modifcations in vivo, and to discover the basis for growth inhibition by misregulation of Pus1-dependent pseudouridylation.

[1] Carlile et al. Nature. 2014 Nov 6;515(7525):143-6.

251 Dynamic regulation of RNA modifications and its role in translational control and disease

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Post-transcriptional RNA modifications are abundant in nature and offer a wealth of chemical diversity to RNA. Noncoding RNAs (ncRNAs) such as ribosomal RNA (rRNA), small nuclear RNA (snRNA), and transfer RNA (tRNA) undergo extensive nucleotide modifications however the full biological relevance of distinct modifications remains elusive. Pseudouridine, the most abundant single nucleotide modification on rRNA, is present in all domains of life and is of great medical relevance as the machinery required for rRNA pseudouridylation is mutated or deregulated in a growing list of human diseases. Previously, our lab has demonstrated that rRNA pseudouridine modifications impart important regulatory potential to the ribosome to control gene expression. Moreover, we have identified an important requirement for pseudouridine modifications in hematopoietic stem cell differentiation and during cellular transformation. To uncover whether rRNA pseudouridylation may be temporally regulated we have identified several novel post-translational modifications that may modulate the activity of the pseudouridine synthase dyskerin. Surprisingly, we have uncovered an exquisite regulation of dyskerin activity downstream of key signaling pathways frequently deregulated in human disease and identified functional roles for distinct post-translational modifications. Coupling this analysis with transcriptome-wide mapping of RNA pseudouridylation we are defining the mechanisms by which H/ACA small nucleolar RNAs and dyskerin dynamically modulate site-specific pseudouridylation and impact ribosome function. Altogether, these approaches will help us functionally characterize the role of specific RNA modifications in gene regulation and reveal novel insights into how perturbations in ribosome modifications may lead to cellular outcomes associated with increased cancer susceptibility and bone marrow failure.

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252 Functional consequences of adenosine methylation in mRNA translation

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Methylation of adenosine residues in mRNA to form N6-methyladenosine (m6A) is a widespread epitranscriptomic modification which was recently shown to occur in thousands of mammalian transcripts. While m6A residues are primarily found near stop codons, they are also abundant within 5'UTRs. Recent studies have begun to elucidate potential roles for m6A residues in regulating splicing or mRNA stability, but these functions are largely thought to be carried out by m6As in the coding sequence or 3'UTR. Thus, the function of m6A residues within the 5'UTR remains poorly understood. Here, we investigate the role of adenosine methylation within the 5'UTR in regulating translation. We find that the presence of m6A residues within the 5'UTR promotes a unique mode of translation initiation which is independent of the 5' cap or cap-binding proteins. This m6A-induced translation initiation is mediated through binding of distinct components of the translation initiation machinery to m6A residues in RNA. Our results uncover a novel function for m6A residues within the 5'UTR and reveal a new mode of translation initiation with important implications for translational regulation and the response to cellular stress.

253 Structural and functional characterization of a unique *Haloferax volcanii* box H/ACA guide RNA involved in sequential pseudouridylation of two residues

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Box H/ACA RNAs are a major class of s(no)RNAs which form s(no)RNP complexes. These RNP complexes direct pseudouridine (Ψ) formation in ribosomal RNAs of Eukarya and Archaea. Archaeal box H/ACA guide RNAs contain structurally conserved features like a proximal stem (P1), a 3' terminal H-box or ACA box and a pseudouridylation pocket (Ψ pocket). A small RNA sR-h45 (~200 nucleotides) was bioinformatically identified and later shown to be responsible for Ψ formation at positions -1940, 1942 and 2605 of *Haloferax volcanii* 23S rRNA. This RNA has two stem loops: a 5'stem loop (SL1) and a 3'stem loop (SL2). A unique feature of this RNA discovered in our studies was the modification of two successive positions (Ψ1940 and 1942) by the same SL2 guide pocket of sR-h45. We are determining the structural features of SL2 stem loop that are involved in two consecutive pseudouridylations. Using recombinant *Methanocaldococcus jannaschii* Box H/ACA proteins for an in vitro pseudouridylation assay it was found that the SL2 Stem loop of sR-h45 could produce both Ψ (1940 and 1942) in vitro. This modification happens in a sequential manner as blocking Ψ1940, drastically reduced the level of the Ψ1942. The results of mutating similar conserved features of the SL2 loop as compared to SL1 (which is responsible for Ψ2605) varied. This tells us that the two loops function differently. As expected the conserved ACA sequence was found to be required for modification for this loop (SL2). However, in our previous studies it was found to be dispensable for the other loop (SL1). To further gain insights into this process guides are also been used wherein the residues in the Ψ pocket have been mutated based on a basic H/ACA guide-target structure. This would determine the specificity of the guide for Ψ1940 and/or 1942. We are currently doing the same work in vivo.

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254 Conserved evolutionary role of ADAR RNA editing enzymes in innate immunity.

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The most abundant base modification in mammalian mRNA and noncoding RNA, is the hydrolytic deamination of adenosine to inosine, catalysed by members of the ADAR family of RNA editing enzymes. In humans less than 1% of all RNA editing events occur within coding sequences. Rather, the RNA editing occurs predominantly within duplex regions formed by *Alu* repetitive elements and in other noncoding sequences. Over a hundred million sites have been reported in the human genome. ADAR1 is responsible for the majority of these promiscuous editing events.

Mutations in the human *ADAR1* cause the autoimmune disease, Aicardi-Goutieres Syndrome which is a rare genetic autoimmune encephalopathy that mimics congenital virus Infection with increased levels of Type1 interferon (IFN). Mice that are *Adar1*$^{-/-}$ die by day E12.5 due to problems with haematopoiesis and increased production of IFN. We have rescued *Adar1* embryonic lethality to live birth in *Adar1*; *Mavs* (mitochondrial antiviral signaling) double mutants in which the antiviral IFN response to cytoplasmic double-stranded RNA (dsRNA) is prevented. This rescue requires the editing activity of an ADAR in the cytoplasm and is not due to site-specific editing.

Drosophila has a single *Adar* gene that is the orthologue of ADAR2. It catalyzes hundreds of site-specific editing events in CNS transcripts. *Adar* mutant flies show reduced viability, uncoordinated locomotion and age-dependent neurodegeneration, consistent with loss of recoding. However, we have found that aberrant induction of innate immune transcripts also occurs in the *Drosophila Adar* mutant. The mutant phenotype is rescued by introducing a second mutation in the innate immune pathway. It should be noted that the *Drosophila* innate immune responses do not involve RLR signaling or IFN. Thus this conserved evolutionary role for ADARs in innate immunity is highly significant.

255 Withdrawn

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256 RNA structure is associated with essential and highly expressed genes on the distal arms of *C. elegans* autosomes.

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Double-stranded RNA binding proteins interact with double-stranded RNAs (dsRNAs) to mediate diverse cellular processes, including mRNA regulation, small RNA biogenesis, and adenosine-to-inosine (A-to-I) RNA editing. Studies of genome-wide A-to-I editing in *C. elegans* have established that dsRNA structures occur in many introns and untranslated regions (UTRs) of coding transcripts. However, the significance of these dsRNA structures in non-coding elements remains poorly understood.

To investigate how structure impacts gene regulation during organismal development, we performed RNA-Seq on total RNA and dsRNA-enriched RNA from three biological replicates of *C. elegans* at each of four broad developmental stages: embryonic, early larval, late larval, and young adult. We used a previously developed computational pipeline to identify 1,523 dsRNAs by defining clusters of A-to-I editing. We observed that these editing-enriched regions (EERs) primarily occur in introns with the majority predicted to fold into intramolecular structures. Expression of individual EERs was dynamic, but collective EER expression peaked in embryos and declined as development progressed. Developmental differences in EER editing correlated with differences in EER expression.

We found that EERs were non-randomly distributed across the genome and were concentrated on distal arms of *C. elegans* autosomes. Previous studies establish that distal arms contain fewer essential genes and are associated with high repeat density and marks of gene silencing. However, despite enrichment of EERs on distal arms, we observed approximately twice as many essential genes associated with EERs than expected by chance. On autosome arms, 26.9% of essential genes were associated with an EER on the same strand. Comparisons of gene expression distributions indicated significantly higher gene expression of EER-associated genes relative to all genes on autosome arms, with over 40% greater median expression in each developmental stage. Genes containing three or more edited features were among the most highly expressed genes on autosome arms. Our results suggest that dsRNA structures positively regulate gene expression in domains of the *C. elegans* genome associated with gene silencing. We are working to directly test the contributions of dsRNA structures to gene expression regulation in autosome arm and body domains of the worm genome.

257 Regulated mRNA pseudouridylation in stress, development and disease

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RNA molecules contain a variety of chemically diverse, post-transcriptionally modified bases. The most abundant modified base found in cellular RNAs, pseudouridine, has recently been mapped to hundreds of sites in mRNAs, many of which are dynamically regulated [1,2,3]. Regulated mRNA pseudouridylation has the potential to significantly affect gene expression through effects on translation initiation efficiency, ribosome pausing, RNA localization, and regulation by RNA interference. In yeast, we have identified more than 400 novel pseudouridylated sites in mRNAs and non-coding RNAs and demonstrated that most are differentially modified between exponential and post-diauxic (nutrient limited) phases of growth. Though the pseudouridine landscape has been determined in only a few cell types and growth conditions, the enzymes responsible for mRNA pseudouridylation are universally conserved and ubiquitously expressed, suggesting many novel pseudouridylated sites remain to be discovered. Here we present for the first time a comprehensive analysis of mRNA pseudouridines in various mouse tissues and disease states using transcriptome-wide pseudouridine profiling (Pseudo-seq). We are creating a complete atlas of mRNA pseudouridine sites in healthy mouse brain, liver and lung. We will also examine mouse disease models for lung cancer and neurodegenerative disorders for perturbations of this mRNA modification landscape. Given the prevalence of pseudouridine regulation in yeast and the genetic evidence linking specific pseudouridine synthases to human disease, we anticipate many interesting findings from the first comprehensive pseudouridine profiling in metazoans.

[1] Carlile et al. Nature. 2014 Nov 6;515(7525):143-6.

[2] Schwartz et al. Cell. 2014 Sep 25;159(1):148-62.

[3] Lovejoy et al. PLoS One. 2014 Oct 29;9(10):e110799.

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258 Cracking the Epitranscriptome

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Over 100 types of distinct modifications are known to be catalyzed post-transcriptionally across different classes of RNA. In an analogous manner to well-studied chemical modifications on DNA and on proteins, RNA modifications are likely to serve fundamental roles in regulating and fine-tuning the incredibly complex and interlinked steps of the RNA life cycle. However, significant challenges in detecting RNA modifications have made it extremely challenging to study their role, with the exception of few highly expressed molecules such as rRNA and tRNA where these modifications have typically been studied. Our work has focused on two RNA modifications: N6-methyladenosine (m6A) and pseudouridine (Ψ). We established experimental methodologies coupled with tailored computational pipelines allowing unbiased transcriptome wide mapping and quantification of these modifications across diverse experimental systems. In both cases, we discovered the modifications to be abundant within mRNAs and to be dynamically modulated in specific conditions, and characterized their positional biases across genes and their evolutionary conservation. We have also studied the mechanistic basis underlying the dynamic onset and offset of modifications, and identified novel factors involved in encoding and decoding of RNA modifications, whose induced expression and redistribution within subcellular compartments underlies the observed condition-specific modification patterns. Our understanding of the universe of RNA modifications - also known as the 'RNA epitranscriptome' - is yet at its infancy, and in future years it will be crucial to determine the functions and mechanisms through which RNA modifications regulate the complex mRNA life cycle and downstream cellular programs.

259 Enzymology and molecular function of 5-methylcytosine (m5C) as a modification of human RNA

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5-methylcytosine (m5C) is a prominent well-understood modification in DNA associated with epigenetic gene regulation in mammalian cells. Moreover, this modification has been documented in all RNA biotypes, though its function remains largely elusive. As a first approach to understand m5C function in RNA, we generated a world-first transcriptome-wide map of m5C residues in human cervical cancer (HeLa) cell RNA (1). This was achieved by performing RNAseq on bisulfite treated RNA (a technique known as bsRNA-seq). Combining this approach with RNAi-mediated knockdown of the two known human RNA m5C methyltransferases NSUN2 and TRDMT1, we identified hundreds of novel targets of these methyltransferases, a subset of which was validated by amplicon sequencing. In our most recent work, we extended the same approach to elucidate targets of the putative human RNA m5C methyltransferase NSUN4 and NSUN5. We have confirmed NSUN4 to function as an RNA m5C methyltransferase, with preliminary work suggesting NSUN4 targets a single site in mitochondrial ribosomal RNA. This finding is in contrast with NSUN2 controlled sites, which are located in all RNA biotypes and in many genomic contexts. Furthermore, analysis of identified m5C sites has resulted in observations that may suggest a function for m5C in RNA. We have previously determined a spatial correlation between m5C and microRNA target sites within mRNAs, showing that Argonaute footprints were enriched just upstream of m5C sites. We have further refined our analysis and found in addition that m5C sites were depleted within conserved microRNA seed matches. These patterns persisted when analysing the NSUN2-modified subset; we also show that NSUN2 does not directly interact with Argonaute in HeLa cell lysates. Altogether, these observations strongly suggest a functional link between mRNA methylation and the microRNA mechanism.

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260 Identification of a new ribose methylation in the 18S rRNA of *S. cerevisiae*

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Methylation of ribose sugars at the 2′-OH group is one of the major chemical modifications in rRNA, and is catalyzed by snoRNA directed C/D box snoRNPs. Previous biochemical and computational analyses of the C/D box snoRNAs have identified and mapped a large number of 2′-OH ribose methylations in rRNAs. In the present study, we systematically analyzed ribose methylations of 18S rRNA in *Saccharomyces cerevisiae*, using mung bean nuclease protection assay and RP-HPLC. Unexpectedly, we identified a hitherto unknown ribose methylation at position G562 in the helix 18 of 5′ central domain of yeast 18S rRNA. Furthermore, we identified snR40 as being responsible to guide snoRNP complex to catalyze G562 ribose methylation, which makes it only second snoRNA known so far to target three ribose methylation sites: Gm562, Gm1271 in 18S rRNA, and Um898 in 25S rRNA. Our sequence and mutational analysis of snR40 revealed that snR40 uses the same D′ box and methylation guide sequence for both Gm562 and Gm1271 methylation. With the identification of Gm562 and its corresponding snoRNA, complete set of ribose methylations of 18S rRNA and their corresponding snoRNAs have finally been established opening great prospects to understand the physiological function of these modifications.

261 Characterization of an *N6*-methyladenosine-dependent RNA structural switch

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N6-methyladenosine (m^6A) is a reversible and abundant internal modification of mRNA and long noncoding RNA (lncRNA) with putative roles in RNA processing, transport, and stability. Although the *N6*-methyl does not preclude Watson-Crick base pairing, m^6A alters the stability of RNA secondary structure. Given that changes in RNA structure can affect diverse cellular processes, the influence of m^6A on mRNA/lncRNA structure has the potential to be an important mechanism for the function of m^6A modification in the cell. In fact, an m^6A site in the lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) was recently shown to induce a local change in RNA structure that exposes a single-stranded U-tract for recognition and binding by the RNA binding protein heterogeneous nuclear ribonucleoprotein C (HNRNPC). This m^6A-dependent regulation of protein binding through a change in RNA structure was termed 'm^6A-switch' (1). To further characterize this first example of a functional m^6A-switch in a cellular RNA, we used nuclear magnetic resonance (NMR) and Förster resonance energy transfer (FRET) to demonstrate the effect of m^6A on a 32-nucleotide RNA hairpin derived from the m^6A-switch in MALAT1. The observed FRET efficiencies and imino proton NMR resonances suggest that m^6A modification destabilizes the upper portion of the hairpin-stem, increasing the solvent accessibility of the neighboring bases while maintaining the overall hairpin structure. The m^6A-induced structural changes in the MALAT1 hairpin serve as a model for a much larger family of m^6A-switches that potentially mediate the influence of m^6A on cellular processes.

(1) Liu et al., *N6*-methyladenosine-dependent RNA structural switches regulate RNA–protein interactions, Nature 518, 560–4 (2015).

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262 Quaking RNA binding proteins regulate RNA metabolism during oligodendrocyte differentiation

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The QUAKING (QKI) isoforms are sequence-specific KH-type RNA binding proteins (RBPs) expressed mainly in the myelinating oligodendrocytes modulating mRNA splicing, export and stability. In the present study, we generated a mouse *qkI* conditional allele, and ablated the expression of the QKI isoforms in oligodendrocytes using Olig2-Cre. The mice displayed pronounced tremors at post-natal day 10 and died in the third post-natal week. Staining for myelin proteins and ultrastructural brain electron microscopy revealed a severe hypomyelination phenotype. To investigate the post-transcriptional regulation events associated with the loss of QKI in oligodendrocytes, we examined the alternative splicing of 1328 events using the LISA platform (Université de Sherbrooke) and performed RNA-seq analysis. The RNA-seq data identified key mRNA transcripts of myelin components such as MBP, PLP, MAG, and MOG to be down-regulated in the absence of QKI, consistent with the oligodendrocyte maturation defects observed. In addition, we identified >30 alternative spliced events regulated with the loss of QKI. *Neurofascin*, is known to be alternatively spliced, leading to Nfasc155 expressed solely in oligodendrocytes, and Nfasc186 expressed only in neurons. These isoforms play key roles in neuroglial interactions. Indeed the $QKI^{FL/FL}$:Olig2Cre mice displayed severe defects in paranodes. In addition, we mapped the RNA elements required for the regulation of Nfasc alternative splicing by QKI using minigene reporters and CLIP. Our data identify the QKI RBPs as early regulators of oligodendrocytes differentiation and as a target for remyelination therapies for multiple sclerosis. This work was supported by a grant from the Multiple Sclerosis Society of Canada.

263 Neuronal activity regulates splicing of retained introns

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In higher eukaryotes the vast majority of introns manifest co-transcriptional splicing. However, a subset of polyadenylated RNAs possess select introns. The biological significance of these retained introns remains poorly understood.

Our project aims to explore potential functions of retaining these introns, focusing on the hypothesis that storage of "almost mature-mRNAs" can represent a mechanism for the cells to quickly respond to their environment. Using a neuronal model, we are investigating whether splicing of retained introns is modulated by neuronal activity. We thus performed deep-RNA sequencing approaches and identified a subset of intron retention events regulated in stimulated neurons. We are defining common features of these regulated introns to guide us toward mechanisms involved in retaining and releasing introns in the RNAs. In parallel, we are also focusing on some specific targets and are studying the consequences of dynamic intron retention for neuronal function.

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264 Generation of human mesenchymal stem cells overexpressing VLA-4 integrin by optimized transfection with ITGA4-mRNA

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Introduction: Stem cell-based regenerative medicine is recently skyrocketing due to the rapidly growing demand of fast ageing societies. The inherent capabilities for promotion of tissue repair and effortless derivation from adult tissues make mesenchymal stem cells (MSCs) a great candidate for therapy. Minimal invasive routes of cell delivery such as intravascular are preferred by patients; however, a lingering problem is insufficient recruitment of these cells to the target tissue. Thus, we decided to borrow an integrin-based mechanism for diapedesis from leukocytes. Due to requirement for only transient expression of integrins, we found that virus-free, clinically applicable mRNA-based transfection perfectly fits our needs. The integrin genes are ca. 3kb, and induction of cellular expression of such large genes by mRNA was not reported as yet.

Methods: A pSP72vector (P2191-Promega) with cloned ITGA4-gene cDNA was used as a template for *in vitro* production of mRNA using the mMessage-mMachine®Kit (AM1344-Ambion) with a poly(A) tailing kit (AM1350-Ambion), or mMessage-mMachine®T7UltraKit (AM1345-Ambion) that included an anti-reverse-cap-analogue (ARCAcap). SSB-protein (S3917-Sigma) was used for stabilization of mRNA. Transfection experiments were carried out in human MSCs (PT2501-Lonza) and HEK293 cells. Lipofectamine®2000 (Invitrogen) was used as a transfection agent. RT-PCR was performed to assess transfection efficiency. Production of ITGA4 protein was confirmed by immunocytochemistry.

Results: We have shown that non-stabilized, 7-methylguanosine-capped ITGA4-mRNA could be effectively delivered to the target cells, but not followed by protein production. Stabilization of ITGA4-mRNA with SSB-protein resulted in ITGA4-protein production exclusively in HEK293 cells. An ARCAcap introduction to ITGA4-mRNA resulted in 24h-long production of ITGA4-protein in MSCs, while ITGA4-mRNA was present in cells for a several times longer period. The temporal pattern of ITGA4-protein location was observed, with perinuclear appearance within 3 hours post transfection, with subsequent displacement reaching cell membrane within next 5 hours.

Conclusions: Translation of the cytoplasm-delivered mRNA, rather than transfection step, seems to be a hurdle preventing ITGA4-protein production in MSCs based on exogenous mRNA delivery, and ARCAcap appears to mitigate this issue. Importantly, the duration of ITGA4-protein presence in MSCs is sufficient to be used to enhance MSCs homing.

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265 Differentially expressed miRNAs in putative grid cells in the entorhinal cortex

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Introduction: Grid cells, so called for their repetitive grid-like firing patterns when an animal explores an environment, are located in the medial entorhinal cortex (MEC) and thought to be involved in spatial navigation. Stellate cells, putative grid cells, are found in layer II of MEC and reside with other specialized cells such as head-direction, border, conjunctive (which have both head-direction and grid-firing properties) cells and interneurons. How specialization of stellate cells occurs during development remains unknown. miRNAs have been shown to be important for the establishment and maintenance of neuronal subtypes, and we therefore asked whether miRNAs are differentially expressed in stellate cells compared to surrounding tissue at stages of development before and after stabilization of grid cell function.

Methods: We used two approaches to find differentially expressed miRNAs. First, we microdissected layer II and the deeper layers of the MEC of rats at postnatal days 2 (P2), 9, 23, and 45. Total RNA was then purified and analyzed on an Agilent miRNA microarray. For our second approach, we labeled the stellate cells by injection of the retrogradely transported dye, DiO, into the dentate gyrus of the hippocampus of rats aged P4/P5. We then sorted labeled from unlabeled cells using FACS. The RNA purified from the two cell populations was analyzed by TaqMan array.

Results and discussion: From the first experiment, we found one miRNA to be differentially expressed between layer II and deep layers at P2, 26 at P9, 11 at P23, and 13 at P45. Eleven miRNAs were differentially expressed between layers at all ages. In the second experiment, seven miRNAs were differentially expressed in stellate cells compared to the surrounding tissue. Two miRNAs were found to be differentially expressed in both experiments. The predicted targets of the differentially expressed miRNAs are involved in pathways for learning and memory, ion transport, and the shaping and connection of neurons, including synaptogenesis. At earlier ages (P2/P9), more predicted targets are enriched in pathways for neuronal development. These results point to fine-tuning of pathways that could be important for the specialized development and maintenance of stellate cell structure and function.

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266 Extended ovarian hormone deprivation alters the effects of 17β-estradiol on microRNA expression in the hypothalamus of aging female rats.

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Administration of 17β-estradiol (E2) has beneficial effects on cognitive function in peri- but not post-menopausal women, yet the molecular mechanisms underlying age-related changes in E2 action remain unclear. We propose that there is a biological switch in E2 action that occurs coincident with age and length of time after ovarian hormone depletion, and we hypothesized that age-dependent regulation of microRNAs (miRNA) could be the molecular basis for that switch. Previously we identified seven miRNAs that are regulated by E2 in an age-dependent manner. Here we tested whether increasing lengths of ovarian hormone deprivation in aged females alters E2 regulation of the seven mature miRNAs. In addition, we determined where along the miRNA biogenesis pathway E2 exerted its effects. Our results showed that age and increased lengths of ovarian hormone deprivation abolished the ability of E2 to regulate mature miRNA expression in the brain and the actions of E2 were mediated primarily through ERβ. Further, we show that E2 regulates the primary and precursor forms of the miRNAs demonstrating that E2 acts at specific points along the miRNA biogenesis pathway. Surprisingly, our results also demonstrate that age dependent alterations in the mature miRNA do not correspond to their primary and precursor expression suggesting that the stability of these miRNAs are changed with aging. Taken together our data demonstrate that miRNA biogenesis in the brain is sensitive to ovarian hormone deprivation and aging.

267 hnRNP-Q/Syncrip regulates neural plasticity and PSD-95 gene expression at the *Drosophila* neuromuscular junction

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Many of the cellular changes that occur during memory acquisition and learning take place pre- and post-synaptically. Transcription and translation of synaptic genes at the cell body are critical during long-term memory but cannot produce rapid local changes in expression at the synapse. Local regulation of translation at the synapse in response to neuronal activation is still not well understood, but certainly requires a diverse range of mRNA binding proteins. Our previous work has shown that the majority of RNA targets of one such RNA binding protein, Syncrip/hnRNP-Q, are known to have specific nervous system functions and that Syncrip mutants exhibit aberrant synapse structure and function at the *Drosophila* larval neuromuscular junction (NMJ). Here, we describe our use of this system to investigate one of the most important Syncrip targets, discs large (Dlg), the *Drosophila* homologue of mammalian PSD-95, which is involved in clustering glutamate receptors in the post synaptic compartments in mammalian brain as well as at the *Drosophila* NMJ. We show that repetitive stimulation in wild type larvae caused a two-fold increase in Dlg protein levels. This increase was abolished in a Syncrip loss-of-function (LOF) mutant and by blocking translation with puromycin. Using Syncrip immuno-precipitation combined with RT-qPCR we determined that Syncrip normally binds *dlg* mRNA, but releases *dlg* upon activation. Collectively these data suggest that stimulus-triggered release of its target allows Syncrip to regulate Dlg expression. We are currently investigating the precise molecular mechanism of how Syncrip regulates Dlg expression in the context of neural plasticity. Given that Syncrip is known to target over 200 genes with specific nervous system functions it will be important to determine if these roles extend to the brain and to other organisms.

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268 Investigating a role for CAPRIN1 in mRNA stability

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RNA binding proteins (RBPs) are RNA-targeting proteins that direct the metabolism of cellular mRNAs and regulate events such as mRNA localization and protein translation. CAPRIN1 is a RBP with disputed molecular function that is localized to cytoplasmic RNA granule and dendritic ribosome complexes. Because RNA granules formation is associated with many neurodegenerative and metabolic diseases, we wanted to know how CAPRIN1 affects RNA metabolism. In order to clarify the molecular role for CAPRIN1 in human cells, we initially overexpressed CAPRIN1 in HEK293T cells, then immunoprecipitated CAPRIN1 complexes and assayed interactors with mass-spectrometry. This approach identified 62 co-precipitated proteins, of which 62% (36) are other RBPs with an enriched function in mRNA processing. Separately, a public CAPRIN1 PAR-CLIP revealed a group of 2900 CAPRIN1 targets with at least one 3'UTR binding site, with enriched association with RNA-processing proteins. Taken together, these findings suggest that CAPRIN1 controls or coordinates the stability of many mRNAs. We are currently validating the mass-spectrometry-defined primary interactors with reciprocal co-IP and we will measure the effect of CAPRIN1 direct binding for a set of mRNA targets.

269 Tau phosphorylation plays a role in mRNA 3' end processing

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Tau is a microtubule-associated protein involved in a number of neurodegenerative disorders, including Alzheimer's disease (AD). In normal individuals, Tau is a highly soluble, non-phosphorylated protein that stabilizes microtubules in the cytoplasm of neurons. However, neurons in AD's contain insoluble hyperphosphorylated-tau aggregates known as paired helical filaments. Previous studies have shown that Pin1, a prolyl-isomerase initially identified as a mitotic regulator overexpressed in most human cancers, is involved in the regulation of Tau phosphorylation under different conditions. Furthermore, Tau has been functionally linked to another Pin1 substrate, the tumor suppressor p53 and some of its isomers. Interestingly, Dr. Kleiman's lab identified p53 as an activator of PARN-dependent mRNA deadenylation in the nucleus as part of the DNA damage response.

As Pin1, p53 and PARN are localized in the nucleus and are involved in the regulation of mRNA 3' end processing under different cellular conditions, we hypothesize that Tau might also localize in the nucleus of non-neuronal cells and functionally overlap with these factors. Our cellular fractionation assays with samples of HCT116 human colon carcinoma cell line indicated that tau isoforms are present in the cell nucleus. Interestingly, nuclear isoform patterns of Tau changed upon Pin1 inactivation, UV irradiation, p53 expression and/or phosphatase treatment. Using co-immunoprecipitation assays, we showed that some Tau isoforms can form (a) complex(es) with p53, Pin1 and PARN, and these interactions change under different cellular conditions. Strikingly, overexpression of Tau, but not of its phosphomimic mutants, induces nuclear deadenylase activity in CHO cells. Finally, siRNA-mediated knockdown of Tau decreases nuclear deadenylation in samples from HCT116 cells under non damaging conditions. Moreover, Tau knockdown further increases the previously described UV-induced deadenylation.

Although more research is necessary, these preliminary results reveal a new potential role for Tau in the regulation of mRNA 3' processing. These findings also support the idea that factors involved in cancer and Alzheimer's disease might play a role in regulating gene expression by a functional interaction with the mRNA 3' processing machinery in different conditions, resulting in specific gene expression patterns.

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270 Timing is everything: extremely short 4-thiouracil labelling to study RNA processing kinetics

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The levels of different RNAs detected at steady state are the product of multiple dynamic processes within the cell. For example, with intron-containing genes, the level of the mature mRNA depends on splicing as well as synthesis and decay. At steady state the abundance of mRNA is generally several orders of magnitude greater than the abundance of the corresponding pre-mRNA, with the rate of production, including splicing, matching that of degradation. This does not allow accurate examination of the rate or efficiency of splicing of individual transcripts. Metabolic tagging with the nucleotide analogue 4-thiouracil (4SU) is one way of determining the relative contributions of RNA synthesis, decay and conversion processes[1]. Labelling RNA *in vivo* with 4SU for very short times (down to 1 min), permits newly synthesised RNA to be affinity-purified, allowing significant enrichment of nascent transcripts before they are processed, so that even highly unstable transcripts such as CUTS can be isolated. For processed transcripts, the rate at which the ratio of precursor/mature RNA species reaches the steady state level is evidently different for different transcripts. Therefore this technique, combined with high throughput sequencing, should allow measurement of transcriptome-wide rates of RNA processing and transcription in *Saccharomyces cerevisiae* with high kinetic resolution.

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271 The role of the mammalian methyltransferase Tgs1 in RNA processing

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The trimethylguanosine synthase 1(Tgs1) is the methyltransferase responsible for the hypermethylation of certain Pol II transcribed RNAs. It converts canonical 7-monomethylguanosine caps of these RNAs into 2,2,7-trimethylguanosine (TMG) caps. snRNAs, snoRNAs and telomerase RNA are among the TMG-capped RNAs. Despite their abundance and roles in many biological events (splicing, rRNA modification, telomere maintenance, et al), limited information is available about how Tgs1 and the resulting TMG cap affect processing of those RNAs.

To gain a comprehensive understanding of the role of Tgs1 in RNA processing, we attempted to knock out Tgs1 in both mouse and human. A mouse Tgs1 hypomorgh cell line exhibits processing defect in U2 snRNA. This phenotype is also seen in a human Tgs1 conditional knockdown cell line. The human cell line was generated using Flp-In/T-REx system combined with CRISPR. A hypomorphic version of Tgs1(H-Tgs1) was integrated into Flp-In 293 T-Rex cell line, whose expression was under the control of a Tet-inducible promoter. The endogenous Tgs1 was subsequently disrupted using CRISPR. The new cell line harbors only H-Tgs1, and after depleting tetracycline in the media, the integrated H-Tgs1 is shut off, resulting in undetectable level of Tgs1 protein. We will present the effects of Tgs1 knockdown on the 3' end processing of snRNAs and snoRNAs.

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272 Towards an Understanding of the Mechanism of CFIm25 in Alternative Polyadenylation

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Alternative Polyadenylation (APA) is a process in which an mRNA undergoes cleavage and polyadenylation at a location other than the expected and most canonical poly(A) site. Our lab and others have recently shown that the Mammalian Cleavage Factor I (CFIm) complex plays an integral role in regulating poly(A) site choice by the cleavage and polyadenylation machinery. Among the three subunits of the CFIm complex (CFIm25, CFIm59, and CFIm68), we observe that the knockdown of CFIm25 causes the most dramatic shift in poly(A) site usage, almost exclusively from a distal site to a more proximal site. Though this activity of CFIm25 is known, the mechanism has not been discerned.

CFIm25 forms a homodimer as well as a hetero-tetramer with two CFIm59 or CFIm68 subunits. The structures of these complexes have been solved but the role that these interactions play in the mechanism of poly(A) site choice is unclear and is the focus of this research. Moreover, we have observed that CFIm25 binding sites are often found at the extremes of 3'UTRs subject to APA suggesting that either two distinct complexes are formed at each poly(A) site or that there is communication between CFIm binding at both positions. We hypothesize that CFIm25 forms a hetero-tetramer with other CFI members, and binds to RNA elements flanking certain proximal poly(A) sites to repress the use of these sites by steric hindrance and decreased recognition of the cleavage and polyadenylation signals. To determine the validity of this hypothesis several tools have been developed. First many mutations have been made of CFIm25 in eukaryotic expression vectors that are predicted to disrupt protein interactions and post-translational modifications that may be key to the mechanism of poly(A) site repression. These constructs will be used in conjunction with an APA reporter that is being developed to interrogate the importance of each element within the CFIm25 structure. It is expected that mutation to those elements required for CFIm25 to interact with its self or other CFI complex members will remove the ability of the complex to repress the proximal poly(A) site usage in the in-vivo reporter system.

273 Intron Retention Is A Major Regulator Of Gene Expression During Terminal Erythropoiesis

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We are studying developmental regulation of pre-mRNA splicing in a unique RNA-seq dataset prepared from five highly purified populations of human erythroblasts, representing the last four to five cell divisions of erythroid progenitors as cells undergo substantial remodeling in preparation for enucleation. Our previous work showed that differentiating erythroblasts execute a robust and dynamic alternative splicing program involving many switches in splicing of cassette exons in conjunction with the last two maturational divisions. Here we used new computational tools to elucidate a novel network of intron retention (IR) events that play an important role in modulating the transcriptome during terminal erythropoiesis. Differences in differentiation stage-specificity, degree of retention, nuclear/cytoplasmic localization, and sensitivity to nonsense-mediated decay (NMD) suggest the existence of multiple IR classes subject to distinct regulatory controls. We analyzed a set of IR events comprised of highly-retained single introns and pairs of introns in otherwise efficiently-spliced transcripts. These stably-expressed IR transcripts were primarily nuclear-localized, where they could avoid NMD and potentially undergo final processing in response to physiological stimuli. Gene ontology (GO) analysis revealed that IR was enriched in major genes for RNA processing, likely influencing expression of numerous downstream splicing targets. Genes with critical functions in iron homeostasis were also affected by IR. High IR was enriched adjacent to alternative NMD-inducing exons, suggesting a mechanistic association with unproductive splicing events; in contrast, retention was lower for introns flanking alternative coding exons and constitutive exons. High IR was also observed in important disease genes including splicing factor SF3B1 (myelodysplasia), transferrin receptor-2 (hemochromatosis type 3); and the RNA binding protein FUS (ALS). Similar to the switches in exon splicing in late stage erythroblasts, many changes in IR also occurred in polychromatic and orthochromatic erythroblasts, representing the two last populations of terminally differentiating erythroblasts. We propose that IR plays a critical role in gene regulation during normal erythropoiesis, and mis-regulation of IR may be responsible for human disease.

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274 Molecular determinants for Archease dependency of the RNA ligase RtcB

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RtcB is a noncanonical RNA ligase that catalyzes the GTP and Mn(II)-dependent joining of either 2′,3′-cyclic phosphate or 3′-phosphate termini to 5′-hydroxyl termini. Archease is a 16-kDa protein that is conserved in all three domains of life. In diverse bacteria and archaea, the genes encoding Archease and RtcB are localized into an operon. We have recently provided a rationale for this operon organization by showing that Archease and RtcB from *Pyrococcus horikoshii* function in tandem. Remarkably, Archease endows RtcB with both ATP and GTP cofactor specificity and also accelerates RNA ligation. However, not all organisms that encode RtcB also encode Archease. Here we demonstrate the existence of Archease-dependent and independent classes of RtcB. The two classes of RtcB are distinguishable by their amino acid sequences, structures and ligation kinetics. Furthermore, we demonstrate that Archease proteins are functionally interchangeable across the domains of life. Archease from archaea can activate RtcB from bacteria, suggesting a high conservation of amino acid residues that form the recognition interface between the two proteins. Our work sheds light on the coevolution of RtcB and Archease and provides insight into Archease function.

275 Dissecting the functional roles of Quaking RNA binding protein isoforms in muscle

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Mammalian RNA binding protein families have members with indistinguishable RNA sequence recognition properties, but different functions, suggesting that important functional elements reside outside the RNA binding domains. Quaking proteins Qk5 and Qk6 arise from one gene through alternative splicing and differ only at the C-termini. Qk5 is nuclear and shuttles whereas Qk6 is predominantly cytoplasmic. The isoform ratios differ between tissues: Qk5 is higher in muscle; Qk6 is higher in oligodendrocytes. We are studying functions of these isoforms and regulatory controls that generate tissue specific isoform ratios. Using isoform-specific RNAi, we find expression of Qk6 requires Qk5, and depletion of Qk6 leads to an increase in Qk5, suggesting that isoform ratios are partially autogenously determined. Genomewide analyses identify numerous changes in splicing and gene expression: some are responsive to Qk5 knockdown (depletes Qk5 and Qk6) but not Qk6 knockdown, whereas others respond to both Qk5 and Qk6 knockdown. To more rigorously identify isoform-specific functions, we deplete endogenous Qk and rescue with a single isoform. This assay shows that Qk5 but not Qk6 is required for splicing at Qk regulated exons. To test whether these roles depend on the isoform-specific C-termini we expressed "tailess" Qk containing only the common 311 amino acids (Qk0), or Qk0 fused to SV40 NLS at its N-terminus (NLS-Qk0) in myoblasts depleted of endogenous Qk. Qk forms that are found in the nucleus (Qk5, NLS-Qk0, and Qk0) promote Qk-dependent splicing, whereas cytoplasmic Qk6 does not, suggesting that Qk6 does not promote splicing because its C-terminus carries a nuclear export signal.

Overall, these data suggest that Qk isoform function is determined by localization, through isoform-specific C-terminal tails, and that interactions with the gene expression regulatory machinery are mediated by common part(s) of the protein. Finally, isoform output is post-transcriptionally controlled in large part by Qk isoforms themselves. We are currently exploring mechanisms by which these unusual auto-regulatory feedback loops are maintained. These findings may provide clues to how dysregulation of Qk expression leads to various pathologies.

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276 TUT-DIS3L2 mediates RNA quality control in mammalian cytoplasm

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The 3'-end RNA uridylation has different roles in different organisms. In mammals, TUTases 4 and 7 catalyze oligo(U) addition to the precursors of certain miRNAs, to mRNAs and U6 snRNA to trigger their degradation (1, 2, 3, 4, 5). On the other hand, the same TUTases monouridylate pre-miRNAs to facilitate their processing by Dicer (6, 7). DIS3L2 is an oligo(U) specific exoribonuclease that degrades uridylated precursors of let-7 miRNAs (8, 9). DIS3L2 has been linked to the Perlman syndrome development and Wilms tumor progression (10). However, no functional link has been made between uridylation and the involvement of DIS3L2 in these diseases.

We have identified that DIS3L2 targets a wide spectrum of uridylated noncoding RNAs. Most of these RNA species contain oligo(U) tail with the average length of 8-10 nucleotides. Most of the DIS3L2 bound RNAs display features of aberrant molecules derived from either aberrant co-transcriptional or posttranscriptional processing. Moreover, we also detect transcripts from pseudogenes of some ncRNAs. Given the broad spectrum of targeted RNA molecules, we propose, that TUT-DIS3L2 activity represents a general RNA degradation and quality control pathway operating in mammalian cytoplasm.

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277 Fine-tuning pre-tRNA processing by environmental conditions in S.cerevisiae.

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In yeast processing of primary tRNA transcript starts with cleavage of 5' leaders followed by removal of 3' trailers and then splicing of introns. We study how end-processing of yeast pre-tRNAs responds to heat shock and changes from fermentable to nonfermentable carbon sources. By northern analysis we show that upon shift to elevated temperatures and/or to glycerol-containing medium the pattern of tRNA precursors changes in a tRNA-type and condition-specific manner. Under stress conditions additional pre-tRNAs accumulate. For pre-tRNA Ile(UAU) and pre-tRNA Lys(UUU), the aberrant forms are unprocessed at the 5' ends; whereas the aberrant pre-tRNA Tyr(GUA) species that accumulates has been processed at the 5' terminus. Similar aberrant precursors for these aberrant tRNAs were detected in the *rex1Δ* strain which is defective in exonucleolytic trimming of pre-tRNA 3' ends. The observations indicated that processing of the 3' ends of tRNA precursors might be inhibited by stress. The additional form of pre-tRNA Ile(UAU) migrates slower than the form previously designated as the primary transcript that contains both the 5' leader and the 3' trailer. Using linker ligation and sequencing we learned that the aberrant slow migrating pre-tRNA Ile(UAU)possesses extended 3' ends. The data indicate that partial processing of the 3' trailers in tRNA Ile(UAU) can occur before removal of the 5' leader. We further show that there is direct correlation between the inhibition of 3'-end processing rate and the stringency of growth conditions. Moreover, under stress conditions Rex1 nuclease seems to be limiting for 3'-end processing of pre-tRNA Tyr(GUA). Thus, our data document complex 3' processing steps that fine-tune levels of functional tRNA in budding yeast in response to environmental conditions.

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278 Alternative Polyadenylation and translation efficiency

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Alternative polyadenylation (APA) can produce mRNA isoforms with different 3'UTR length, which are very common in eukaryotic cells. APA was found associated with serveral biological phenomena, such as cancer, immune response and embyro development, and 3'UTR takes important roles in mRNA stability, translation efficiency and subcelluar localization. However, there is still lack of genome wide evidences for APA effects on these functions of 3'UTR. Furthermore, contradictary results of APA effects on the translation efficiency emerged recently. Here we developed a new low-input method to sequencing APA sites (IVT-SAPAS). And with IVT-SAPAS and polysome profiling, we found that, in breast cancer cells, more ribosomes were bound on the mRNA isoforms with shorter 3'UTRs, suggesting that mRNA isoforms with shorter 3'UTR have higher translation efficiency.

279 The cleavage/polyadenylation complex in *Trypanosoma brucei*: novel components identification and function analysis

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3' polyadenylation is an essential step for most eukaryotic mRNAs maturation, but little is known about the composition, structure and function of polyadenylation complex of trypanosomes. Trypanosomes are evolutionarily ancient unicellular protozoan parasites with genes clustered into polycistronic transcription units. The mRNA 3'-end formation of a gene was found to be coupled with trans-splicing which add a 5' cap structure to the adjacent downstream gene transcript. Therefore, the 3'-processing complex in trypanosomes is undoubtedly endowed with some characteristics distinct from other eukaryotes. Here we reported the purification and subsequent proteomic and functional characterization of *Trypanosoma brucei* mRNA 3' processing complex. Nine core cleavage/polyadenylation factors were identified in the purified 3' processing complexes, including previously experimentally determined CPSF30 and Fip1-like, computer predicted CPSF160, CPSF100, CPSF73, and CstF50, and three novel proteins, designated as CPC159, CPC30 and CPC15, which have no obvious homology to the proteins outside the kinetoplastides. RNAi knockdown the expression of CPSF160, 100, 73, CstF50 and CPC159 inhibited growth, resulted in the decline of matured α- and β-tubulin mRNAs and accumulation dicistronic tubulin transcripts, indicating that they are each essential for cell viability and mRNA processing in both procyclic and bloodstream forms of *T. brucei*. However, a weak knockdown the expression of CPC30 and CPC15 did not affect growth and tubulin mRNA splicing. Remarkably, disruption of CPSF73 and CstF50, especially the latter, caused more usage of proximal poly(A) sites, strongly suggesting an involvement of these two core factors in the polyadenylation site selection in *T. brucei*. Unexpectedly, CPSF160 or CPSF73 depletion by RNAi dramatically down-regulated the mRNA abundance but up-regulated the proteins level of the reporters in a dual reporter system mimicking the expression of the endogenous α- and β-tubulin, strongly suggesting that CPSF160 and CPSF73 may function in the translation control. This hypothesis was further supported by the comparison of endogenous tubulin mRNA and protein level upon depletion of core poly(A) factors. All together, these results suggest that the cleavage/polyadenylation complex in *T. brucei* may regulate the gene expression at multiple levels, including poly-cistronic transcripts splicing, poly(A) site selection, and translation efficiency.

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280 Characterization of double-stranded RNA cleavage by the inside-out mechanism of Ribonuclease III

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Double-stranded RNA (dsRNA) plays critical roles in many biological progresses, especially gene expression regulation. Ribonuclease III (RNase III) selectively recognizes and cleaves dsRNA by hydrolyzing phosphodiester bonds, resulting in dsRNA fragments that have two-nucleotide 3′ overhangs. In Escherichia coli, RNase III functions as a molecular ruler: associates the ends of the substrate, measures a relatively fixed distance from the ends, and performs the cleavage, generating products ranging from 10-15 base pairs (bp) in length. Interestingly, a single amino acid substitution (E38A) of E. coli RNase III promotes an alternative mode of cleavage similar to the inside-out mechanism of Dicers from budding yeast: multiple RNase IIIs perform cleavage concurrently while sitting next to each other on the substrate dsRNA, creating longer products around 23 bp in size.

In an enzyme engineering effort aimed to alter the product size of the inside-out processing, we further characterized this mode of dsRNA cleavage by E. coli RNase III. Using a long dsRNA (~1.6 KB) generated by in vitro transcription from the firefly luciferase sequence as the substrate, we measured the cleavage activities of wild-type (WT) and mutant RNase III proteins by gel electrophoreses and validated by deep sequencing. To our surprise, a small portion (0.5%) of the WT enzyme cleavage products was 22-bp, indicating that the inside-out type of cleavage may happen under physiological conditions. While the mutants generated more 22-bp duplexes during cleavage, the majority of the products were still 10-12 bp in length. Interestingly, products with different sizes were generated from different regions of substrate, which was consistent between the WT and all mutant enzymes tested. These observations suggest that RNase III decides its mode of cleavage and size of products based on local structure and sequences.

This study provided new insights into the mechanism of RNase III cleavage. It also shed lights on cost-effective approaches to generate therapeutic small interfering RNAs by prokaryotic RNase III enzymes.

281 Structural and functional comparison of nuclear and organellar *Arabidopsis thaliana* proteinaceous RNase Ps give an insight of their evolution

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RNase P catalyzes the removal of 5' leader sequence of immature tRNAs. The canonical RNase P is a ribozyme associated with one to ten proteins depending on the organism. However, protein only RNase Ps (PRORPs) were discovered recently in Eukaryotes. *Arabidopsis thaliana* PRORP1 and PRORP2 enzymes are both nuclear encoded but have different cellular localization; PRORP2 localizes to the nucleus, PRORP1 to the chloroplast and the mitochondria. Here we were able to capture a snapshot of the early specialization of these *A. thaliana* PRORPs by comparing *in vitro* functional data and the crystal structures of PRORP1 and PRORP2. Mg^{2+} dependent activities under single turnover conditions of PRORP1 and PRORP2 show high similarity with substrates of distinct origin, although PRORP2 processes mitochondrial substrates slightly slower than nuclear ones. We obtained a 3.3 Å resolution crystal structure of PRORP2 and compared it to the previously published structure of PRORP1. Both of the proteins have an overall V-shape with similar structural features; however PRORP2 adopts a more open conformation than PRORP1. The overall similarities but minor differences of PRORP1 and PRORP2 suggest that these enzymes are in the early steps of specialization after localization into different cellular compartments.

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282 Understanding the Role of Post-Translational Modifications in the Activity of Splicing Factor PTBP1

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Signal-induced alternative splicing is an important mechanism for regulating protein isoform expression in response to changing cellular environments. Splicing changes in response to extracellular stimuli can be mediated by post-translational modifications (PTM's) in splicing factors which alter the proteins' concentration, RNA binding properties, cellular localization, and/or protein-protein interactions. Despite the functional importance, molecular mechanisms linking extracellular stimuli to pre-mRNA splicing; the connection between post-translational modifications in splicing factors (such as phosphorylation and acetylation) and alterations in the cellular splicing pattern is not well understood.

The Polypyrimidine Tract Binding Protein 1 (PTBP1) is a well-studied splicing factor that has served as an informative model in understanding how RNA binding proteins affect spliceosome assembly1. PTBP1 has 4 RNA recognition motifs (RRMs') connected by 3 linker regions and an N-terminal region with a nuclear localization signal. PTBP1 functions primarily to repress inclusion of regulated cassette exons in the final mRNA transcript. The N1 exon of the c-src pre-mRNA is a well-studied model system used to characterize the splicing activity of PTBP1.

Proteome wide mass spectrometry results of post-translational modifications highlight modifications including phosphorylation, acetylation and ubiquitination in PTBP1 from several cell lines2. We note that modifications are restricted to the RRM's, N-terminal region of the protein and modified residues include those that participate in RNA binding. Modifications also alter cellular localization, as for example phosphorylation of serine16 mediates nuclear-cytoplasmic shuttling of PTBP13. The roles of all other post translational modifications of PTBP1 are not understood. To examine the biological significance of these modifications we are currently conducting an alanine scan of the modified residues and assaying the mutants for splicing activity.

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283 Cellular site of UPF1 phosphorylation in nonsense-mediated mRNA decay

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Nonsense-mediated mRNA decay (NMD) is an mRNA quality control mechanism that detects and degrades aberrant mRNAs harboring a premature termination codon (PTC). NMD also targets normal physiologic mRNAs, contributing to the maintenance of cellular homeostasis in a changing environmental milieu. A central NMD factor is the ATP-dependent RNA helicase UPF1, which is activated by SMG1-mediated phosphorylation to trigger targeted mRNA degradation[1,2]. During mammalian-cell NMD, phosphorylated UPF1 (p-UPF1) mediates translational repression of the NMD target[3] and recruits endonucleolytic and/or exonucleolytic activities[4,5]. Although UPF1 phosphorylation is a key event to initiate targeted mRNA decay, where within the cell UPF1 phosphorylation occurs has been debated. Some data indicate that p-UPF1 preferentially interacts with many nucleus-associated RNA-binding proteins, suggesting that UPF1 phosphorylation occurs prior to when UPF1-bound mRNPs are exported to the cytoplasm[6]. Other data indicate that p-UPF1 in the form of exogenously expressed ATPase-deficient UPF1variants localize predominantly to cytoplasmic processing bodies (P-bodies)[7-9], indicating that UPF1 phosphorylation occurs in ribosome-free P-bodies[10,11]. Here we show that endogenous p-UPF1exists on cytoplasmic polysomes in human cells. Using immunofluorescence, endogenous p-UPF1 is consistently found to reside diffusely in the cytoplasm and is not enriched in P-bodies (the latter, as defined using anti-DCP1a). The same result was obtained when the concentration of cellular p-UPF1 was elevated by exposing cells to okadaic acid. From these and other results, we conclude that p-UPF1 exists in the translationally active cytoplasmic fraction of cells, supporting that UPF1 phosphorylation occurs co-translationally.

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284 Generation of functionally distinct isoforms of PTBP3 by alternative splicing and translation initiation

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Polypyrimidine tract binding protein (PTBP1) is a widely expressed RNA binding protein that acts as a regulator of alternative splicing regulator and of cytoplasmic mRNA functions. Vertebrates contain two closely related paralogs with >75% amino acid sequence identity. PTBP2 plays a key role in neuronal differentiation; early replacement of PTBP1 by PTBP2 leads to a concerted set of splicing changes. By comparison, little is known about the physiological roles of PTBP3, although its expression and conservation throughout the vertebrates suggests that it may play a role in haematopoietic cells.

We have characterized the mRNA and protein isoform complement of PTBP3 (also known as ROD1). Combinatorial alternative splicing events at the 5´ end of the gene allow for the generation of three major protein isoforms, with individual mRNAs able to generate up to three protein isoforms via alternative translation initiation. The shortest isoform arises via initiation at AUG11 and lacks most of the first RNA binding domain, yet binds to a number of RNAs with a higher affinity than some of the longer isoforms, and shows identical in vitro splicing repressor activity upon FAS exon 6. The PTBP3 isoforms vary in their RNA binding properties and nuclear/cytoplasmic distribution. The AUG4 and AUG11 isoforms are evenly distributed between nucleus and cytoplasm suggesting that PTBP3 may have major post-transcriptional cytoplasmic roles.

In order to further study the role of PTBP3 proteins in the hematopoietic system we are generating conditional knock out mouse models and carrying out genome wide analyses in order to identify binding and functional targets of PTBP3 proteins.

285 Quantifying the Effects of MDS Mutations in HSH155 on Splicing of Yeast Pre-mRNAs

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The spliceosome is responsible for the excision of introns from pre-mRNA in eukaryotes and alterations of the spliceosome can cause serious consequences. Recently, studies have shown mutations in certain genes associated with the spliceosome are linked with certain types of Myelodysplastic Syndromes (MDSs). Specifically, mutations in SF3B1, an U2snRNP associated splicing factor, have been found in approximately 20% of MDS cases. In our research, we will investigate the corresponding mutations in the *S. cerevisiae* homolog, HSH155, which is well-conserved between humans and yeast. We have introduced MDS-related mutations into *S. cerevisiae* HSH155 and showed the cells have no significant growth differences than the wild type. However, these cells show a change in the fidelity of branchsite selection: several MDS mutations decrease the splicing of ACT1-CUP1 reporters with nonconsensus splice sites (see abstract by Carrocci et al.). Based on this result, we expect splicing of only a subset of yeast transcripts with nonconsensus splice sites to be affected by the mutations, resulting in an increased ratio of pre-mRNA to mRNA. In order to check this hypothesis, we are carrying out Reverse-Transcription Polymerase Chain Reaction (RT-PCR) on six different genes (SUS1, DYN2, GLC7, YFR045w, PMI40, MATa1) to see if such differences exist between wild-type and mutants. In addition, we are quantifying the levels of ACT1-CUP1 pre-mRNA and mRNA in our reporter system by primer extension assays. Determining how these mutations affect splicing in yeast will help us not only understand the essential roles of the HSH155 protein but also provide clues to how these mutations may cause MDS in humans.

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286 A Recurrent Gene Fusion Event Generates a Novel 3´UTR for Cyclin D1 in Mantle Cell Lymphoma

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The three human D-type cyclins activate cdk4/6 and are rate-limiting for G1-S-phase cell cycle transition. Of the three cyclins, deregulated levels of cyclin D1 result in early onset of oncogenesis and increased metastasis. There are two cyclin D1 splice variants, which occur because of a common G870A polymorphism found at the exon 4 splice site. The cyclin D1a (cyclin D1) isoform contains five exons (~4.5kb) and cyclinD1b (~1.7kb) contains exons 1-4 and a short sequence derived from intron 4. The cyclin D1b isoform is limited to only a few tumors, whereas cyclin D1 is more widely overexpressed in cancer.

Cyclin D1 is overexpressed in >90% of the clinically incurable B-cell lymphoma subtype, Mantle Cell lymphoma (MCL) cases and is used in disease diagnosis. Normal B-cells do not express cyclin D1. However, in MCL a t(11:14) (q13;q32) chromosomal translocation event places the *CCND1* oncogene under the control of the IgH enhancer region resulting in cyclin D1 transactivation. Despite this activation, the normal half-life of full length cyclin D1 mRNA is only 30 minutes. Some MCL patients have tumors that express cyclin D1 transcripts with truncated 3´UTRs that are highly proliferative resulting in a two-year reduction in survival. Although the coding region remains unaltered, truncation of the 3´UTR eliminates numerous AU-rich elements and miRNA binding sites. In some MCL patients, diverse mutations generate a premature canonical polyadenylation signal (PAS) thus facilitating alternative polyadenylation. The rest of the 3´UTR truncations are posited to result from unknown chromosomal deletions.

We have identified a novel *CCND1-MRCK* gene fusion in MCL. The coding region is unaltered, but the 3´UTR consists of sequences from both *CCND1* and *MRCK* and uses a canonical PAS derived from *MRCK's* intron. Consequently, the resulting fusion transcript is refractory to miRNA regulation. RNAi that targerts either *CCND1* or the *MRCK* sequence results in comparable levels of cyclin D1 protein knockdown. Considering that cyclin D1 overexpression is an early event in MCL oncogenesis and a current therapeutic target, identification of all the possible oncogenic transcripts is essential for optimal design of RNAi therapeutics and amplicons for PCR detection.

287 *In vivo* characterization of the *Drosophila* mRNA 3' end processing core cleavage complex (CCC)

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A core cleavage complex (CCC) consisting of CPSF73, CPSF100 and Symplekin is required for co-transcriptional 3' end processing of all metazoan pre-mRNAs, yet little is known about the *in vivo* molecular interactions within this complex. The CCC is a component of two distinct complexes, the cleavage/polyadenylation complex and the complex that processes non-polyadenylated histone pre-mRNAs. RNAi-depletion of CCC factors in *Drosophila* culture cells causes reduction of CCC processing activity on histone mRNAs, resulting in read through transcription. In contrast, RNAi-depletion of factors only required for histone mRNA processing allows use of downstream cryptic polyadenylation signals to produce polyadenylated histone mRNAs. We used Dmel-2 tissue culture cells stably expressing tagged CCC components to determine that amino acids 272-1080 of Symplekin and the C-terminal ~200 amino acids of both CPSF73 and CPSF100 are required for efficient CCC formation *in vivo*. Additional experiments reveal that the C-terminal 241 amino acids of CPSF100 are sufficient for histone mRNA processing indicating that the first 524 amino acids of CPSF100 are dispensable for both CCC formation and histone mRNA 3' end processing. CCCs containing deletions of Symplekin lacking the first 271 amino acids resulted in dramatic increased use of downstream polyadenylation sites for histone mRNA 3' end processing similar to RNAi-depletion of histone specific 3' end processing factors FLASH, SLBP and U7 snRNA. We propose a model in which CCC formation is mediated by CPSF73, CPSF100 and Symplekin C-termini, and the N-terminal region of Symplekin facilitates co-transcriptional 3' end processing of histone mRNAs.

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288 RNA-Binding-Protein-Modifying (RBPM) Enzymes can Guide the Function of RNP Complexes *In Cis*.

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RNA-binding proteins control gene expression in all living cells by regulating all aspects of RNA biology. A typical RNA-binding protein has thousands of RNA targets. Strikingly, many RNA-binding proteins are multifunctional and, depending on their substrates, can have positive, negative or passive effects on the same molecular process. For example, Lin28a protein inhibits the processing of pre-let-7 and pre-miRNA-9 but has no effect on numerous other miRNAs, despite efficient binding to their precursors.

Recent high-throughput studies identified many new RNA-binding proteins that were not known for their RNA-binding properties and bear no identifiable RNA-binding domains. Among them are protein-modifying enzymes such as kinases, phosphatases and E3 ubiquitin ligases. This raises the possibility of the spatial control of RNA processing through post-translational protein modifications by RNA-Binding-Protein-Modifying (RBPM) enzymes.

We show that E3 ubiquitin ligase Trim25 is a RNA-dependent co-factor for Lin28a/TuT4-mediated uridylation of let-7 precursors. This demonstrates that an RBPM enzyme can guide the function of RNA-protein complexes *in cis*. We hypothesize that RBPM enzymes provide an additional layer of control over canonical RNA-binding proteins, and can dictate their function. Our working model is that when an RBPM enzyme binds RNA in the vicinity of a canonical RNP complex, it will modify it, thereby altering or fine-tuning its molecular function. This model could explain a long-standing conundrum of why some RNA-binding proteins are multifunctional and, depending on the substrate they bind, have positive, negative or passive effects on the same RNA processing event.

289 Identification of a new domain in RNA polymerase II CTD-interacting protein Pcf11

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In eukaryotes, poly(A) tails are added to nuclear pre-mRNA 3'-ends in the two steps of cleavage and polyadenylation. This co-transcriptional processing requires the activity of a large protein complex comprising at least 20 different polypeptides in yeast organized primarily into the two factors CF IA and CPF. We are interested in the functional characterization of Pcf11 and also the interaction and cooperation with the other protein components of CF IA (Cleavage Factor IA): Rna14, Rna15 and Clp1.

The Pcf11 protein is organized into different functional domains: the first 130 amino acids of the protein have been shown to interact directly with the carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II. This interaction is an essential feature in the coupling between 3'-end processing and transcription termination. Interestingly, the CTD-interacting domain (CID) of Pcf11 is dispensible for mRNA 3'-end maturation. Other characterized regions in Pcf11 include the interaction domain with Rna14 and Rna15 found in the central part of the protein, and Clp1 makes contacts with the C-terminal region.

In spite of the numerous functions already ascribed to Pcf11, there is still a large portion of the polypeptide that has not yet been characterized. For example the region from the end of the CID to the beginning of the Rna14/Rna15 interaction domain has no known function. A notable uninterrupted stretch of 20 glutamine residues is present within the region. The remainder we have now determined by NMR spectroscopy to correspond to a folded domain composed of three α-helices. Apart from the confirmed presence of a folded structure, this newly identified domain in Pcf11 does not exhibit any diagnostic features that would suggest a specific biological role.

To gain insight into the function of this new domain, we have employed a systematic strategy of mutagenesis, either by deletion or via point mutations. Using these mutant strains and mutant proteins, we are investigating effects in viability, pre-mRNA 3'-end processing and transcription termination.

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290 Conserved regions preceding and within the flexible tether affect tRNase Z enzyme activity

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tRNase Z, a member of the metallo-β-lactamase family of enzymes, plays a central role in precursor tRNA (pre-tRNA) maturation by endonucleolytically cleaving the 3' trailer, leaving a OH group ready for CCA addition and aminoacylation. tRNase Z has two forms, a short form, tRNase Z^s that is found mainly in bacteria and archaebacteria, and a long form, tRNase Z^L, which is found only in Eukaryotes. tRNase Z^L arose from a tandem duplication of tRNase Z^s, with an amino and carboxy domain linked together by a ~80 residue tether. The tether was found to be the most flexible region in tRNase Z^L based on limited proteolysis and mass spectroscopy analysis (Wilson et al., PLoS ONE 8(7), 2013). The flexibility of this region may help explain the ~2000-fold greater catalytic efficiency of tRNase Z^L compared with tRNase Z^s. While tRNase Z^s functions as a homodimer of identical subunits, tRNase Z^L, with its two domains linked by a flexible tether, is less restricted in its interactions, which could contribute to the higher catalytic efficiency observed. This hypothesis was explored by ala-scanning through two conserved regions, $N_{dom}T_{prox}$ which resides on the amino side in close proximity to the tether (H_{315} - G_{333}), and the T_{flex} region (M_{376} - R_{384}) which is found directly in the tether. Initial results in $N_{dom}T_{prox}$ show significant impairments in a small region, V328 - L331, with k_{cat}/K_M impairing between a ~130 and 300-fold relative to wild type. In the T_{Flex} region, the most notable results of alanine substitutions occur at N_{378}, which shows a 3-fold higher catalytic efficiency compared with wild type, and R_{382} which shows a ~350-fold decrease in catalytic efficiency. These impairments in catalytic efficiency indicate the important role the tether plays, not only as a linker between the amino and carboxy domain of tRNase Z^L, but also in the enzyme's function in pre-tRNA maturation.

291 Comprehensive mapping of alternative tissue-specific 3′UTR isoforms across Drosophila species

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Cleavage and polyadenylation is an essential step in the maturation of most mRNAs. Recent studies have shown that ~50% of all transcripts in diverse metazoans exhibit cleavage and polyadenylation at two or more sites, leading to the formation of alternate length 3′ untranslated regions (3′UTRs). Recently our lab has shown that alternative cleavage and polyadenylation (APA) in the nervous system of D. melanogaster typically generates a large population of mRNA isoforms bearing 3′UTRs of exceptional length. Conversely, mRNAs often express specific very short isoforms in testes. The functional consequences of this dramatic regulation of 3′UTR length between tissues remain elusive.

To start understanding the role of 3′UTR expression diversity in Drosophila, we have generated high-resolution maps of functional polyadenylation signals (PAS) from RNA extracted from heads, ovaries and testes of D. melanogaster using a combination of RNA-seq and 3′end sequencing. To assess conservation and divergence of 3′UTR expression dynamics, we characterized similar tissue APA maps from two different species of Drosophila that diverged from D. melanogaster 5 mya and 40 mya. These datasets expand our understanding of APA patterns in D. melanogaster and for the first time allow us to map 3′UTRs in two related species and assess the rate of PAS recognition gain or loss throughout evolution.

Our analyses show broad conservation of exceptional 3′UTR lengths in the nervous system. Surprisingly, nucleotide-level conservation of 3′UTRs increases with length, suggesting that these long 3′UTRs might have functional roles in the nervous system. We observe conservation of tissue-specific 3′UTR isoforms expression for a majority of genes in all three species. However, a number of genes exhibit species-specific PAS recognition, resulting in novel 3′UTR isoforms that are expressed predominantly in testes. Additionally, using highly conserved, tissue-specific PAS, we identify motifs that are likely involved in tissue-specific PAS recognition. Finally, we have developed a genome browser to enable simultaneous visualization and comparison of genomic data from different related species that aims to facilitate the identification of evolutionarily conserved and novel aspects of gene expression and regulation.

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292 Integrator subunit 4 functions as a "Symplekin-like" scaffold for the Integrator Cleavage Factor

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RNA Polymerase II (RNAPII) exists with either of two mutually exclusive complexes during the process of transcription: Mediator or Integrator. Much is known about the mechanism and function of Mediator; however, we have very little information about the more recently identified Integrator. The Integrator complex, thus far, has been shown to be critical for the 3' end formation of uridine-rich small nuclear RNA (U snRNA). Recently, however, we and others have demonstrated that Integrator plays a critical role in the release of paused RNAPII in metazoans. This new information was as unexpected as it was provocative leading to the question of how Integrator differentially functions at distinct gene types and whether its endonuclease factor is universally required for its activity.

Here, we have conducted a modified two-hybrid screen looking for Integrator subunits that interact with the heterodimeric Integrator cleavage factor that is composed of subunits 9 and 11 (IntS9/11). We identified that IntS4 specifically interacts with IntS9 and IntS11 when expressed together and fails to interact with either subunit alone. We have conducted systematic dissection of each of the subunits to determine regions required to mediate the formation of the hetertrimer. These analyses have identified that the C-terminal motif of IntS11 is necessary and sufficient for heterodimer formation (9/11) as well as the formation of the heterotrimer. Further, we have identified that N and C terminal regions of IntS4 mediate its ability to bind to IntS9/11. We will present results investigating the functional relevance of these interactions in both U snRNA processing and pause release. These results shed light on the inner mechanics of a poorly understood complex and indicate a similarity in how the pre-mRNA CPSF cleavage complex (Symplekin/CPSF73/100) and the Integrator cleavage complex assemble.

293 Genome-wide screen identifies novel pathways for tRNA nuclear export and important factors for cytoplasmic splicing

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tRNAs are major components of the cell's protein synthesis machinery. In yeast, tRNAs are transcribed in the nucleus. After the removal of 5' and 3' ends and the addition of CCA and some modifications, end-matured tRNAs are exported to the cytoplasm and delivered to the mitochondrial surface where intron splicing occurs. Nuclear-cytoplasmic movement of intron-containing tRNAs involves the initial nuclear export of tRNAs by Los1, retrograde nuclear import of cytoplasmic tRNAs, and re-export of the imported tRNAs back to the cytoplasm by Los1 and Msn5. However, many aspects of tRNA metabolism and subcellular movement remain unknown. For example, there is least one unknown nuclear export pathway for intron-containing tRNAs in yeast in addition to the Los1-dependent pathway. To identify all the missing gene products involved in tRNA biology, we conducted a systematic and unbiased genome-wide screen in budding yeast utilizing a new method that allows large-scale analysis of tRNAs. The complete set of 4848 deletion strains and two collections that together contain 1091 strains with temperature-sensitive mutations of essential genes were analyzed for defects in tRNA biology. Analyses of some of the identified mutants have provided surprising insights. For example, our studies implicate the roles of Ran GTPase-dependent karyopherin Crm1/Xpo1 and the mRNA and ribosomal export machinery in tRNA nuclear-cytoplasmic dynamics. Similar to *los1Δ* cells that have defect in initial tRNA export, cells that have a ts mutation of the karyopherin *CRM1 (crm1-1)* accumulate end-matured intron-containing tRNAs. Cells missing both of the two previously described tRNA nuclear exporters, *i.e.*, *los1D msn5D*, are viable, but the *crm1-1 los1Δ msn5Δ* triple mutant is not, suggesting that cells require at least one of these nuclear export pathways for viability. We identified components of the mRNA and ribosomal export machinery that also accumulate unspliced tRNAs, likely because the tRNAs are unable to access the cytoplasmic tRNA splicing endonuclease. We also learned that proper localization of the tRNA splicing endonuclease complex onto the mitochondrial surface requires outer mitochondrial TOM and SAM proteins. In sum, our genome-wide screen has led to discoveries of novel gene products that function in eukaryotic tRNA processing and subcellular dynamics.

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294 mRNA 3'UTR shortening is a new mTORC1-activated molecular signature defining the specificity in ubiquitin-mediated proteolysis

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Mammalian target of rapamycin (mTOR) enhances translation from a subset of mRNAs containing distinct 5'-untranslated region (UTR) sequence features. Here we found 3'UTR shortening in mRNAs as a new molecular signature of the mTOR-activated transcriptome that promotes translation. Using genetic or chemical modulations of mTOR activity in cells or mouse tissues, we show that the cellular mTOR activity is crucial for 3'UTR shortening. While long 3'UTR-containing transcripts minimally contribute to translation, 3'UTR-shortened transcripts efficiently form polysomes in the mTOR-activated cells, leading to increased protein production. Strikingly, selected E2 and E3 components of ubiquitin ligase complexes are enriched by this mechanism and consequently, the turnover of their substrates is elevated upon mTOR activation. Together, these findings identify a new role of mTOR in the regulation of ubiquitin-mediated proteolysis pathway and suggest a cellular mechanism of how mTOR determines selective protein degradation by modulating 3'UTR length in mRNAs.

295 Structure and function analysis of Drosha

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Drosha, along with its partner DGCR8 in mammals, cleaves primary microRNA transcripts to produce precursor microRNA transcripts in the canonical microRNA processing pathway. While DGCR8 has been reasonably well characterized, the structure and function relationship of Drosha has not been reported. Our current study aims to understand the domain structures and functions of the human Drosha protein.

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296 Complementary sequence-mediated exon circularization

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Exon circularization has been identified from many loci in mammals, but the detailed mechanism of its biogenesis has remained elusive. By using genome-wide approaches and circular RNA recapitulation, we demonstrate that exon circularization is dependent on flanking intronic complementary sequences. Such sequences and their distribution exhibit rapid evolutionary changes, showing that exon circularization is evolutionarily dynamic. Strikingly, exon circularization efficiency can be regulated by competition between RNA pairing across flanking introns or within individual introns. Importantly, alternative formation of inverted repeated Alu pairs and the competition between them can lead to alternative circularization, resulting in multiple circular RNA transcripts produced from a single gene. Collectively, exon circularization mediated by complementary sequences in human introns and the potential to generate alternative circularization products extend the complexity of mammalian posttranscriptional regulation.

297 Effects of Local RNA Environments on Ribonuclease P Processing Specificity

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The affinity and kinetics of RNA and protein association depend on the stability of the RNA-protein complex as well as the stability the free RNA and protein. Ribonuclease P (RNase P) is a ribonucleoprotein enzyme involved in tRNA processing that relies on protein subunit (C5 protein) forming essential contacts to a linear sequence of 3-8 nucleotides in the 5' leader of ptRNA. Therefore binding is likely to be influenced by the presence of competing secondary structure in the free RNA. To address this issue, we compared the kinetics of cleavage in monocistronic and polycistronic ptRNA. For the polycistronic valV/W operon we observed directional cleavage in a 3' to 5' manner. Kinetics analysis on valV and valW individually suggests that structure and sequences in the leader sequences might affects RNase P processing. To understand further, we design different structure and leader sequence of valV and perform single turnover kinetics to test our hypothesis. In a parallel experiment we designed three different ptRNA substrates in which different 21 nucleotides extensions are appended upstream of the RNase P leader binding site to test the effect of secondary structure on processing. The sequence of 21A was designed to have transversion mutations at all 21 nucleotides compared to the 21B. The 21C was designed to form a stable stem loop and should insulate it from inhibitory interactions with additional leader sequences. Using new high-throughput methods we determined the rate constants for all sequence variants at either N(-1) to N(-6) and N(-3) to N(-8) in the 21A, B and C backgrounds. Comparison of initial HTS-KIN data suggests formation of inhibitory secondary structure influences the association kinetics of subset of substrates. Current studies focus on completing the comparative HTS-KIN analyses and developing methods for quantitatively analyzed these data for contributions from complex stability versus formation of competing structure in the free RNA ligand.

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298 Fluorescent Broccoli RNA AptamerExhibits G-quadruplex Character

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Fluorophore-binding RNA aptamers can serve as RNA mimics of fluorescent proteins. These aptamers are sequences selected for their ability to cause fluorescence when bound to DFHBI, a GFP fluorophore analog, and related small molecules. Structures solved for the "Spinach" aptamer have recently revealed the presence of a non-canonical G-quadruplex core. Here we find that "Broccoli," an RNA aptamer selected for activation of DFHBI fluorescence using cell-based selection, exhibits signature G-quadruplex properties. These include a strong dependence on potassium (K^+) or rubidium (Rb^+), but not cesium or lithium ions, for fluorescence and signature G-quadruplex chemical shifts in the imino proton region of NMR spectra. Thermal denaturation studies indicated minimal effects on global RNA structure upon DFHBI and K^+/Rb^+ binding. Mutagenesis revealed key domains required for fluorescence. Additionally, fluorescent RNA aptamers reported to have significantly lower efficiencies for DFHBI activation did not exhibit quadruplex character. These results suggest that G-quadruplex structures may be a recurring structural motif among successful DFHBI-activating fluorescent RNA aptamers. Incorporation of sequences and conditions that promote quadruplex formation may be a strategy for improved evolution and selection of fluorescence-activating aptamers.

299 Topological structure determination of RNAs using small angle X-ray scattering

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The manifold roles of non-coding RNAs in regulation of gene expression and human biology as a whole, have led to an increasing interest in understanding the structural and molecular basis of their diverse functions. However, our knowledge of three-dimensional structure of RNA is very limited compared to protein counterpart, primarily due to the technical limitations of the current methods. We have developed a powerful computational technique called R2DSAXS3D for the de novo topological structural determination of RNAs with a variety of architectural forms, by using SAXS data and RNA secondary structure information. Search for the structure is carried out at two levels of coarse graining by representing each nucleotide as a single glob at the first stage and then splitting into subglobs at the second stage. Beginning from an arbitrary initial 3D structure that represents the complete secondary structure in an open conformation, the system traverses through carefully selected natural hierarchical moves restrained by the experimental SAXS data. Our method has been validated to reproduce ~5 Å resolution topological structures of several RNAs that represent several major types of folds and complexities in the structure database. Conceptual simplicity that incorporates the secondary structure motifs, choice of independent set of moves while avoiding the conventional fragment replacement bias, and flexibility to capture a variety of tertiary interactions are some key novel approaches adopted in our algorithm. In addition, our method can also assimilate any topological structural information obtained from other methods to further enhance the resolution of the calculated structures.

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300 Insight into the biological functions of retroelement's RNA through the predictions and analyses of their RNA 3D structures.

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RNA is a key molecule acting in cellular processes as a carrier of genetic information or catalyst. Those two RNA functions are strictly related to its structure. Insights into RNA's three-dimensional structures are limited by RNA flexibility and size. Therefore new methods for RNA structure determination and/or prediction are required to avoid those difficulties. Methods for computer modeling of the RNA 3D structures can be improved by experimental data. Recent method called RNAComposer [1], available with user-friendly servers (http://rnacomposer.ibch.poznan.pl and mirror http://rnacomposer.cs.put.poznan.pl) allows for fully automated prediction of RNA 3D structures based on sequence, secondary structure and tertiary contacts if provided.

RNAComposer allows predictions of 3D structures of RNA up to 500 nts. Accuracy of the RNAComposer predictions was tested using known 3D structures of riboswitches [2], RNA molecules with extensive complexity. Here we present the workflow of RNAComposer based procedure illustrated using lysine riboswitch. Several important points resulted from those analyses. To obtain correct 3D structure of the RNA of interest, the RNA secondary structure model should be reinforced through experimental data. Moreover, information about experimentally supported tertiary contacts increases the accuracy of predicted models.

Retroelements like Ty1 or HIV-2 have an RNA genome. The 5'UTR play multiple regulatory roles in their replication cycles. 2D structures of those regions were obtained using SHAPE methodology and mutational analysis and 3D structures of those previously undetermined large RNA (388 and 560 nts) were predicted. Insight into 3D structure of Ty1 and HIV-2 RNA helps to answer important biological questions. In combination with the hydroxyl radical mapping of the protein binding sites provide a platform to model structures of RNA/protein complexes.

This work was supported by the National Science Center Poland [2012/06/A/ST6/00384], [2011/01/D/NZ1/03478] and Ministry of Science and Higher Education [0492/IP1/2013/72].

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301 From crystal structures to trajectories: hopping on the PDB to model conformational transitions in RNA

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Structure and function of RNA molecules are often dictated by a diverse repertoire of base-base interactions such as Watson-Crick base pairing, base stacking and non-canonical interactions. We recently introduced a minimalistic description of RNA three-dimensional structure consisting in one oriented bead per nucleotide that effectively describes these fundamental interactions [1]. This molecular representation makes it possible to define a metric for measuring distances between RNA conformations. The accuracy of the metric is assessed with respect to its ability to find motifs within the available databases of known RNA 3D structures. Interestingly, this metric can be successfully employed to construct dynamical trajectories utilizing static snippets from crystallographic structures. The approach is very general and is used for characterizing important conformational transitions in RNA such as duplex and hairpin formation, providing pathways that can be readily compared with atomistic molecular dynamics simulations.

[1] Bottaro S, Di Palma F,and Giovanni Bussi. Nucleic Acids Res 2014; 42:13306-13314

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302 ChemModSeq in action: close-up on ribosomal RNA structural rearrangements during eukaryotic 40S subunit assembly

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Ribosome maturation is a highly complex and dynamic process that involves the tight coordination of (1) ribosomal RNA processing and folding events, (2) sequential binding of ribosomal proteins and (3) the input of approximately 200 trans acting ribosome assembly factors. Despite having a fairly complete picture of what assembly factors interact with the ribosomal intermediates and when, we still lack detailed knowledge of the RNA folding steps required for ribosome synthesis and the role the ribosomal assembly factors play in these RNA restructuring events. To address this we have developed ChemModSeq a method that combines SHAPE chemical probing, high-throughput sequencing and statistical modeling. ChemModSeq was applied to the yeast 40S assembly intermediates and generated the first nucleotide resolution map of ribosomal RNA flexibility, revealing structurally distinct pre-40S intermediates and mechanistic insights into assembly dynamics (Hector et al., 2014). We demonstrate that ChemModSeq is a robust and reliable method for the analysis of RNA secondary structure both *in vivo* and *in vitro.* The ChemModSeq protocol and the statistical methods are applicable to any ribonucleoprotein complex.

Hector, R. D., Burlacu, E. et al. (2014). Snapshots of pre-rRNA structural flexibility reveal eukaryotic 40S assembly dynamics at nucleotide resolution. *Nucleic Acids Research* 42: 12138–12154

303 Conformational changes in the adenine riboswitch using molecular dynamics and enhanced sampling techniques: understanding the role of ligand and Mg^{2+}

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Riboswitches are known to regulate the genetic expression by means of conformational changes triggered by highly specific interactions of the aptamer with the sensed metabolite. The non-coding sequence in the mRNA of *add* gene from *V. vulnificus* contains an adenine responsive riboswitch. Classical molecular dynamics (MD) simulations of its aptamer have been performed starting from the experimental crystal structure, both in the presence and the absence of its physiological ligand and of monovalent (K^+) or divalent (Mg^{2+}) cations. We first use steered MD to induce the opening of the P1 stem and investigate its stability. Our results show that the ligand directly stabilizes the P1 stem by means of stacking interactions quantitatively consistent with thermodynamic data [1]. Then, using a combination of umbrella sampling, metadynamics, and replica exchange, we show that the formation of the L2-L3 kissing complex cooperates with ligand binding and we are able to quantify the ligand-induced stabilization and the effects of the divalent cations [2,3]. Conformational changes are characterized using a recently introduced technique [4]. Results are in agreement with experiments and shed a new light on the ligand-dependent folding mechanism of the adenine riboswitch.

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[3] Di Palma, Bottaro, and Bussi 2015 (in preparation)
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304 Three-dimensional conformation of the human U2-U6 snRNA complex analyzed by fluorescence resonance energy transfer.

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U2 and U6 small nuclear (sn)RNA molecules are the only snRNA components of the spliceosome directly implicated in pre-mRNA splicing catalysis . Different stereochemistry observed for the two steps[1]suggests conformational change between them. We have identified two folds for the protein-free human U2-U6 snRNA complex in vitro, a predominant four-helix fold and a minority three-helix conformation[2]that undergo dynamic exchange[3]. To investigate the global conformation of the human U2-U6 snRNA complex in the lowest energy state we are using time resolved fluorescence resonance energy transfer (trFRET) to measure distances between fluorophores at termini of the U6 snRNA intramolecular stem loop (ISL) and termini of neighboring helices. In the absence of Mg^{2+}, the mean distance between ISL and Helix III is 79.6±6.7 Å, and between ISL and Helix II is 73±2 Å; using these values and the lengths of the helices, we estimate corresponding angles of 131.5±23° and 122.2±6°, respectively. The distance between the 5' and 3' termini of the U2 snRNA fragments is ~90 Å, corresponding to an angle of ~107° between Helix III and Helix II. Taken together, these data suggest the protein-free complex has roughly tetrahedral geometry; the narrow distribution of data suggests similar overall geometries for the four- and three-helix folds. Addition of Mg^{2+} results in significant shortening of the distances between ISL and the neighboring helices, suggesting compaction of structure around the junction, in agreement with measurements by analytical ultracentrifugation[1], but contrary to conclusions from FRET studies in yeast[4]. Similar analysis of a mutant complex favoring the four-helix fold resulted in a lesser change upon addition of Mg^{2+}, suggesting that Mg^{2+}-dependent compaction of the wild-type U2-U6 RNA complex involves contributions from both conformational change within the junction and junction-independent change in the relative orientation of the stems. These findings may help explain how conformational change facilitates splicing activity.

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305 Structural imprints *in vivo* decode RNA regulatory mechanisms

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Visualizing the physical basis for molecular behavior inside living cells is a grand challenge in biology. RNAs are central to biological regulation, and RNA's ability to adopt specific structures intimately controls every step of the gene expression program1. However, our understanding of physiological RNA structures is limited; current in vivo RNA structure profiles view only two of four nucleotides that make up RNA2,3. Here we present a novel biochemical approach, In Vivo Click SHAPE (icSHAPE), that enables the first global view of RNA secondary structures of all four bases in living cells. icSHAPE of mouse embryonic stem cell transcriptome versus purified RNA folded in vitro shows that the structural dynamics of RNA in the cellular environment distinguishes different classes of RNAs and regulatory elements. Structural signatures at translational start sites and ribosome pause sites are conserved from in vitro, suggesting that these RNA elements are programmed by sequence. In contrast, focal structural rearrangements in vivo reveal precise interfaces of RNA with RNA binding proteins or RNA modification sites that are consistent with atomic-resolution structural data. Such dynamic structural footprints enable accurate prediction of RNA-protein interactions and N6-methyladenosine (m6A) modification genome-wide. New icSHAPE data will be presented exploring RBP-RNA and RNA-RNA interactions transcriptome-wide.

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306 Dissecting nucleic acids electrostatics

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The ionic atmosphere surrounding nucleic acids remains partially understood at atomic level-detail. However, recent advances in experimental and theoretical approaches create the prospects for the first steps towards dissecting nucleic acids solvation. I will present an assessment of current molecular mechanics models used in conjunction with molecular dynamics simulations (MD) and 3D-RISM by carrying out careful comparisons with "ion counting" and NMR relaxation experiments. I will show that while both MD and 3D-RISM can predict experimentally observed trends, there are ionic strength and temperature ranges for which values of preferential interaction parameters and overall tumbling times deviate from experimentally derived estimates. Finally, I will illustrate distinctive features of nucleic acids ionic atmosphere such as territorial and site binding using novel ways to map ion and solvent densities.

307 Mapping the ion atmosphere around nucleic acids

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The ion atmosphere surrounding the nucleic acids affects their folding, condensation and binding to other molecules. It is thus of fundamental importance to gain predictive insight into the formation of the ion atmosphere and thermodynamic consequences when varying ionic conditions. One step towards this goal is gaining predictive theoretical insight into the composition and spatial extent of the ion atmosphere. For this we employ state of the art methods such as molecular dynamics and 3D-RISM that show promising predictive power when compared against experimental "ion counting" measurements. Using three-dimensional distributions of ions and solvent around typical rigid nucleic acid conformations we monitor the extent of the ion atmosphere when varying salt concentration and composition or nucleic acid size. Our results should bring new insights into understanding the accumulation of ions around finite size nucleic acids, aiming to provide a molecular level interpretation of how Coulombic end-effects influence ion binding.

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308 Development of a viroid structural compendium

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Viroids are single stranded, circular RNA with the size range of 246-401 nucleotides. They are the smallest known phytopathogens and are causing a wide array of symptoms. One of the most interesting characteristics of viroids is their lack of coding capacity for any pathogens specific proteins. This has for consequence that they depend on their sequence and their structure to interact with their host, replicate and propagate. In general, the secondary structures of viroids have been predicted solely based on their sequence using thermodynamics-based RNA folding programs, which have been shown to possess several important limitations. The predicted structure of a viroid needs to receive physical support prior to its use in the accurate interpretation of any mechanistic studies. Recently, we have adapted SHAPE probing coupled with fluorescence sequencing technique and computer-assisted structure prediction for the probing of viroid. By means of this technique, we have elucidated the structure of one variant for each viroid species of the *Avsunviroidae* family and most viroid of the *Pospiviroidae* family. By comparing the structure of viroids genomes predicted only by bioinformatics with the viroids' structures predicted with the help of SHAPE data many differences were seen. This is a good indication of the importance of integrating in solution data to the prediction of the RNA structure. With this compendium of viroid structure, we are providing a better way to look at the viroid genome structure in solution.

309 Probing RNA structure and dynamics using NMR solvent paramagnetic relaxation enhancements

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RNA plays essential roles in virtually all aspects of gene regulation. RNA *cis* elements typically provide binding sites for RNA binding proteins to regulate the maturation and processing of mRNAs, or long non-coding RNAs (lncRNAs) adopt higher order architectural folds. To understand the underlying molecular mechanisms of these RNAs information about their tertiary structure is required. Recent developments with structure probing using chemical modification (i.e SHAPE) or enzymatic methods provide insight into accessibility of bases and functional groups at low resolution. However, these methods are affected by flexibility and dynamic properties of the RNA which can lead to incorrect interpretation of the data.

NMR spectroscopy provides a wide range of tools and parameters to characterize the structure and dynamics of RNA molecules in solution and can ultimately be used to determine high-resolution structures of RNA. NMR methods can probe the solution conformation of an RNA and thereby complement crystallographic studies and validate structure-probing methods.

Here we demonstrate that NMR solvent paramagnetic relaxation enhancements (sPRE) are an efficient novel method to investigate the solvent accessible surface of RNA molecules. The surface of a biological macromolecule is probed by the chemically inert paramagnetic Gd^{3+} complex Gd(DTPA-BMA) (Gadodiamide, Omniscan) which induces relaxation enhancements for NMR signals in a distance-dependent manner.

We demonstrate this approach for different RNA molecules, including a 14-mer well characterized RNA UUCG stem-loop. The experimental sPRE data are in excellent agreement with the solvent accessible surface area and sPRE effects back-calculated from an available structure of the RNA.

The acquisition of NMR sPRE data is straightforward as the inert Gd^{3+} complex is simply added to the RNA solution. The solvent accessibility determined form sPRE data can be efficiently used as restraints in structure calculation and computational modelling of RNA structures. The method is perfectly suited to simplify and accelerate structure determination of RNAs, in particular when only sparse data is available.

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310 rRNA GTPase center: Kinetics of tertiary structure formation

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The 60 nucleotide prokaryotic rRNA GTPase center adopts an intricate tertiary structure that requires Mg2+ ions. Its ion requirements have been extensively studied by the Draper lab, and here we extend those studies to observe the kinetics of tertiary structure formation using stopped-flow fluorescence. Using RNAs that contain a single 2-aminopurine (2AP) nucleobase, we monitor its fluorescence changes upon addition of Mg2+. Different sites of 2AP substitution reveal how the elements of the RNA (hairpin loop, internal loop, and 3-way junction) respond to addition of Mg2+ and suggest how the elements are coordinated during the global folding process. Timescales of folding events range from < 1 ms to > 5 seconds. We hope to model the pathway of folding.

311 Generalized Manning Condensation Model Captures the RNA Ion Atmosphere

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RNA is highly sensitive to the ionic environment, and typically requires Mg^{2+} to form compact structures. There is a need for models capable of describing the ion atmosphere surrounding RNA with quantitative accuracy. We present a model of RNA electrostatics and apply it within coarse-grained molecular dynamics simulation. The model treats Mg^{2+} ions explicitly to account for ion-ion correlations neglected by mean field theories. Since mean-field theories capture KCl well, it is treated implicitly by a generalized Manning counterion condensation model. The model extends Manning condensation to deal with arbitrary RNA conformations, non-limiting KCl concentrations, and the ion inaccessible volume of RNA. The model is tested against experimental measurements of the excess Mg^{2+} associated with the RNA, Γ_{2+}, because Γ_{2+} is directly related to the Mg^{2+}-RNA interaction free energy. The excellent agreement with experiment demonstrates the model captures the ionic dependence of the RNA free energy landscape.

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312 Thermodynamic contribution of nebularine-uridine base pairs in RNA duplexes

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Nonstandard nucleotides are ubiquitous in RNA. Thermodynamic studies with RNA duplexes containing nonstandard nucleotides whether incorporated naturally or chemically, can provide insight into the stability of Watson-Crick pairs and the role of specific functional groups in stabilizing a Watson-Crick pair. For example, an A-U, inosine-ŸU, and AŸ-pseudouridine pair each form two hydrogen bonds. However, an RNA duplex containing a central I-ŸU pair or central A-ŸpseudoU pair is 2.4 kcal/mol less stable or 1.7 kcal/mol more stable, respectively, than the corresponding duplex containing and A-U pair. In the nonstandard nucleotide nebularine (Neb), or purine, a hydrogen replaces the exocyclic amino group of A. This replacement results in a Neb-ŸU pair containing only one hydrogen bond. Optical melting studies were performed with RNA duplexes containing Neb-ŸU pairs adjacent to different nearest neighbors. The resulting thermodynamic parameters were compared to RNA duplexes containing A-U pairs in order to determine the contribution of the hydrogen bond involving the exocyclic amino group. Preliminary results indicate a loss of 1.8 kcal/mol when Neb-ŸU replaces A-U in an RNA duplex. Nearest neighbor parameters will be derived for use in free energy and secondary structure prediction software.

313 Crystal structure of a c-di-AMP riboswitch reveals an internally pseudo-dimeric RNA

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Cyclic diadenosine monophosphate (c-di-AMP) is an essential bacterial second messenger that regulates the expression of bacterial genes governing cell wall homeostasis, metabolism, and sporulation. In addition to interacting with receptor proteins, c-di-AMP modulates gene expression by directly binding to c-di-AMP riboswitches found in the 5′ untranslated regions of mRNAs. Riboswitches are structured RNA motifs that bind to small molecules or metabolites and regulate gene expression. Riboswitches consist of an "aptamer" domain that specifically recognizes the ligand and an "expression platform" that transmutes ligand binding into a genetic output. Using X-ray crystallography to determine the structure of the Bacillus subtilis c-di-AMP riboswitch aptamer domain, we have recently shown that the riboswitch binds two copies of c-di-AMP in a striking pseudosymmetrical fold (Jones CP and Ferré-D'Amaré AR, EMBO J (2014)). Each half of the riboswitch consists of a three-helix junction that folds into a critical tetraloop-like motif, which directly interacts with the two bound c-di-AMP molecules. The structure also reveals that specificity to c-di-AMP over cyclic diguanosine monophosphate (c-di-GMP) is achieved by steric exclusion of c-di-GMP and specific interactions with the adenine nucleobases of c-di-AMP. Using c-di-AMP analogs and point mutations of residues surrounding the ligand binding sites, we have uncovered the contribution of each interaction between c-di-AMP and the riboswitch via isothermal titration calorimetry. To better understand how ligand binding affects gene expression, we examined the c-di-AMP riboswitch in the presence and absence of ligand using small-angle X-ray scattering, which reports on the global shape of a molecule in solution. These experiments suggest that the c-di-AMP-bound riboswitch is more compact than the free RNA, consistent with a large conformational change upon ligand binding. This structural change likely couples c-di-AMP binding to regulation of gene expression. As pseudosymmetry of the c-di-AMP riboswitch is rare among RNA structures, we also compare the structure of the c-di-AMP riboswitch to those of the pseudosymmetrical flavin mononucleotide (FMN) and glycine riboswitches.

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314 Identification of Novel Yeast Introns and Common Secondary Structure Features in *in vivo* Assembled Spliceosomal Complexes

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The spliceosome is the compositionally and structurally dynamic RNA-protein machine responsible for faithfully removing introns from pre-messenger RNAs (pre-mRNAs) to yield the continuous protein-coding segment of an mRNA. As a finely tuned process of great complexity and critical importance to the diversification of the proteome, it is thought that up to 50% of all mutations connected to human disease act through disruption of the splicing code. The structure and conformation of the RNA components of the spliceosome are central to its function. Proper assembly and catalytic activation require an elaborate sequence of RNA:RNA and RNA:protein rearrangements as well as specific pre-mRNA substrate sequences that serve as a scaffold to which splicing factors and regulators bind to ensure splicing fidelity. Single-molecule tools have begun to uncover the complex series of pre-mRNA conformational rearrangements required for efficient spliceosome assembly and catalysis. Interestingly, our prior work revealed that the efficiently spliced Ubc4 intron exhibits significant secondary structure in the absence of all spliceosomal components, which places the flanking exons, as well as the 5'SS and BP regions, much closer than expected from their linear sequence distance. This observation indicates that the secondary structure of well-splicing introns may have evolved to favor juxtaposition of the splice sites in support of spliceosomal catalysis, an idea that was first proposed in yeast more than 20 years ago and that supports a model in which the intron plays a more active role in positioning the 5'SS and BP close to one another, similar to the function of self-splicing group I and II introns.

To understand the underlying structure-dynamics-function relationships and test the hypothesis that specific intron secondary structures dominate in spliceosomal cycle intermediates, we have applied selective 2'-hydroxyl acylation analyzed by primer extension and mutational profiling (SHAPE-MaP) to the RNA contained in the immediately pre-catalytic spliceosomal B^{act} complex isolated directly from yeast. This approach allows for a detailed analysis of the secondary structures of the approximately 270 spliceosomal substrates found in B^{act}, providing a high-throughput approach for drawing conclusions about universal intron structure-function relationships in yeast spliceosomal complexes. The most recent results will be presented.

315 Influenza segment 5 (+)RNA - how the RNA folds

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Influenza A virus causes seasonal epidemics and occasional, but deadly, pandemics. Influenza virus antigenic drift and antigenic shift result in new strains that are particularly dangerous to humans. Therefore, it is important to make an effort to create new therapeutic strategies for combating influenza. RNA is essential in the virus life cycle and is thus an attractive target for new therapeutics. Knowledge of influenza RNA structure is limited, but this information is crucial in designing RNA-targeting anti-influenza drugs.

Here, we present a model of the secondary structure of the influenza virus type A segment 5 (+)RNA. Segment 5 encodes nucleoprotein (NP), which is a structural protein and also takes part in the regulation of transcription and replication. NP with viral polymerase and genomic (-)RNAs form eight ribonucleoprotein complexes that are packaged into virions. Segment 5 (+)RNA structure was biochemically mapped in vitro. Based on the in vitro mapping data we showed that segment 5 (+)RNA has extensive and thermodynamically-stable secondary structure. Folding of (+)RNA may be important in the regulation of several processes: e.g. protein expression. The identified conserved secondary structure motifs provide ideal leads for targeting RNA sequences/structures.

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316 Structure and sequence elements of the CR4/5 domain of medaka telomerase RNA important for telomerase function

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Telomerase is a unique reverse transcriptase that maintains the 3' ends of eukaryotic chromosomes by adding tandem telomeric repeats. The RNA subunit (TR) of vertebrate telomerase provides a template for reverse transcription, contained within the conserved template/pseudoknot domain, and a conserved regions 4 and 5 (CR4/5) domain, all essential for catalytic activity. We report the nuclear magnetic resonance (NMR) solution structure of the full-length CR4/5 domain from the teleost fish medaka (Oryzias latipes). Three helices emanate from a structured internal loop, forming a Y-shaped structure, where helix P6 stacks on P5 and helix P6.1 points away from P6. The relative orientations of the three helices are Mg^{2+} dependent and dynamic. Although the three-way junction is structured and has unexpected base pairs, telomerase activity assays with nucleotide substitutions and deletions in CR4/5 indicate that none of these are essential for activity. The results suggest that the junction is likely to change conformation in complex with telomerase reverse transcriptase and that it provides a flexible scaffold that allows P6 and P6.1 to correctly fold and interact with telomerase reverse transcriptase. Our solution NMR structure of the CR4/5 domain will be compared with the recent x-ray crystal structure, and the role of CR4/5 will be discussed.

317 Study of Stability and Folding of GNRA and UNCG Tetraloops Using Microseconds Molecular Dynamics and Well Tempered Metadynamics

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UNCG and GNRA tetraloops represent an important class of RNA structural motifs. Despite their small size, a wealth of experimental data and recent progress in theoretical simulations of their structural dynamics and folding, our understanding of the folding and unfolding processes of these small RNA elements is still limited. Theoretical description of the folding and unfolding processes requires broad and robust sampling, which can be achieved by using either an exhaustive timescale in classical molecular dynamics simulations or sophisticated enhanced sampling methods, such as well-tempered metadynamics, to accelerate sampling in a few chosen collective variables. Here, we present structural dynamics of both UNCG and GNRA tetraloops revealed by 15-ms-long simulations and a series of well-tempered metadynamics. Although we showed that selection of the collective variable for description of the full folding and unfolding processes was not straightforward and formation of the loop appeared to be a hidden variable in such an approach, both classical and biased simulations provided new insights into the conformational behavior of the studied tetraloops. We observed that the unfolding mechanism of the GNRA tetraloop agreed with the reverse folding mechanism suggested by recent REMD simulations.[1] Further, the orientation of the glycosidic bond of the G_{L4} nucleobase was critical for UUCG tetraloop folding. The simulation data strongly supported the hypothesis that G_{L4}-*anti* forms a kinetic trap along the tetraloop folding pathway. Finally, we identified limitations of the methods based on sampling along a few collective variables for description of complex folding and unfolding processes.

1. Kuhrova, P.; Banas, P.; Best, R. B.; Sponer, J.; Otyepka, M. *J. Chem. Theory Comput.* **2013**, *9*, 2115-25.

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318 On-Enzyme Refolding Permits Small RNA and tRNA Surveillance by the CCA-Adding Enzyme

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Transcription in eukaryotes produces a number of long noncoding RNAs (lncRNAs). Two of these, MALAT1 and Menβ, generate a tRNA-like small RNA in addition to the mature lncRNA. The stability of these tRNA-like small RNAs and bona fide tRNAs is monitored by the CCA-adding enzyme. Whereas CCA is added to stable tRNAs and tRNA-like transcripts, a second CCA repeat is added to certain unstable transcripts to initiate their degradation. Here, we characterize how these two scenarios are distinguished. Following the first CCA addition cycle, nucleotide binding to the active site triggers a clockwise screw motion, producing torque on the RNA. This ejects stable RNAs, whereas unstable RNAs are refolded while bound to the enzyme and subjected to a second CCA catalytic cycle. Intriguingly, with the CCA-adding enzyme acting as a molecular vise, the RNAs proofread themselves through differential responses to its interrogation between stable and unstable substrates.

Reference:

Claus-D. Kuhn, Jeremy E. Wilusz, Yuxuan Zheng, Peter A. Beal, and Leemor Joshua-Tor (2015) On-Enzyme Refolding Permits Small RNA and tRNA Surveillance by the CCA-Adding Enzyme. Cell 160, 1-15.

319 The Biological Impacts of RiboSNitches: Linking RNA Structural Changes with Molecular Phenotypes

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Single nucleotide variants that affect RNA structure (riboSNitches) were recently discovered through next-generation sequencing structure determination in humans. What phenotypic changes do these riboSNitches cause within a cell? There is evidence that RNA structure is important for both function and regulation. Thus, we hypothesize that riboSNitches can affect RNA stability, translational efficiency and protein binding, and that the majority of riboSNitches will have a functional impact on their transcript. We are currently testing our hypothesis with a multi-pronged approach using genotyped human lymphoblastoid cells. First, we are identifying riboSNitches with allele-specific selective 2′-hydroxyl acylation analyzed by primer extension and mutational profiling (SHAPE-map) structure determination. Then we are testing RNA stability with a sequencing method based on the classical pulse-chase assessment with bromouridine - 5'-bromo-uridine immunoprecipitation chase-deep sequencing analysis or BRIC-Seq. In addition, we are determining translational efficiency with polysomal fractionation. Finally, we are looking at global changes between RNA folding in lysates versus the test tube with the goal of identifying and testing specific protein binding sites in vitro. Done in tandem, in an allele-specific manner, these studies will allow us to determine whether riboSNitches alter the stability, translational efficiency or protein-binding characteristics of their transcripts. Connecting riboSNitches to biological mechanisms is an essential step toward understanding how genotype leads to phenotype and personal variation leads to disease risk.

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320 Context-sensitivity of Isosteric Substitutions of non-Watson-Crick Basepairs in Recurrent RNA 3D Motifs

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Sequence variation in a widespread, recurrent RNA structural motif, the Sarcin/Ricin (S/R) motif, was studied to address two related questions: First, what are the effects on the stabilities of conserved RNA 3D motifs of isosteric and non-isosteric substitutions in non-Watson-Crick (non-WC) basepairs? Second, is there selection for particular base combinations in non-WC base pairs, depending on the temperature regime to which an organism adapts? A survey of large and small subunit rRNAs from organisms adapted to different temperatures revealed the presence of systematic sequence variations at many non-WC paired sites of S/R motifs. For example, a preference for AG trans Hoogsteen-Sugar (tHS) basepairs is observed in organisms adapted to high temperatures and motifs with this base combination at tHS pairing positions tend to be more stable, as measured by UV melting of oligonucleotides containing the motif. More stable motifs also tend to be more rigid, as reflected in resistance to enzymatic digestion. The thermodynamic measurements show that base substitutions at non-Watson-Crick pairing sites can significantly affect the thermodynamic stabilities of S/R motifs, even for isosteric substitutions that minimally distort the 3D structure of the motif. Moreover, these effects are highly context specific, as the same base substitutions can have very different effects on stability, indicating the importance of base-stacking and base-phosphate interactions on motif stability. This study highlights the importance of non-canonical base pairs and their contributions to modulating the stability and flexibility of RNA molecules.

321 Comparative Structural and Sequence Analysis of RNA 3D Motifs from ribosomal RNA: Hairpin loops, internal loops, and extended helical elements

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The RNA 3D Motif Atlas is an automated pipeline for extracting hairpin and internal loops from representative RNA 3D structures in the NDB/PDB and clustering them by structural similarity. An assessment of the reliability of the automated pipeline to asses the structural conservation of homologous motifs from SSU and LSU rRNA structures will be presented. To facilitate comparative structural analysis of large RNA 3D structures such as the rRNAs, software tools have been developed to consistently color helical elements in 2D and 3D structures using a palette of colors that reproduce well on screen or paper. These tools color SSU and LSU rRNAs from diverse organisms represented in NDB/PDB in a consistent manner using nucleotide mappings to helical elements to facilitate structural comparisons and will be made available for Pymol and Chimera and in 2D formats.

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322 High-throughput probing of human lncRNA secondary structures

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Long noncoding RNAs (lncRNAs) comprise a large proportion of the human transcriptome. Over the past decade, lncRNAs have been increasingly recognized as important regulators of multiple gene expression processes, and a growing number have been associated with human development and diseases. Unlike protein coding mRNAs, lncRNA structures and their interactions with proteins are believed to be crucial for their regulatory functions. To experimentally determine the secondary structures of lncRNAs, we applied a recently developed method, Mod-seq[1]. In Mod-seq, RNAs are chemically probed with SHAPE reagents, and modification sites are identified using high throughput sequencing. Mod-seq provides substantial improvements in throughput than traditional RNA secondary structure determination methods, allowing rapid probing of lncRNAs that are thousands nucleotides long. We applied Mod-seq to two *in vitro* transcribed lncRNAs, sno-lncRNA and NEAT1. sno-lncRNA is deleted in an important human disease, Prader-Willi Syndrome (PWS). Sno-lncRNAs are produced from introns with two imbedded snoRNA genes, which leads to transcription of lncRNAs flanked by snoRNA sequences at both 5' and 3' ends. Our Mod-seq probing showed that the 5' and 3' snoRNA sequences in sno-lncRNA have similar secondary structural patterns, and that 4 Rbfox protein binding sites share a structure motif that resembles internal loop. NEAT1, another nuclear lncRNA, serves as the structural scaffold for nuclear granules called paraspeckles. We are applying Mod-seq probing of human and mouse NEAT1 to reveal stable secondary structures and conserved secondary structural motifs across species.

1. Talkish, J., May, G., Lin, Y., Woolford, J. L. & McManus, C. J. Mod-seq: high-throughput sequencing for chemical probing of RNA structure. *RNA* 20, 713-20 (2014).

323 Towards computing the effective concentrations that define RNA tertiary structure

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Many functions of RNA molecules are encoded in their tertiary structures. Here we describe and present initial tests for a novel method to predict RNA tertiary contacts based on topological constraints inherent in the secondary structure. First, we are generating a large number of low resolution models with Rosetta Fragment Assembly of RNA and the RNAmake modeling/design software for a given sequence and secondary structure to investigate possible contacting helices. Next, we count all base pairs between two helices that are in correct distance and orientation. Based on these data, we can rigorously compute effective molarity between all doublets of base pairs, highlighting motifs that are poised to form tertiary interactions. We will present results on (1) a well-characterized tectoRNA construct [1] that consists of two RNA molecules held together by two tetraloop-tetraloop receptor interactions (2) a glycine riboswitch and (3) the P4-P6 domain of the Tetrahymena group I intron. Our next step will be blind predictions of tertiary folds of RNA with potential A-minor interactions. We hope that the improvement in RNA 3D modeling process itself might lead to better understanding of folding of RNA molecules and how they function in nature.

[1] Jaeger, L., Westhof E., Leontis NB., TectoRNA: modular assembly units for the construction of RNA nano-objects, Nucleic Acids Research, 2001, Vol. 29, No. 2, (2001)

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324 Direct Observation of Guanine Riboswitch Folding using Optical Tweezers

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Guanine riboswitches are present in the 5'- untranslated region of mRNAs involved in purine metabolism. While the basis of ligand-binding and molecular discrimination has been studied to atomic details, the associated structural rearrangements and their kinetics are poorly understood. We recently built a high resolution optical-tweezers that can measure fluctuations with high accuracy and precision at a temporal resolution of sub-milliseconds. Using the custom-built instrument, we followed the structural rearrangements in single-molecules of guanine riboswitch that take place immediately upon ligand binding. Equilibrium sampling methods revealed a short-lived intermediate state occurring at < 1 s. Further characterization indicated that the intermediate plays a key role in the RNA compaction and triggers series of tertiary and secondary structure rearrangements. Equilibrium sampling methods offer direct insights into the kinetics of the intermediate and its effect on the barrier height, which impedes receptor formation. By coupling binding energy with the structural rearrangements, the nascent RNA remodels the activation barrier to favor the receptor conformation; a mechanistic framework that may be applicable to other RNA-based regulatory systems.

325 Whole-transcript SHAPE-MaP analysis of the LHR mRNA reveals structural features determining ZFP36L2 binding specificity

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ZFP36L2 is an RNA-binding protein containing a tandem zinc finger domain of the CCCH type. It belongs to the TTP-family of proteins which binds to AU-rich element-containing (ARE) transcripts and leads to mRNA instability and degradation. Using a mouse model in which ZFP36L2 is expressed at lower levels we observed the phenotype of female infertility. Further analysis of our mouse model revealed that Luteinizing Hormone Receptor (LHR) mRNA is a specific target transcript of ZFP36L2 and this interaction has an important physiological role. Interestingly, another protein Luteinizing Hormone Receptor Binding Protein (LRBP) has also been described to bind to the LHR mRNA leading to instability of this transcript. We performed whole-transcript SHAPE-MaP (Selective 2' Hydroxyl Acylation by Primer Extension-Mutational Profiling) to understand the structural determinants of binding specificity of ZFP36L2 and LRBP to the LHR mRNA. We obtained high-resolution SHAPE data on the full-length transcript (2.5kB) and used this to develop a structural model of this transcript. We were particularly interested in understanding the structural features that determine which ARE is accessible to ZFP36L2. We find that presenting in a hairpin loop does not seem to be sufficient to allow binding to a particular ARE sequence, but that the neighboring nucleotides must also have high flexibility (SHAPE reactivity). Furthermore, we identified highly reactive nucleotides located at the polypyrimidine tract in the coding sequence of the LHR mRNA that corresponds to the RNA sequence recognized by LRBP. Thus, LHR mRNA like *c-fos* mRNA has destabilizing sequences at the 3'UTR and at the coding sequence.

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326 Global RNA Fold and Molecular Recognition for a *pfl* Riboswitch Bound to ZMP, a Master Regulator of One Carbon Metabolism

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ZTP, the pyrophosphorylated analog of ZMP (5-amino-4-imidazole carboxamide ribose-5'-monophpsphate), was initially identified as an alarmone that senses 10-formyl-tetrahydroflate deficiency in bacteria. Recently, a *pfl* riboswitch has been identified that selectively binds ZMP and regulates genes associated with purine biosynthesis and one-carbon metabolism. We report on the structure of the *T. carboxydivorans pfl* riboswitch sensing domain in the ZMP bound state, thereby defining the pseudoknot-based tertiary RNA fold, the binding pocket architecture and principles underlying ligand recognition specificity. Molecular recognition involves shape complementarity, with the ZMP 5-amino and carboxamide groups paired with the Watson-Crick edge of an invariant uracil, the imidazole ring sandwiched between guanines, while the sugar hydroxyls form intermolecular hydrogen bond contacts. The burial of the ZMP base and ribose moieties, together with coordination of the carboxamide by Mg^{2+}, contrasts with exposure of the 5'-phosphate to solvent. Our studies highlight the principles underlying RNA-based recognition of ZMP, a master regulator of one-carbon metabolism.

327 Influence of Multiple Bulge Loops on the Stability of RNA Duplexes

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36 RNA duplexes containing two single nucleotide bulge loops were optically melted in 1M NaCl in order to determine the thermodynamic parameters $\Delta H°$, $\Delta S°$, $\Delta G°_{37}$, and T_M for each duplex. Because of the large number of possible combinations and lack of sequence effects seen previously (Blose et al., *Biochemistry 46*, 15123 (2007), we limited our initial investigations to adenosine bulges, the most common naturally occurring bulge. For example, the following duplexes investigated :

5'GGC**A**XY**A**GGC 5'GGC**A**XY GCC 5' GGC XY**A**GCC
CCG YX CCG CCG YX**A**CGG CCG**A**XY CGG

The identity of XY (where XY are Watson Crick base pairs) and the total number of base pairs in the central stem were varied. In addition the length and composition of the terminal stems were varied. As observed for duplexes with a single bulge loop, the influence of the multiple bulge loops on duplex stability is primarily influenced by non-nearest neighbor interactions. In particular, the stability of the central and terminal stems influence the destabilization of the duplex by the inserted bulge loops. The model proposed to predict the influence of multiple bulge loop on duplex stability suggests that the destabilization of the bulge is related to the stability of the adjacent stems. A database (Andronescu et al., *BMC Bioinformatics 9*, 340 (2008)) of RNA secondary structures was examined to determine the naturally occurring abundance of duplexes containing multiple bulge loops. Of the 2000 examples found in the database, over 65% of the two bulge loops occur within 3 base pairs of each other. A database (Popenda et al., *BMC Bioinformatics 11*, 231 (2010)) of RNA 3-dimentional structures was examined to determine the structure of duplexes containing two bulge loops. The structures of the bugle loops are described.

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328 hiCLIP reveals the atlas of mRNA secondary structures recognized by Staufen 1

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The structure of mRNAs is essential for post-transcriptional regulation, largely because it affects binding of trans-acting factors. However, little is known about the *in vivo* structure of full-length mRNAs. Here we present hiCLIP, a high-throughput technique to identify RNA secondary structures that interact with RNA-binding proteins. We applied the technique to Staufen 1 (STAU1) to investigate global features of the RNA structures it binds. In doing so, we uncover a dominance of intra-molecular RNA duplexes, a depletion of duplexes from coding regions of highly translated mRNAs, an unforeseen prevalence of long-range duplexes in 3′ untranslated regions (UTRs), and a decreased incidence of SNPs in duplex-forming regions. We also demonstrate that a long-range secondary structure spanning 858 nt in the 3′ UTR of the X-box binding Protein 1 (XBP1) mRNA regulates its stability and cytoplasmic splicing. Our study reveals the fundamental role of mRNA structure in regulating gene expression and highlights hiCLIP as a robust, widely applicable method for discovering novel RNA secondary structures.

329 Informa: a sequence-based, rational approach to design small molecules that target RNA.

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RNA plays important roles in cells well beyond translation or encoding protein. RNA regulates many major diseases. Some RNA motifs create unique niches susceptible to small molecules, the binding of which may modulate the RNA's function. The ability to predict RNA structure from sequence could be powerful to design small molecules that target RNA. As compared with traditional methods, a computational approach called Informa provides a more efficient method of designing small molecules to target RNA. By comparing the structural motifs in a user-determined target RNA to the motifs that can be targeted in a database of RNA motif-small molecule interactions, Informa identifies small molecule candidates for RNAs of interest. An update of the Informa database was performed to include interactions discussed in the literature. Additionally, a lead optimization tool was implemented to identify compounds with high similarity to Informa hits, thus increasing the number of compounds that can be pursued as lead compound candidates. All of these hits can be tested experimentally to examine biological function of RNA or to examine the ability of the compounds to act as therapeutic agents.

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330 Addressing the Major Limitations of RNA Secondary Structure Prediction Software

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There are several methods available to predict RNA secondary structure from sequence. The most popular approach is to predict the lowest free energy structure with a dynamic programming algorithm. Surprisingly, there are many RNA structures that are still predicted rather poorly, suggesting that there is still room for improvement. Three limitations of RNA secondary structure prediction software have been identified and addressed: (1) frequently occurring RNA secondary structure motifs were identified and thermodynamically characterized, and updated models were derived, (2) the effect of Na+ concentration on the thermodynamic parameters for Watson-Crick pairs was determined and correction factors were derived, and (3) nearest neighbor parameters for common non-standard nucleotides were derived. Free energy parameters, correction factors, and predictive models based on these data can be incorporated into prediction software to improve prediction of RNA secondary structure from sequence.

331 Searching for Structural Patterns in RNA 3D Structures

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RNA secondary structure is important for designing therapeutics, understanding protein-RNA binding, and predicting tertiary structure of RNA. The RNA Characterization of Secondary Structure Motifs (RNA CoSSMos) Database is a freely accessible and searchable online database and website of three dimensional characteristics of secondary structure motifs. To create the RNA CoSSMos Database, 2,156 PDB files were searched for internal, bulge, and hairpin loops, and each loop's structural information, including sugar pucker, glycosidic linkage, hydrogen bonding patterns, and stacking interactions, were included in the database. False positives were defined, identified, and reclassified or omitted from the database to ensure the most accurate results possible. Users can search via general PDB information, experimental parameters, sequence, and specific motif, and by specific structural parameters in the subquery page after the initial search. Returned results for each search can be viewed individually or a complete set can be downloaded into a spreadsheet to allow for easy comparison. Once developed, the RNA CoSSMos Database was used to find, annotate, and compare the structural features of bulges in 3D structures.

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332 Controlling mRNA stability and translation with the CRISPR endoribonuclease Csy4

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The CRISPR endoribonuclease, Csy4 (Cas6f) has recently been described as a potential tool for modifying RNA processing in cells. Csy4 recognizes a substrate RNA through a specific 28 nucleotide hairpin sequence and cleaves it at the 3' end of the stem. To explore applicability in mammalian cells, we introduced this hairpin at various locations in mRNAs derived from reporter transgenes and systematically evaluated the effects of Csy4-mediated processing. Placing the hairpin in the 5' UTR or immediately after the start codon resulted in efficient degradation of target mRNA by Csy4 and knockdown of transgene expression by 20 to 40-fold. However, when the hairpin was incorporated in the 3' UTR prior to the poly(A) signal, the mRNA was cleaved but there was only a modest decrease in transgene expression (~2.5 fold) observed. In the absence of a poly(A) tail, Csy4 rescued the target mRNA substrate from degradation and also resulted in expression of the protein suggesting that it also allowed translation of the cleaved mRNA. In contrast, neither catalytically-inactive (H29A) nor binding-deficient (R115A/R119A) Csy4 mutants were able to exert any of the above-described effects. Generation of a similar 3' end by RNase P-mediated cleavage was unable to rescue transgene expression independent of Csy4. These results support the idea that the selective generation of the Csy4/hairpin complex resulting from cleavage of the mRNA might serve as a functional poly(A)/Poly-A Binding protein (PABP) surrogate, stabilizing the mRNA and supporting mRNA translation. Although the exact mechanism(s) remain to be determined, our studies expand the potential utility of CRISPR nucleases as tools for controlling mRNA stability and translation.

333 Protein synthesis by ribosomes with tethered subunits

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The ribosome is a ribonucleoprotein machine responsible for protein synthesis. In all organisms, it is composed of two subunits, each built on its own ribosomal RNA (rRNA) scaffold. The ability of the subunits to reversibly associate and dissociate at initiation and termination steps of translation, respectively, and move relative to each other during elongation is considered a fundamental property of the ribosome. Expanding the repertoire of ribosome substrates and functions would be greatly beneficial for the advancement of systems and synthetic biology. However, as with any biological system, engineering objectives are often completely opposed to the growth and reproduction objectives of the organism. This problem can be solved in Escherichia coli through the use of a specialized ribosome that translates only a specific type of engineered messenger RNAs (mRNAs) and avoids translation of native cellular mRNAs. So far, efforts to construct such orthogonal ribosomes have focused on modifying the small subunit alone because orthogonality is endowed by modifying the Shine-Dalgarno sequence of an mRNA and the corresponding complementary sequence in the small subunit 16S rRNA. Unfortunately, free exchange between the subunits has meant the large subunit, responsible for peptide bond formation and protein excretion, could not be extensively engineered. Here we show that ribosomes with tethered and thus inseparable subunits (termed Ribo-T) are capable of successfully carrying out protein synthesis. By engineering a hybrid rRNA composed of small and large subunit rRNA sequences, we produced a functional ribosome in which the subunits are covalently linked into a single entity by short RNA linkers. Strikingly, Ribo-T was not only functional in vitro, but was able to support cell growth even in the absence of wild type ribosomes. We used Ribo-T to create the first fully orthogonal ribosome-mRNA system and demonstrated its evolvability by selecting otherwise dominantly lethal rRNA mutations in the peptidyl transferase center that facilitate translation of problematic protein sequences. Ribo-T can be used for exploring poorly understood functions of the ribosome, enabling orthogonal genetic systems, and engineering ribosomes with new functions.

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334 Regulation of -1 Programmed Ribosomal Frameshifting Efficiency by Synthetic Riboswitches

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Riboswitch, structured RNA capable of binding cellular metabolites to control downstream gene expression by ligand-dependent RNA conformational change, has been characterized in a variety of prokaryotic systems. However, few successful riboswitch applications in mammalian cells have been reported. -1 programmed ribosomal frameshifting (-1 PRF) affects gene expression at the level of protein synthesis and requires a hepta-nucleotides slippery sequence (XXXYYYZ) followed by an appropriately spaced RNA pseudoknot to cause a fraction of ribosomes to shift into the -1 reading-frame during translation. We have demonstrated that a stable RNA hairpin structure upstream of slippery site caused an attenuation effect on the efficiency of -1 PRF in SARS coronaviruses. Based on the similarity between this co-translational attenuation hairpin and the co-transcriptional ρ-independent transcription termination hairpin, we have recently shown that the -1 PRF attenuation event could be regulated by a synthetic riboswitch that modulates the formation of upstream attenuation hairpin. We have also applied this engineered ligand-dependent attenuator upstream of a downstream SAH responsive -1 PRF stimulator pseudoknot to achieve synergetic -1 PRF regulation by two different ligands. However, the application of SAH responsive-pseudoknot is limited by the leaky regulation background caused by endogenous SAH in cells.

To resolve this problem, we developed a strategy for engineering a synthetic riboswitch pseudoknot. Here, we presented our designing principle of the synthetic riboswitch pseudoknots, aiming to regulate the efficiency of -1 PRF in mammalian cells by non-metabolite lignads. We will also demonstrate that these engineered riboswitch pseudoknots can be combined with ligand-dependent upstream attenuator to achieve synergetic regulation of -1 PRF. In addition to extending the riboswitch application to a new gene expression platform to enrich the gene regulation repertoire in mammalian cells, the use of a synthetic ligand prevents regulation leakage from the endogenous cellular metabolites.

335 Synthesis and applications of RNAs with position-selective labeling and mosaic composition

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The structural, dynamic study and the applications of RNAs could be significantly enhanced by methods that enable to introduce isotope-labeled or modified nucleotides to specific positions. However, the methods that can synthesize tens of milligrams of such RNAs with reasonable cost have not been reported. We developed a method, PLOR (Position-selective Labeling Of RNA), that allows selective labeling of RNA molecules in desired regions, or residues, for studies by Nuclear Magnetic Resonance (NMR) and single molecule Förster resonance energy transfer (smFRET). PLOR is based on the ability to stall and re-start *in vitro* transcription with phage T7 RNA polymerase and solid-phase immobilized DNA templates. We demonstrate the utility of this labeling technology by preparing various isotope- or fluorescently-labeled 71nt-RNA samples for NMR and smFRET. We also developed an automated platform for production of selectively labeled RNAs by following the principles of PLOR.

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336 Expression kinetics of nucleoside-modified mRNA formulated with lipid nanoparticles and administered into mice by various delivery routes

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In vitro transcribed messenger RNA (mRNA) represents a novel, promising therapeutic platform for a wide variety of clinical applications. Several strategies have been employed to increase mRNA translation and half-life, including the use of lipid nanoparticles (LNP). LNPs have been extensively used for systemic delivery of small interfering RNA and are now being investigated as mRNA delivery vehicles. In this study, we evaluate the in vitro and in vivo transfection efficiency of LNP-encapsulated mRNA encoding luciferase (LNP-Luc). Whereas LNP-Luc efficiently transfected HEK 293T cells, transfection of human dendritic cells was shown to be ApoE-dependent. In vivo, LNP-Luc was more efficient than commercially available lipid-based complexing agents regardless of injection route. Systemic injection resulted in luciferase expression in the liver, with the highest expression observed after intravenous administration of LNP-Luc. Subcutaneous and intradermal injection resulted in local expression and significantly more durable luciferase expression than systemic injection. These results demonstrate that LNPs are viable mRNA delivery vehicles and support further development of LNP-encapsulated mRNA as therapeutic agents.

337 Design and characterization of a non-canonical bacterial translation initiation element using phenotypic sorting and high-throughput sequencing.

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Manipulation and fine-tuning of gene expression is critical for a wide range of cellular behaviors and synthetic biology applications. Regulation can be achieved on the transcriptional and the translational level. Transcriptional promoters and regulatory factors can be manipulated and changed. Alternatively, translation initiation is a highly regulated phase during protein biosynthesis and is therefore a promising target for expanding our toolbox of cellular control devices, as well as understanding the mechanistic and structural basis of translation regulation through non-canonical translation initiation regions (TIR) located in the 5'-UTR of mRNAs. Besides providing mechanistic insight into the underlying principles of translation initiation regulation, it is of great value for bioengineers to have a tool analogous to virus IRESs for use in prokaryotes that enables rational fine-tuning of translation efficiencies other than through the classical Shine-Dalgarno sequence. To this end we have designed standardized TIR constructs based on an existing highly structured *Escherichia coli* 5'UTR and generated a TIR library using saturation mutagenesis followed by investigating the sequence - translation efficiency relationship using phenotypic cell sorting and high-throughput next generation sequencing. A 3 dimensional structural model of the TIR generated using small angle x-ray scattering data (SAXS) and molecular dynamics based flexible fitting will be presented. This TIR library with a wide range of translation efficiencies has been submitted to the Registry of Standard Biological parts in BioBrick format.

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338 *In vitro* selection of RNA aptamers for benzylguanine SNAP ligands for visualization of RNAs in living cells

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To illuminate various aspects of protein function, labeling of fusion proteins with small tags (such as the SNAP-tag) by organic dyes has been widely applied. Small molecule fluorophores have many advantages over the use of fluorescent proteins, such as much greater brightness. Like proteins, RNA also exhibits a diverse range of functions in biological systems. However, imaging of RNA in living cells is challenging. We have developed RNA aptamers capable of binding benzylguanine SNAP tag ligands. At least one RNA sequence (JX1) is capable of not only benzylguanine binding but also binding of fluorescent benzylguanine derivatives, with a Kd value of 219.1±7.8 nM. Moreover, a fluorescence polarization competition assay indicated that the JX1 RNA has specific binding affinity for benzylguanine over guanine analogues. RNA structure analysis by in line probing and nuclease digestion assays showed conformational changes in the JX1 RNA induced by benzylguanine interaction. Current work is focused on using the JX1 RNA as a tool for imaging RNA in living cells.

339 RNAMake: an Automated Design Toolkit for RNA 3D Structure

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Engineered RNA and RNA/protein complexes are offering new routes for deciphering genetic regulation and for the eventual rational design of diagnostics and therapeutics. These designed or repurposed molecules harness RNA's ability to adopt complex 3D shapes, to perform catalysis, to guide pairing to DNA or RNA, and to change shapes in response to cellular and viral molecules. Development of RNA's potential as a design medium is hindered by inaccurate models of RNA folding and design, necessitating time-consuming selection methods, expert inspection, and/or trial-and-error refinement. To accelerate the generation of RNA-based technology, we are developing and rigorously testing RNAMake, an automated 3D architectonics toolkit for searching combinations of canonical helices and noncanonical 3D motifs to satisfy a large range of design problems. Building on pioneering work from Jaeger and colleagues, we are curating an experimentally determined database of 'modularity scores' for all known structural motifs. We have developed an unambiguous reporting system for characterizing modularity by designing scaffolds that connect the segments of a tetraloop-tetraloop receptor tertiary contact, performing massively parallel chemical profiling, and then integrating these data to parameterize a 'nearest-neighbor' model for 3D motif modularity. We are then testing automated designs for two practical applications: improving a small aptamer's affinity for its target small molecule by reducing its binding conformational entropy and generating an RNA 'claw' that binds another RNA through rigid struts interconnecting A-minor motifs. RNAMake helps test principles and automates a significant part of 3D RNA design, solidifying and accelerating our conceptual foundation for rational RNA engineering.

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340 Tandem "Spinach" Array for mRNA Imaging in Living Cells

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Live cell RNA imaging is important for understanding RNA activities and dynamics. Compared to the commonly applied indirect labeling strategies that engineer target RNAs with recognition sequences which bind fluorescent protein-fused RNA binding proteins, using fluorogenic small molecules and RNA aptamers might cause less perturbation to the native RNA characteristics. In a recently developed "Spinach" system, the 24-2 RNA aptamer binds and induces EGFP-like fluorescence for 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI). The system shows potential for live cell RNA imaging with high binding specificity, good cell permeability and low cytotoxicity of DFHBI, and great fluorescence enhancement upon binding to RNA aptamer. The Spinach system with one aptamer per RNA is sufficient for imaging highly abundant nontranslated RNAs (tRNAs, rRNAs, trinucleotide repeats,*etc.*), but showe low fluorescence signal inadequate for mRNA imaging. In this work we constructed Spinach arrays containing multiple aptamers (8 - 64 aptamer repeats) in tandem for mRNA labeling, resulting in as high as 12 fold brightness enhancement for cellular mRNA imaging. In combination with strong laser excitation and pulsed illumination strategy, we could observe mRNAs labeled with the Spinach array in bacteria to the level of 50 - 180 copies per cell. In addition, transcriptional fusion to the Spinach array did not affect mRNA transcription, translation or degradation. Therefore, the aptamer array might be a generalizable labeling method for high-performance and low-perturbation labeling of RNA for live cell RNA imaging.

341 Genomic alterations dysregulate cancer genes by modulating microRNA activity

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MicroRNAs (miRNAs) play key roles in cancer etiology. Recent evidence suggests that targets of the same miRNA programs are stoichiometrically coupled via a competitive endogenous RNA (ceRNA) mechanism. Consequently, aberrant expression of multiple targets of the same miRNA program dysregulate coupled ceRNA-oncogenes and tumor suppressors (henceforth cancer genes). Indeed, we computationally infer and experimentally validate that coordinated copy number and methylation alterations (genomic alterations) contribute to physiologically significant *in-trans* dysregulation of hundreds of coupled cancer genes in multiple tumor contexts. Both high-throughput perturbation assays and low-throughput mutational assays confirmed significant ceRNA-mediated regulation of cancer genes in eight tumor contexts, including ESR1 and APC in breast and colon cancer, respectively. Our analysis infers roles for previously uncharacterized genomic alterations and it suggests that ceRNA-mediated interactions account for a substantial fraction of cancer's missing genomic variability.

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342 Evolution of mobile group II introns towards inactivation in the bacterial genome

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Group II introns are self-splicing catalytic RNAs that probably originated in bacteria and act as mobile retroelements. The dispersal and dynamics of group II intron spread within a bacterial genome are thought to follow a selection-driven extinction model. Likewise, various studies on the evolution of group II introns have suggested that they are evolving toward an inactive form by fragmentation, with the loss of the intron 3´-terminus, but with some intron fragments remaining and continuing to evolve in the genome. RmInt1 is a mobile group II intron that is widespread in natural populations of *Sinorhizobium meliloti,* but some strains of this species have no RmInt1 introns. We studied the splicing ability and mobility of the three full-length RmInt1 copies harbored by *S. meliloti* 1021, and obtained evidence suggesting that specific mutations may lead to the impairment of intron splicing and retrohoming. Our data suggest that the RmInt1 copies in this strain are undergoing a process of inactivation that may precede intron fragmentation. Based on these and previous observations, we suggest a model for the evolution of group II introns in the bacterial genome.

343 Splicing promotes the nuclear export of *β-Globin* mRNA by overcoming nuclear retention elements

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Most current models of mRNA nuclear export in metazoan cells suggest that an mRNA must have mRNA nuclear export-promoting signals in order to be exported from the nucleus to the cytoplasm. Under such a scenario, mRNAs that lack these specialized signals would be shunted into a default pathway where they are retained in the nucleus and are eventually degraded. For example, it has been shown that splicing promotes the nuclear export of several model mRNAs such as human β-*Globin*, and that in the absence of splicing, the cDNA-derived mRNA is retained in the nucleus and degraded. We discovered that the intronless β-*Globin* mRNA contains an RNA element that retains the mRNA in the nucleus and inhibits its export to the cytoplasm. Intriguingly, we found that this nuclear retention element can be overcome by either increasing the length of the mRNA or by including an intron. Our results suggest that in contrary to those current paradigms, the default pathway for most mRNAs is to be exported from the nucleus, unless the mRNA contains elements that actively promote its nuclear retention. These results provide novel insights into how eukaryotic cells selectively regulate the expression of different mRNAs.

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344 Aptamer-Mediated Delivery of Large Functional RNA

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Cell-penetrating RNAs bind to cell surface receptors and internalize through endocytosis, which can either be triggered by ligand binding or occur as a normal consequence of membrane and receptor recycling. Cell-type-specific aptamers have been used to deliver a variety of molecular payloads, such as small molecule drugs, imaging agents and small interfering RNAs. Although larger RNAs, such as lncRNA and engineered RNA nanostructures, offer tremendous potential as therapeutic and regulatory agents, their aptamer-mediated delivery into cells has not yet been demonstrated. Here we report on a plug-and-play platform for the aptamer-mediated delivery of increasingly large RNA payloads into targeted cell types. Various cell-penetrating aptamers ("delivery modules") were annealed to the fluorescent RNA aptamers, Spinach and Broccoli ("payload modules"). The impact of the size of the RNA payload (~30-190 kDa) was explored through multimerization of the payload aptamers and was accomplished through several designs. The relatively small size of the dimeric Broccoli aptamer facilitated engineering of a tetrameric aptamer by inserting dimers into each end of a thermodynamically stable three-way junction (3WJ) from bacteriophage pRNA. A much larger octomer was formed by incorporating two tetramers into a single transcript separated by a 5s rRNA 3WJ. Formation of the self-assembling aptamer-aptamer complexes was confirmed through electrophoretic mobility shift assays and productive folding of the annealed payload was validated with spectrofluorometry. Fluorescence confocal microscopy was used to monitor delivery of functional RNA to a range of mammalian cells and demonstrated specificity of targeting aptamers for their intended cell-types. Delivered complexes exhibited punctate intracellular distribution and demonstrated co-localization in endosomal compartments. Our findings demonstrate that large functional RNAs can be internalized into living cells and provides a modular, reprogrammable platform for aptamer-mediated aptamer delivery and several multimerized fluorescent aptamer constructs that offer improved signal and stability. Importantly, because fluorescent signal is only evident while the RNA remains intact and correctly folded, this strategy provides a powerful method to monitor the real-time integrity, delivery and localization of large and functional RNA cargoes.

345 mRNA translation within Spreading Initiation Center regulate cellular adhesion

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Metastatic cancer cells are cancer cells that gained the ability to disseminate and invade distant organ with growth favorable microenvironment. Sam68, an RNA-binding protein was found to play a crucial role in tumor progression and metastasis in many type of cancer, mainly by affecting the adhesion and migration potential of cancer cells.

We found that Sam68 localization within Spreading Initiation Centers (SICs), a premature adhesion structure, could directly affect the adhesion potential of seeding cell. SICs are transient structures found in seeding cell, localized on top of newly formed adhesion complexes. These structures are distinct from newly formed focal adhesion complex and gradually disappear with the formation of stable adhesion site during the initial phase of adhesion/spreading. Herein, we examine how RNA-binding proteins activities are modulated by the microenvironment and how this affects cellular adhesion and focal adhesion maturation during SICs formation.

Our work shows a dynamic recruitment, within these structures, of specific RNA-binding protein known for their ability to regulate mRNA translation (FMRP, G3BP1, Sam68). Concomitantly, we found that polyadenylated co-localized with these RNA-binding proteins and that inhibition of their translation caused an increase in the number of SICs positive cells showing a significant decrease in adhesion strength and limited spreading capacity. Surprisingly, these effects were restricted to fibroblastic-like cell lines and cancer cell that underwent epithelial to mesenchymal transition (EMT), while translation inhibition did not affect these cellular process in cancer cell lines that retained an epithelial-like morphology.

Herein, we showed that SIC processing can be regulated by a select number of RNA-Binding proteins and their ability to translate SIC-bound mRNA. Hence, we propose a mechanism by which SICs can act as a premature adhesion checkpoint modulating the adhesion, spreading and invasion of metastatic cell that underwent EMT.

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346 Sub-cellular localization of human motor mRNAs at the level of single molecules

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Localization of mRNAs is a key post-transcriptional process by which a cell transports specific RNAs to predetermined locations in the cytoplasm. It is observed from bacteria to humans, and it is involved in many processes such as local translation and cell polarity. Motor proteins such as kinesins and myosins play essential roles in transporting various cargo throughout the cell and in establishing and maintaining cell polarity. Despite these important roles, it is not known whether mRNAs encoding motor proteins can be localized and locally translated. This would however provide an elegant mechanism to drive cell polarity since the locally translated motor protein could then move back and forth on the cytoskeleton to carry various substrates to where it was initially translated. In order to test this hypothesis, we have performed a systematic study in which we analyzed the intra-cellular localization of all human mRNAs coding for motor proteins, at the level of single molecules using smFISH (single molecule FISH). Results showed that among a total of 79 genes analyzed, 7 mRNAs were found to display a non-random localization pattern in HeLa cells: four were localized to pseudopodia and cellular extensions, one to the cell periphery, one to several cytoplasmic aggregates, and one to a single cytoplasmic spot. Examination of additional cell types showed that localization of most of these mRNAs was widespread as it also occurred in myoblasts and neurons. In one case, the encoded motor protein colocalized with its mRNA, suggesting a pattern of local translation consistent with our initial model. In the other cases, the localization of the other proteins was unrelated to that of their respective mRNAs. Functional experiments are currently ongoing to address the role of mRNA localization for specific motor proteins.

347 HAC1 mRNA: A Model mRNA to Decipher Mechanisms of mRNA Relocation and Translation

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In yeast Saccharomyces cerevisiae, the HAC1 mRNA remains in a translationally inert form in the cytoplasm wherein an intervening sequence (intron) base pairs with the 5'-untranslated region (5'-UTR). Under conditions of cellular stress, an endoplasmic reticulum (ER)-resident protein Ire1 cleaves out the intron from the HAC1 mRNA. Following ligation by tRNA ligase, a matured mRNA is formed with a new open reading frame, which translates the Hac1 protein. The HAC1 mRNA cleavage essentially requires intracellular relocation and co-localization with the Ire1 protein, which has been reported to be mediated by the 3'-UTR. Recently, our lab has shown that the 3'-UTR also activates translation of the HAC1 mRNA; however, mechanisms of mRNA relocalization and/or translational activation remain largely unknown. Furthermore, our lab has discovered that two yeast kinases Kin1 and Kin2 modulate both mRNA targeting and translation. We are studying the mechanisms of intracellular relocation of HAC1 mRNA.

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348 Egalitarian regulates the transport, anchoring, and translation of *oskar* mRNA.

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The proper functioning of most eukaryotic cells requires microtubule-based transport of cargoes to specific cellular sites. The mechanism by which cargoes are recognized and coupled to motors remains poorly understood. The *Drosophila* oocyte provides a genetically tractable model for investigating this important topic. The polarity of the *Drosophila* oocyte is established by the localization of *oskar* mRNA to the posterior pole. The localization of *oskar* mRNA, coupled with translational regulation, results in restriction of Oskar protein to the oocyte posterior. Oskar is a morphogen that establishes the polarity of the oocyte and future embryo. Previous studies have shown that the localization of *oskar* mRNA requires microtubules and the motors Kinesin-1 and Dynein. The goal of this study was to determine the mechanism by which *oskar* mRNA is linked to these microtubule motors. The Egalitarian (Egl) - BicaudalD (BicD) complex has been shown to be required for the localization of several mRNAs in the *Drosophila* embryo. Egl is an RNA binding protein that physically interacts with Dynein. We therefore hypothesized that Egl couples *oskar* mRNA to the Dynein motor. In support of this hypothesis, we demonstrate that Egl is present in a complex with *oskar* mRNA in vivo. Furthermore, depletion of Egl resulted in *oskar* mRNA delocalization. Under normal circumstances, *oskar* mRNA is only translated into protein once it is correctly localized. Thus, in most mutants, delocalized *oskar* mRNA is not translated. Surprisingly, upon Egl depletion, the delocalized *oskar* mRNA was precociously translated into protein. This suggests an unanticipated role for Egl in regulating the translation of *oskar* mRNA. Finally, in order to test the significance of the Egl-BicD interaction, we examined oocytes expressing a mutant isoform of Egl that is not capable of binding BicD. Although *oskar* mRNA was enriched at the oocyte posterior in this mutant, it was not anchored. This suggests that the Egl-BicD interaction is less important for transport, but rather functions within the context of anchoring. In conclusion, our results identify a central role for the RNA binding proteins Egl in all phases of *oskar* mRNA localization; transport, anchoring and translational regulation.

349 Protein arginine methyltransferase CARM1 attenuates the paraspeckle-mediated nuclear retention of mRNAs containing IR*Alu*s

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In many cells, mRNAs containing inverted *Alu* repeats(IR*Alu*s) in their 3′-untranslated regions (3′-UTRs) are inefficiently exported to the cytoplasm. Such nuclear retention correlates with paraspeckle-associated protein complexes containing $p54^{nrb}$. However, nuclear retention of mRNAs containing IR*Alu*s is variable and how regulation of retention and export is achieved is poorly understood. Here we show one mechanism of such regulation via the arginine methyltransferase CARM1. We demonstrate that disruption of CARM1 enhances the nuclear retention of mRNAs containing IR*Alu*s. CARM1 regulates this nuclear retention pathway at two levels: CARM1 methylates the coiled-coil domain of $p54^{nrb}$, resulting in reduced binding of $p54^{nrb}$ to mRNAs containing IR*Alu*s; CARM1 also acts as a transcription regulator to suppress *NEAT1* transcription, leading to reduced paraspeckle formation. These actions of CARM1 work together synergistically to regulate the export of transcripts containing IR*Alu*s from paraspeckles under certain cellular stresses, such as poly(I:C) treatment. This work demonstrates how a post-translational modification of an RNA binding protein affects protein-RNA interaction and also uncovers a mechanism of transcriptional regulation of the long noncoding RNA *NEAT1*.

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350 Prrp: An RNA Binding Protein that Anchors mRNA to the Actin Cytoskeleton

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Cytoplasmic RNA localization is a widely-conserved mechanism for spatial regulation of gene expression. In Xenopus laevis, local expression of Vg1 protein, a TGF-ß growth factor family member, is essential for embryonic patterning. This spatially-restricted expression is achieved by transport of Vg1 mRNA to the vegetal cortex of the oocyte. Although Vg1 mRNA localization occurs during early oogenesis, active translation does not occur until much later, during late oogenesis and early embryogenesis. Because the time between RNA localization and protein expression is on the order of weeks, it is critical that the mRNA remain in place at the cortex. This subcellular retention of localized RNA is termed anchoring. Although RNA anchoring is critical to ensure spatially-restricted expression of the localized mRNA, almost nothing is known about its mechanism. Using a combination of biochemical and in vivo imaging approaches, we have probed the connections between Vg1 mRNA and the cortical cytoplasm. Our results reveal that Vg1 mRNA is immobile at the cortex during oogenesis and that the RNA-binding protein, Prrp (proline rich RNA binding protein), is a strong candidate for a Vg1 mRNA anchoring protein. Prrp (also known as DAZAP1) is comprised of two RNA binding domains and a profilin-interacting proline-rich domain. Prrp is expressed throughout oogenesis and colocalizes with Vg1 mRNA at the vegetal cortex during oogenesis. We have found that the RNA binding domains of Prrp bind directly and specifically to Vg1 mRNA. To investigate the role of Prrp in RNA anchoring we have used a dominant-negative approach with a mutant form of Prrp (dnPrrp) designed to interfere with function. Our results demonstrate that dnPrrp significantly alters the normal anchoring pattern of Vg1 RNA, implicating Prrp as a crucial component of a cortical RNA anchoring complex.

351 Exu structure reveals dimerization as a key requirement for anterior localization of *bicoid* mRNA in *Drosophila*.

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In *Drosophila*, Exuperantia (Exu) has an essential role in the determination of anterior-posterior polarity by ensuring the proper localization of *bicoid* (*bcd*) mRNA at the anterior pole of the oocyte. Exu is required during early stages of oogenesis for the establishment of *bcd* mRNA localization. Despite having been known for almost three decades, Exu's function in this process is still unclear.

To gain insights into the molecular mechanism of *bcd* mRNA localization, we solved the crystal structure of Exu. The protein contains two structured domains: a 3'-5' exonuclease (exo)-like domain, and a helical sterile alpha motif (SAM)-like domain. We show that the active site in the exo-like domain is disrupted, and the protein is therefore inactive. The structure reveals that Exu forms a stable homodimer *in vitro*; dimerization is required *in vivo*, since monomeric mutants fail to rescue *bcd* mRNA localization in Exu-null flies. We further observed that Exu binds RNA *in vitro* in a non sequence-specific manner and with high affinity. Deletion of the SAM-like domain causes a dramatic drop in the RNA-binding affinity, suggesting that this domain is the major site of RNA interaction.

All together, our results support the view that Exu is a RNA binding protein. Interaction with the RNA is mediated mainly by the SAM-like domain, while the exo-like domain might act as a platform to recruit additional protein factors, probably required to define target specificity *in vivo*. Finally, we show that Exu is a dimer; though dimerization is not required *in vitro* for interaction with the RNA, it is necessary *in vivo* for *bcd* mRNA localization.

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352 Isolation and characterization of RNA transport granules reveals both molecular motors and stress granule components.

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Localization of mRNA within the cytoplasm is a powerful post-transcriptional regulatory mechanism that acts to control gene expression both spatially and temporally. Among vertebrates, localization of maternal mRNAs to the vegetal cortex of the *Xenopus* oocyte is an important model for understanding how RNA molecules can be targeted to specific regions of the cell cytoplasm to generate spatially restricted gene expression. One well-characterized example is *Vg1* mRNA, whose vegetally-restricted expression is critical for embryonic patterning. Through recognition of *cis*-acting RNA localization elements by *trans*-acting RNA binding proteins, Vg1 mRNA is transported by molecular motors to the vegetal cortex in large ribonucleoprotein (RNP) complexes, termed RNA transport granules. Although formation of a localization-competent RNP granule is believed to be an early or initiating event in the RNA localization pathway, the molecular nature of these RNP granules and their relationship to other types of cytoplasmic granules (such as p-bodies, stress granules, etc.) is not yet clear. To investigate these questions, we have developed procedures for isolation of vegetal RNA transport granules and analyzed the composition of these granules by mass spectrometry. Our results have revealed new components of the transport machinery, many of which are conserved amongst members of the large family of cytoplasmic RNA granules—specifically, stress granules and other maternal RNP granules. After validation by immunolocalization and biochemical approaches, we are working to investigate the specific roles candidate proteins play in RNP granule assembly. Our preliminary results promise new insights into the mechanisms that regulate RNA localization and the interactions that underlie RNP dynamics and activity.

353 Abstract Withdrawn

354 Characterization of a new cis-acting signal required for the Anxa2 mRNA axonal localization by SMN

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Spinal muscular atrophy (SMA) is a recessive disease affecting motor neurons of the spinal cord and leading to muscular atrophy. This pathology is caused by mutations and/or deletions of the survival motor neurons gene (SMN1), which lead to reduced levels of SMN protein and to a selective dysfunction of motor neurons. Besides its function in the biogenesis of spliceosomal snRNPs, it has been proposed that SMN might possess a motor neuron specific role and could function in the transport of axonal mRNA and in the modulation of local protein translation. We recently found that SMN colocalizes with axonal mRNAs in neurites and axons of differentiated NSC34 motor neuron-like" cell line and that SMN depletion gives rise to a decrease in the axonal transport of Anxa2 and Cox4i2 mRNAs (Rage et al., 2013 RNA. 2013 (12): 1755-66).

In order to characterize the structural features of Anxa2 mRNA required for its axonal targeting by SMN, we constructed reporters carrying different RNA regions in the pcDNA3-lacZ-24xMS2 vector. After transfection in NSC34 cells, the detection of the transcripts was followed by FISH in differentiated axon-like structures. We found that a G-rich region, overlapping the end of the coding region and the start of the 3'UTR is important for axonal localization of Anxa2 mRNA. Although this G-rich motif contains a consensus sequence for a putative G-quadruplex, preliminary results using a reverse transcription assay show that it is not folded into a G-quadruplex-forming structure, a motif that has been shown to have properties of zip-code for dendritic localization. While a structural definition of this region is in progress by chemical and enzymatic probing, a prediction of the secondary structure by RNAfold suggests that this G-rich region is able to fold into a stem-loop structure, conserved in mouse and human. Moreover, mutations in this stem-loop abolish targeting of Anxa2 reporter mRNAs in axon-like structures. In addition, localization of both wt and mutated Anxa2 reporters is restricted to the cell body in SMN depleted cells. Our studies suggest the existence of a novel and essential determinant for axonal localization of mRNAs that is mediated by the SMN complex.

355 Withdrawn

356 Nuclear export of spliceosome-discarded intermediates

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The precise removal of introns is essential for cell survival. To promote fidelity in nuclear pre-mRNA splicing, the spliceosome rejects and discards suboptimal splicing substrates that have engaged the spliceosome. Although intron-containing transcripts can be retained in the nucleus for degradation, spliceosome-discarded species, including the 5' exon and lariat intermediate, can be exported into the cytoplasm for degradation, as revealed by translation of the discarded species or degradation by cytoplasmic nucleases. However, it is unknown how these suboptimal intermediates are exported for turnover.

Here, we used single molecule RNA-FISH to provide direct evidence that mutated lariat intermediates are exported into the cytoplasm. Disruption of the discard pathway, mediated by the DEAH-box ATPase Prp43p, blocks the export of mutated lariat intermediates. Further, we demonstrated that nucleoporins (Nup2p and Nup60p), the mRNA export factor Mex67, and export adaptors play an essential role in the export of mutated lariat intermediates. Surprisingly, we found evidence that Mlp1p, a factor implicated in retaining unspliced pre-mRNA in the nucleus, is required for the export of mutated lariat intermediates, and the Nab2p-interacting domain of Mlp1p is required for this function. Together, our findings establish the importance of the mRNA export pathway in exporting spliceosome-discarded intermediates for degradation and implicate a dual role for Mlp1p in not only retention of unspliced transcripts but also export of partially spliced transcripts.

357 A large-scale functional tethering screen of RNA binding proteins reveals novel regulators of RNA turnover and translational control

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The fate of RNAs in the cell is determined by RNA binding proteins (RBPs), which control all aspects of RNA metabolism including splicing, subcellular localization, and stability. Advances in high-throughput sequencing and large-scale quantitative mass spectrometry have enabled experimental identification of hundreds of RBPs; a recent census estimates the number of high-confidence human RBPs to be around 1500. For a majority of these RBPs, specific roles in RNA processing remain to be elucidated. Here, we have begun to functionally categorize these RBPs by generating a library of 955 RBPs fused to the bacteriophage MS2 coat protein (MCP), which recognizes the MS2 hairpin RNA motif. We have used this library in a large-scale luciferase-based screen to identify candidate RBPs that affect RNA stability and/or translational efficiency when recruited to the 3' untranslated region (3' UTR). MCP-fused RBPs were exogenously expressed in HeLa cells along with inducible destabilized luciferase constructs harboring MS2 binding sites in their 3' UTRs. Out of 955 RBPs screened, 223 and 32 significantly upregulated and downregulated, respectively, levels of both firefly and *Renilla* luciferases. Among the RBPs with the strongest negative effect were poly(A)-specific ribonuclease (PARN) and several members of the Ccr4-NOT cytoplasmic deadenylation complex and the decapping complex, indicating that our screen robustly recovers known RNA degradation factors. Analogously, several RBPs previously reported to activate mRNA translation in specific contexts, such as boule-like protein (BOLL) and La-related protein 4B (LARP4B), effected strongly increased luciferase levels. We are currently investigating a set of *bona fide* RBPs that have emerged as hits in our tethering screen but whose role in RNA metabolism is unknown. We are also combining our approach with measuring luciferase transcript levels by qPCR to dissect RBP effects on RNA stability *versus* translation efficiency. We anticipate that our library of MCP-RBP fusions will serve as a valuable resource to the RNA community and that its application in similar screens, in combination with RBP knockdown assays and RNA target identification by CLIP-seq, will provide deeper insights into RBP function.

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358 Identification of mRNA Targets of Human PUF Proteins

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Members of the PUF family of RNA binding proteins inhibit translation and stimulate mRNA decay of specific target mRNAs by binding to sequence specific elements in the 3'-Untranslated Regions (3'-UTRs). Human PUF proteins, PUM1 and PUM2, are potent repressors that reduce abundance of reporter mRNAs that possess high affinity 3'-UTR PUM Response Elements (PREs). Very few natural target mRNAs that are regulated by PUM1 and PUM2 have been identified. Here, we utilize RNA Seq and BruChase-Seq to globally identify mRNA targets that are differentially expressed and/or stabilized, respectively, upon knockdown of PUM1 and PUM2 in cultured Human cells. A group of high confidence mRNA targets was identified with three criteria: transcripts significantly increased in abundance in response to knockdown of both PUM1 and PUM2, contained at least one PRE, and were bound by either PUM1 or PUM2 as determined in previously published RIP-chip and PAR-CLIP data sets (Galgano *et al* 2008, Morris *et al* 2008, and Hafner *et al* 2010). We identified 258 transcripts fit these criteria. This list contains four of nine previously identified PUM target mRNAs, illustrating the effectiveness of our approach. We then performed pulse-chase labeling of RNAs using Bromo-Uridine (BruChase) followed by RNA Seq to measure relative synthesis and decay rates of all expressed RNAs. As a result, we identified hundreds of mRNAs that are specifically destabilized by PUM1 and PUM2. Gene ontology categorization of the high-confidence PUM target mRNAs illustrates the breadth and diversity of the cellular pathways and processes that are regulated by human PUF proteins including: neurogenesis, cell migration, cell proliferation, and specific developmental processes. These results identify natural target mRNAs and provide new insights into the biological functions of human PUM proteins and their impact on protein expression and mRNA stability.

359 Dynamics, Mechanism and Regulation of Autophagy

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Stress granules and P bodies are conserved cytoplasmic aggregates of non-translating messenger ribonucleoprotein complexes (mRNPs). They are implicated in the regulation of mRNA translation and decay and are related to RNP granules in embryos, neurons, and pathological inclusions in degenerative diseases such as amyotrophic lateral sclerosis, frontotemporal lobar degeneration and multisystem proteinopathy. Using baker's yeast, 125 genes were identified in a genetic screen that affected the dynamics of P bodies and/or stress granules. Analyses of such mutants, including CDC48 alleles, provided evidence that stress granules can be targeted to the vacuole by autophagy, in a process we termed "granulophagy". Moreover, stress granule clearance in mammalian cells is reduced by inhibition of autophagy or by depletion or pathogenic mutations in p97, the human ortholog of CDC48. We are currently focused on identifying candidate factors in yeast and human cell lines that physically link stress granules to the core autophagic machinery, as well as defining conditions under which granulophagy is most active. An emerging model based on studies by many labs is that impaired autophagic clearance of stress granule-related and pathogenic RNP granules may underpin the onset of several degenerative diseases. An understanding of granulophagy may therefore elucidate new therapeutic options to reduce degenerative disease pathology.

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360 Modular Xrn1-Resistant RNAs Prevent 5'→3´ Decay in Budding Yeast

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A discrete, structured RNA element in the 3´ untranslated region of arthropod-borne Flaviviruses adopts a unique three-dimensional fold to resist degradation by the 5'→3' exoribonuclease Xrn1 (Chapman et al. Science, 2014 and Chapman et al. eLife, 2014). Here we show that these Xrn1-resistant RNAs (xrRNAs) can be used as modular elements to protect mRNAs from 5´→3´ exonucleolytic decay in Saccharomyces cerevisiae. We tested the ability of xrRNAs installed in pre-mRNA to prevent the decay of splicing intermediates by the debranching and decay pathway that is typically responsible for recycling lariat introns formed during pre-mRNA splicing. We show that these modular xrRNA structures operate to protect a series of splicing reporters from 5'→3' decay by Xrn1 and facilitate persistent IRES-driven translation of a downstream β-galactosidase. Specifically, installation of xrRNA structures in these reporters yields an extension in their mRNA half-life and the accumulation of partially-decayed intermediates. These intermediates harbor functional IRES's that allow them to re-enter the mRNP cycle and be translated into functional proteins. Together these findings demonstrate that RNA structures from diverse (+)-strand RNA viruses have the potential to stabilize arbitrary mRNAs in vivo. This strategy may be useful in characterizing the role played by temporally expressed coding and non-coding RNAs as well as in creating new types of RNA therapeutics.

361 NOT2,3, and 5 physically link mRNA decapping to the deadenylation complex.

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Decay of mRNA is essential for the efficient regulation of gene expression. A major pathway of mRNA degradation is initiated by the shortening of the poly(A) tail via the CCR4/NOT deadenylase complex. Deadenylation is followed by removal of the 5' cap (i.e. decapping) and then 5' to 3' exonucleolytic decay of the message body. The highly conserved CCR4/NOT deadenylase complex consists of the exonucleases CCR4 and POP2/CAF1, as well a group of four or five (depending on the organism) accessory factors of unknown function, i.e. NOT proteins. In this study, we see no role of NOT2 or NOT3 and NOT5 (close paralogs of each other) in poly(A) tail shortening in budding yeast; this is despite their well established association with the deadenylase complex. Rather, we find that NOT2, 3, and 5 are directly involved in promoting mRNA decapping. While NOT2 and 5 have a general role in promoting mRNA decapping, NOT3 is message specific. We saw no association of NOT2 with known mRNA decapping factors by yeast-two hybrid, although NOT3 and 5 were found to bind to the decapping activator protein PAT1. Together, these data implicate the deadenylase complex in coordinating the downstream decapping reaction via NOT2, 3, and 5. This suggests that the coupling of deadenylation with decapping is, in part, a direct consequence of coordinated assembly of decay factors.

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362 Codon Optimality is a major determinant of mRNA decay

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Messenger RNA degradation represents a critical regulated step in gene expression. While the major pathways in turnover have been identified, accounting for disparate half-lives has been elusive. We show that codon optimality is one feature that contributes greatly to mRNA stability. Genome-wide RNA decay analysis revealed that stable mRNAs are enriched in codons designated optimal, whereas unstable mRNAs contain predominately non-optimal codons. Substitution of optimal codons with synonymous, non-optimal codons results in dramatic mRNA destabilization, while the converse substitution significantly increases stability. Further, we demonstrate that codon optimality impacts ribosome translocation, connecting the processes of translation elongation and decay through codon optimality. Finally, we show that optimal codon content accounts for the similar stabilities observed in mRNAs encoding proteins with coordinated physiological function. This work demonstrates that codon optimization exists as an evolutionary mechanism to finely tune levels of mRNAs, and ultimately, proteins.

363 Control of mRNA Decay by Puf Proteins Regulates Ribosome Biogenesis

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The Puf Family of eukaryotic RNA-binding proteins plays roles in stem cell maintenance, cell development, and differentiation by binding conserved elements within the 3'UTR of target mRNAs, typically resulting in mRNA degradation and/or translational repression. Pufs are characterized by repeat domains comprising eight tandem repeats of ~36 residues; each repeat contacts one base within an 8-12 nt target sequence containing a conserved UGU, called the Puf response element (PRE). Once bound, Puf proteins elicit mRNA repression through inhibition of cap-binding events or recruitment of mRNA decay factors. Saccharomyces cerevisiae expresses six Puf proteins; hundreds of candidate Puf mRNA targets have been predicted from physical association/bioinformatic screens. We focus on understanding mechanistic roles of Pufs in regulating target mRNAs. One of our studies analyzed several predicted Puf4p targets containing a consensus binding sequence for Puf4p; all are involved in various steps of ribosome biogenesis. Analysis of target mRNA steady-state levels and half-lives in wild-type versus *puf4Δ* strains revealed small differences between the two, prompting examination of these targets in other PUF deletion strains. Only in a *puf4*, *puf5* double deletion strain, not in single deletion strains, are target mRNAs stabilized, suggesting redundancy of these two Puf proteins to stimulate decay of targets. Surprisingly, deletion of PUFs 1-5 destabilizes target mRNAs, resembling wild-type decay. Mutation of the consensus UGUA to ACAC within the PRE stabilizes target mRNAs as seen in the *puf4*, *puf5* double deletion, even when PUFs 1-5 are deleted, suggesting whatever mechanism is destabilizing the mRNAs in the absence of PUFs 1-5 utilizes the PRE. As multiple mRNA targets of Puf4p and Puf5p regulation are involved in ribosome biogenesis, we determined the role of Pufs in globally regulating ribosomes. Over-expression of Puf4p causes delays in 35S precursor rRNA processing, as well as nuclear accumulation of pre-ribosomal subunits. We hypothesize this is due to increased repression of mRNA targets whose protein products process 35S precursor rRNA and export pre-ribosomal subunits from the nucleus. Current work is focused on understanding how Puf proteins interact with other 3' UTR binding proteins, and how precise regulation of Puf activity impacts cell-wide protein production.

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364 Regulated endocleavage balances the degradation of nonsense-mediated decay substrates

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Nonsense-mediated mRNA decay (NMD) is a eukaryotic mRNA surveillance mechanism that maintains the fidelity of gene expression by targeting aberrant transcripts. NMD degrades transcripts containing premature termination codons (PTCs) and thereby prevents the synthesis of C-terminally truncated proteins with potentially unphysiological or deleterious functions.

Activation of NMD by aberrant translation termination triggers the assembly of a surveillance complex onto the mRNA, consisting of the proteins UPF1, UPF2, and UPF3. Subsequently, the kinase SMG1 phosphorylates the central NMD factor UPF1 within N- and C-terminal extensions, which function as binding sites for SMG6 and the SMG5-SMG7 heterodimer. The degradation of NMD targets occurs via endonucleolytic cleavage (SMG6) or deadenylation and decapping (SMG5/7). However, the contribution of SMG5/7 and SMG6 to the degradation of individual substrates and its regulation by UPF1 remains unresolved.

Here, we dissect the regions of key NMD factors required for endocleavage and find that two NMD reporter mRNAs exhibit striking differences. Our results reveal transcript-specific modes of SMG6 activation by UPF1 and identify a minimal C-terminal fragment of SMG6 that exhibits full endocleavage activity. Furthermore we map the sites of endocleavage and the NMD eliciting features of endogenous NMD substrates via 3′ fragment capture and degradome sequencing. Using endogenous and reporter NMD targets, we observe that SMG7 regulates the endocleavage efficiency of NMD substrates with uORFs and long 3′UTRs.

Our results suggest that the mRNA architecture determines the extent to which SMG6 is regulated by SMG7, initiating either a degradative or regulatory route of NMD. While NMD has been originally considered as a mechanism to degrade faulty mRNAs, our results highlight its emerging function as a master regulator of gene expression.

365 Zinc finger RNA binding protein (ZFR) protects mRNAs from degradation

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Interactions between mRNAs and trans-acting regulatory factors influence every step of gene expression, from transcription to decay. In particular, we are interested in understanding how differential rates of mRNA turnover are used to determine cellular responses to changing physiological conditions. As one part of this effort, we are using a combination of biochemical and functional assays to identify proteins that modulate mRNA degradation to maintain appropriate cellular levels of specific mRNAs.

Here, we developed a purification scheme to isolate proteins assembled on reporter RNAs with distinct structural features and translational efficiencies. In this way, we identified the evolutionarily conserved Zinc finger RNA binding protein (ZFR) to be specifically purified with an mRNA that is poorly translated due to a highly structured 5'UTR element. To identify cellular mRNAs regulated by the ZFR protein, we first performed transcriptome-wide RNAseq studies in cells depleted of ZFR or control cells, observing that ZFR regulates the abundance of a wide diversity of mRNAs. Further biochemical assays show that ZFR is directly associated with a subset of regulated mRNAs and that these mRNAs become highly unstable in the absence of ZFR. We are actively pursuing both biochemical and bioinformatic approaches to elucidate the molecular mechanisms of ZFR-mediated mRNA stabilization.

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366 YbeY and RNase R in Ribosome Degradation

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In *Escherichia coli,* ribosome degradation occurs both during stress and under normal growth conditions. The exact mechanisms of ribosome degradation and the RNase(s) initiating rRNA degradation remain unknown. We are interested in the roles of ribonucleases RNase R and YbeY in ribosome degradation. Together, these two enzymes have been associated with 70S ribosome quality control. We present the effects of protein synthesis inhibitors chloramphenicol and erythromycin on wild type, YbeY and RNase R deletion strains. In the presence of chloramphenicol and erythromycin ribosome degradation increases in RNase R and wild type strains. Also, the accumulation of abnormal ribosomal particles is detected in RNase R and wild type strains. In contrast, antibiotic treatment of the YbeY strain did not lead to accumulation of abnormal ribosomal particles. We measured rRNA degradation directly to find that mutant rRNAs that are degraded in wild type strains are stabilized in both YbeY and RNase R strains. These results suggest an important role for YbeY in ribosome degradation, possibly in the early steps of it.

367 RNA 5′-kinase-mediated eukaryotic mRNA decay

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Intrinsic and endoribonuclease-catalyzed RNA cleavage produces RNA fragments with 5′-hydroxyl (5′-OH) and 2′,3′-cyclic phosphate termini. Modification of 5′-OH termini to 5′-PO_4 by 5′-kinase activities promotes their repair by ligation or decay by 5′->3′ exoribonucleases. We developed a new method to identify 5′-OH RNAs that couples the unique specificity of *E. coli* RtcB RNA ligase with massively parallel DNA sequencing. Application of the method to budding yeast identified numerous 5′-OH fragments in cellular RNAs. Many 5′-OH fragments derived from mRNA accumulate upstream of codons for polyelectrostatic peptides, suggesting that they are created during translation. The 5′-kinase activity of multifunctional tRNA ligase Trl1 is required for exon ligation during tRNA splicing and also promotes rapid 5′->3′ decay of spliced tRNA introns by Xrn1 (Wu and Hopper 2014). We analyzed the accumulation of 5′-OH RNAs in *trl1*Δ cells that express either *E. coli* RtcB RNA ligase (Tanaka and Shuman 2011), or a construct encoding 10 "intron-less" tRNAs (adapted from Kosmaczewski *et al.* 2014).

We also studied two specific examples of 5′-kinase-mediated mRNA turnover. In the unfolded protein response (UPR), the Ire1 kinase/endoribonuclease responds to ER stress by cleaving *HAC1* pre-mRNA, creating exon products with 5′-OH termini (Gonzalez *et al*. 1999). Modified of the 5′-OH on cleaved *HAC1* 3′-exon by Trl1 promotes its ligation and subsequent translation (Sidrauski *et al*. 1996). We found that cells that lack Xrn1 or 5′-kinase activity exhibit constitutive UPR activation, indicating that Xrn1 5′->3′ decay is required to prevent *HAC1* ligation and unintended UPR activation. We also show that the endonucleolytic products of no-go mRNA decay (NGD) have 5′-OH termini, which are subsequently phosphorylated by Trl1 5′-kinase and decayed 5′->3′ by Xrn1 (Doma and Parker 2006). Insofar as the source of endonucleolytic activity during NGD is unknown, the 5′-OH terminus of NGD cleavage products suggests recruitment of a metal-independent endoribonuclease during NGD, or intrinsic RNA cleavage caused by ribosome stalling.

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368 Autoregulation of the *RTR1* mRNA by Rtr1p involves a novel mRNA degradation mechanism

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Rtr1, (Regulator of transcription 1), has previously been identified as a phosphatase that dephosphorylates Ser5 and Tyr1 of the RNA polymerase II CTD tail, thus establishing a role for this protein in regulating transcription elongation and termination. Here, we show that Rtr1 autoregulates its own mRNA post-transcriptionally. Previously, an RNA binding protein (RBP) binding site has been identified in the *RTR1* 3'UTR by the gPAR-CLIP approach, in which ubiquitous RBP sites were identified genome-wide. We utilized a construct with a GFP ORF and the 3'UTR and terminator of *RTR1* (*GFP-3'UTR/*T_{RTR1}) to determine the effect of the deletion of this RBP binding site in various backgrounds. The *GFP-3'UTR/*T_{RTR1} mRNA displayed increased abundance and stability when the genomic *RTR1* gene was deleted. However, deletion of the RBP binding site in the *GFP-3'UTR/*T_{RTR1} mRNA resulted in increased abundance in a wild-type background, but not the *rtr1Δ* strain. RNA immunopreciptation (RIP) with a 3X-FLAG tagged Rtr1 showed that Rtr1 associates (directly or indirectly) with the wildtype *RTR1* 3'UTR-containing mRNA but not the *RTR1* 3'UTR-containing mRNA with the RBP site deletion.

To further characterize the degradation of *RTR1,* we analyzed downstream mRNA factors that may be responsible for the degradation of *RTR1* upon Rtr1p binding. Intriguingly, our results suggest that *RTR1* is degraded downstream by the Rex2 and Rex3 3'-5' exonucleases. Deletion of the Rtr1 binding site in the *RTR1* 3'UTR did not show any synergistic increase in mRNA abundance in a *rex2Δrex3Δ* strain. Further, *RTR1* transcripts showed increased stability in the *rex2Δrex3Δ* background based on transcription shut off assays. Rex proteins are RNaseD type exonucleases which are known to be involved in the 3'-end processing of ncRNAs like snRNAs, the 5S and 5.8S rRNAs, and the RNA component of RNase MRP, but not in the degradation pathway of mRNAs. Thus, we hypothesize that the autoregulation of *RTR1* by its own protein product results in the targeting of the mRNA to a previously unidentified mRNA degradation pathway.

369 A novel isoform of HBS1L provides a link between the cytoplasmic exosome and a SKI complexes in human cells.

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Regulation of gene expression relies heavily on RNA metabolism. Many proteins, including nucleases, are involved in RNA decay and quality control in eukaryotes. A multi-subunit exosome complex is one of the major nucleases present in the cytoplasm in human cells. The exosome function in this compartment depends strictly on cooperation with the SKI protein complex, which has RNA helicase activity. Biochemical experiments in yeast proved that the SKI heterotetramer is responsible for delivering RNA substrates directly into the exosome channel and that the exosome/SKI complex cooperation requires the presence of the Ski7 protein, which is a factor linking both complexes. Mechanisms of cooperation between the SKI and exosome complexes in human cells remain unknown, since the human genome does not encode an ortholog of the yeast Ski7 protein.

An analysis of cDNA clones suggested that the *HBS1L* gene expression can give rise to several mRNAs coding for different putative protein variants, however none of the alternative isoforms has been tested so far.

We have confirmed that a short protein variant, which is called HBS1LV3 herein, does indeed exist within human cells. We have conducted a co-IP analysis which revealed that the unexplored HBS1LV3 protein specifically interacts with the cytoplasmic exosome complex, since in addition to the exosome core we identified exclusively cytoplasmic DIS3L exoribonuclease (but not its nuclear paralogue DIS3 protein, or the nucleolar RRP6). Moreover, all three cytoplasmic SKI complex components copurified with HBS1LV3. In order to characterize functions of the HBS1LV3 in RNA degradation we have conducted RNA-seq experiments of samples isolated from HEK293 cells in which expression of the *HBS1L* gene has been silenced by introducing siRNAs. The data presented herein show that knocking down *HBS1LV3* expression results in global increase in mRNA levels.

Overall, our data indicate that the human HBS1LV3 protein acts as a functional homologue of the yeast Ski7 protein and enables proper cytoplasmic mRNA decay, being a factor that mediates the interaction between the SKI and exosome complexes in human cells.

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370 Regulation of mRNAs Involved in Copper Homeostasis by the Nonsense-mediated mRNA Decay Pathway

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The nonsense-mediated mRNA decay (NMD) pathway is an mRNA degradation pathway that degrades mRNAs that prematurely terminate translation. These mRNAs include mRNAs with premature termination codons as well as many natural mRNAs. In *Saccharomyces cerevisiae*, a number of features have been shown to target natural mRNAs to NMD. However, the extent to which natural mRNAs from the same functional group are regulated by NMD is not widely known. We examined mRNAs involved in copper homeostasis and predicted to be regulated by NMD by genome-wide studies. A subset of these mRNAs was found to have a long 3'-UTR that could target them for degradation by NMD. In the conditions tested we found that three of these mRNAs are direct NMD targets. Additionally, the long 3'-UTR of one of the mRNAs was found to contribute to the regulation of the mRNA by the pathway. We also found that the transcript that codes for the copper sensing transcription factor, *MAC1* was regulated by NMD based on the growth conditions.

371 A systematic analysis of the *Hmga2* 3′UTR reveals a high density of regulatory sequence elements but very few regulatory interactions between elements

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Post-transcriptional gene regulation is often controlled through *trans*-acting regulatory factors (including microRNAs and RNA binding proteins) that bind to sequence elements within the 3′ untranslated regions (3′UTRs) of mRNAs. To better understand the role 3′UTRs play in gene regulation, we performed a detailed analysis of a single mammalian 3′UTR. Our principal goals were to identify the complete set of regulatory elements within the 3′UTR and to determine the extent to which elements interact with and affect one another. We chose the long and well-conserved 3′UTR of *Hmga2*, which is an oncogene whose overexpression in cancers often arises from mutations that remove 3′UTR regulatory sequences. We used reporter assays in cultured cells to generate complete maps of the regulatory information across the *Hmga2* 3′UTR at different resolutions, ranging from 50- to 400-nucleotides. We found many previously unidentified regulatory sites, most of which were up-regulating. Also, their location and impact was conserved between different species (mouse, human and chicken) and robust to different cellular environments. By systematically comparing the regulatory impact of 3′UTR segments of different sizes we were able to determine that the majority of regulatory sequences function independently, with only a very small number of segments showing any evidence of interactions. These findings were also true for another 3′UTR we examined, that of *PIM1*, indicating these findings may be generalizable. We therefore believe that our complete characterization of one 3′UTR can help us better understand the principles of 3′UTR mediated gene regulation.

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372 LARP4B is an mRNA stability factor that acts via AU-rich sequence elements

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mRNAs are key molecules in gene expression and subject to diverse regulatory events. Regulation is accomplished by distinct sets of trans-acting factors that interact with mRNAs and form defined mRNA-protein complexes (mRNPs). The resulting "mRNP code" determines the fate of any given mRNA and thus controlling gene expression at the post-transcriptional level. The La-related protein 4B (LARP4B) belongs to an evolutionarily conserved family of RNA binding proteins characterized by the presence of a La-module implicated in direct RNA binding. Biochemical experiments have shown previously direct interactions of LARP4B with factors of the translation machinery. This finding along with the observation of an association with actively translating ribosomes suggested that LARP4B is a factor contributing to the mRNP code. To gain insight into the function of LARP4B in vivo we tested its mRNA association at the transcriptome level and its impact on the proteome. PAR-CLIP analyses allowed us to identify the in vivo RNA targets of LARP4B. We show that LARP4B binds to a distinct set of cellular mRNAs by contacting their 3´UTRs. Biocomputational analysis combined with in vitro binding assays identified the LARP4B binding motif on mRNA targets. The reduction of cellular LARP4B levels leads to a marked destabilization of its mRNA targets and consequently their reduced translation. Our data identify LARP4B as a component of the mRNP code that influences the expression of its mRNA targets by affecting their stability.

373 Interaction between the RNA-dependent ATPase and poly(A) polymerase subunits of the TRAMP complex is mediated by short peptides and important for snoRNA processing

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The RNA exosome is one of the main 3' to 5' exoribonucleases in eukaryotic cells. Although it is responsible for degradation or processing of a wide variety of substrate RNAs, it is very specific and distinguishes between substrate and non-substrate RNAs as well as between substrates that need to be 3' processed and those that need to be completely degraded. This specificity does not appear to be determined by the exosome itself but rather by about a dozen other proteins. Four of these exosome co-factors have enzymatic activity, namely, the nuclear RNA-dependent ATPase Mtr4, its cytoplasmic paralog Ski2 and the nuclear non-canonical poly(A) polymerases, Trf4 and Trf5. Mtr4 and either Trf4 or Trf5 assemble into a TRAMP complex. However, how these enzymes assemble into a TRAMP complex and the functional consequences of TRAMP complex assembly remain unknown. Here, we identify an important interaction site between Mtr4 and Trf5, and show that disrupting the Mtr4/Trf interaction disrupts specific TRAMP and exosome functions, including snoRNA processing.

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374 The human La-related protein-4 (LARP4) coding sequence contains a translation-sensitive mRNA instability element that limits LARP4 levels; overcoming this uncovers a new mRNA-related activity

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The La module is comprised of a La motif (LAM) followed by a RNA recognition motif (RRM) that work together in the nuclear La protein to bind oligo(U)-3'OH and stabilize its small nascent RNA targets against 3' exonucleolytic decay. Human La-related protein 4 (LARP4) was previously shown to i) be a cytoplasmic mRNA-associated factor, ii) exhibit sequence-specific binding to poly(A), iii) independently associate with poly(A)-binding protein, PABC1 through a PAM2 motif, and iv) increase stability of its target mRNAs (Yang et al MCB 2011). LARP4 shares homology with the related factor, LARP4B. Insertion of only the ORF sequences for La, LARPs 4, 4B, 6 and 7, into a plasmid bearing a minimal promoter-enhancer, FLAG tag and short UTRs, led to a ~50-fold range in accumulation of the LARP mRNAs with similar differences among the FLAG-tagged proteins. Using this system we found that LARP4, but not 4B, is limited to low level accumulation in the transfected cells. The ~50-fold difference in the FLAG-tagged LARP4 and 4B levels is reflected by accumulation levels of the mRNAs , also consistent with differences in their half-lives. We found that LARP4 mRNA contains a coding region determinant (CRD) of instability which when deleted increases expression of the mutated LARP4 mRNA and the corresponding protein fragments. Mutagenesis of the identified CRD nucleotide element by swapping synonymous codons to preserve amino acid identity led to significant increases in the LARP4 mRNA and protein levels. Transferring the LARP4 CRD sequence to a heterologous tet-off reporter increases the mRNA decay rate when it is part of the open reading frame but not when placed in the 3' UTR downstream of the stop codon. The collective data establish the existence and identification of the LARP4 mRNA CRD of mRNA instability. Plans to identify trans-acting factors that control and/or regulate these CRD-mediated effects are underway.

Use of the full-length LARP4 codon swapped expression construct overcomes the CRD-mediated limit on cellular LARP4 protein levels with a consequent manifestation of a new mRNA-related activity. The striking effect of this new reagent on the metabolism of polyadenylated mRNAs will be revealed.

375 Evolution via alternative splicing and gene duplication in *SKI7* and *HBS1*

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In *Saccharomyces cerevisiae*, the paralogs *SKI7* and *HBS1* have similar but distinct functions in mRNA surveillance pathways. Ski7 is involved in normal mRNA degradation and nonstop decay, where it is believed to recognize the stalled ribosome and then recruit the Ski complex and exosome to degrade the nonstop transcript. Hbs1 is a GTPase that binds to Dom34 and together these proteins bind to stalled ribosomes and recycle them in many pathways, including no-go and nonfunctional 18S rRNA decay. The *SKI7* and *HBS1* genes are paralogs that are the result of a whole genome duplication event that occurred in an ancestor of *S. cerevisae*. Our lab has demonstrated that *SKI7* and *HBS1* were subfunctionalized following duplication, meaning that each paralog retained only one function of the ancestral gene. Recently, we have shown that the *SKI7/HBS1* gene of *Lachancea kluyveri*, a model of the ancestral gene, is alternatively spliced to produce two proteins with distinct Ski7 and Hbs1 functions. Alternative splicing of a single *SKI7/HBS1* gene is conserved in many animals and fungi, including humans. It has previously been suggested that Ski7 function arose in *S. cerevisiae*, but our finding that Ski7 function is older than the duplicated *SKI7* gene suggests *SKI7* function is likely to be widespread. Currently, we are using the single *SKI7/HBS1* of *L. kluyveri* to better understand the functional divergence of *SKI7* and *HBS1* and to better understand the specific role of Ski7 in nonstop decay. Specifically, we are examining the effect of disrupting the conserved GTPase domain in both Ski7 and Hbs1 on specific decay pathways.

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376 The mammalian La-related protein 4 (LARP4) mRNA contains a functional AU-rich element and its protein levels are regulated by Tristetraprolin

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La-related protein 4 (LARP4) is a poly(A) binding protein involved in the metabolism and translation of mRNA. To learn more about its function, we generated a LARP4 KO mouse, and are also examining the regulation of its mRNA, the topic of this abstract. The online ARED organism search engine predicts an AU-rich element (ARE) in the 3'-UTR of LARP4 mRNA. AREs are mammalian cis-acting sequences of 50-150 nt within the 3'-UTRs of 5-8% of human mRNAs that mostly encode proteins that need to be tightly regulated, e.g. transcription factors, cytokines and cell-cycle genes.

To assess if the ARE is functional, we adopted a Tet-off system to monitor the stability of a specific mRNA reporter under a dox-repressible promoter in HeLa Tet-Off cells. Inserting the putative LARP4 ARE in the 3'-UTR of the reporter decreased the mRNA half-life from over 6 hours to 100 minutes. Point mutations to the ARE rescued the mRNA stability. Several reported ARE-binding proteins were assessed for a role in LARP4 mRNA metabolism. RNA co-immunoprecipitation and other approaches indicate that one of these, Tristetraprolin (TTP), binds LARP4 mRNA *in vivo* and regulates it expression. Over-expression of wild-type TTP, but not its RNA binding mutant or other proteins tested, decreases LARP4 protein but surprisingly not LARP4 mRNA levels. Consistent with this, mouse LARP4 protein levels are higher in a TTP knock out cell line vs. a wild type control. When these cells are stimulated with TNFα to induce TTP, LARP4 protein levels are down regulated in a TTP-dependent manner. Our hypothesis is that TTP decreases the translational efficiency of LARP4 mRNA. Preliminary analysis of polysome profiles is consistent with this.

In conclusion, we are the first to show that the expression of a La-related protein is regulated by TTP. And, more strikingly, TTP-mediated down regulation of the protein level occurs without apparent effects on mRNA decay. To our knowledge, this is the first example of regulation by TTP exclusively at the protein level and not by mRNA decay and is therefore is a good model for studying TTP-dependent translational regulation.

377 Not1 regulates the DEAD-box ATPase Dhh1 to control Processing Body formation and cytoplasmic mRNA fate

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Rapid modulation of gene expression is critical for cells to respond to environmental challenges and initiation developmental programs. Cells employ a large number of mechanisms to achieve tight regulation of gene expression, including post-transcriptional control of active messenger RNA (mRNA) levels by inhibition of translation or by mRNA degradation. While mRNA production via transcription has been extensively characterized, our understanding of how mRNAs are partitioned between an actively translating state and an inactive state remains limited. We show that the evolutionarily conserved DEAD-box ATPase, Dhh1, is an important regulator of cytoplasmic mRNA fate by controlling mRNA localization to Processing Bodies (PBs) - cellular structures involved in storage or degradation of mRNAs. Purification of TAP-tagged wild-type and mutant Dhh1 messenger ribonucleoprotein (mRNP) complexes followed by analysis of their interactomes by mass spectrometry identified that a mutant of Dhh1 that cannot bind to ATP is unable to interact with the Ccr4-NOT deadenylase complex - an intracellular machine involved in transcriptional regulation and mRNA turnover. Furthermore, disrupting conserved residues of Dhh1 that are critical for binding to Not1 causes constitutive formation of PBs - similar to an ATPase-dead allele of Dhh1. GFP-tagged Not1-binding mutants of Dhh1 also no longer dynamically shuttle to and from PB foci, as demonstrated by Fluorescence Recovery After Photobleaching (FRAP), much like ATPase-dead Dhh1. In addition, disruption of RNA binding in these Dhh1 mutants blocks constitutive PB formation, suggesting RNA-binding is critical for the entry of Dhh1 into PBs, while ATPase activity is critical for release. These results suggest the ATPase activity of Dhh1 is regulated in vivo, and that this regulation may ultimately determine the fate of cytoplasmic mRNA - whether it is actively translated in the cytoplasm, or delivered to Processing Bodies for degradation or storage.

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378 The RNA helicase Skiv2l2 works to maintain proliferation in mammalian cell lines

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Transcriptional errors and RNA processing result in aberrant RNAs that must be degraded to prevent their detrimental accumulation in the cell. In mammals, the helicase Skiv2l2 unwinds targeted RNAs to facilitate entry into the nuclear exosome, resulting in the degradation of both nucleolar RNAs via the TRAMP complex and nuclear RNAs via the NEXT complex. While studies have discovered RNA surveillance targets, Skiv2l2's influence on cellular processes like differentiation remains unclear. In development, stem cells must maintain a pluripotent and proliferative state before differentiating into a specific cell type. Both the maintenance of stem cells and the initiation of differentiation require the regulation of certain RNAs in the cell. By controlling which RNAs are degraded, Skiv2l2 determines which RNAs persist in the cell, including long non-coding RNAs and microRNAs that regulate gene expression during development. When differentiation is induced in murine neuro2A (N2A) and P19 embryonic carcinoma cells, Skiv2l2 is downregulated, suggesting that Skiv2l2 may be involved in maintaining either the pluripotent or proliferative nature of these cancer cells. RNAi knockdown of Skiv2l2 results in enhanced expression of neuronal cell markers in N2A cells and cardiac muscle markers in P19 cells as measured via qRT-PCR. Since siRNA knockdown of Skiv2l2 enhanced differentiation in two cell types, it was concluded that Skiv2l2 works to maintain cells in a multipotent, proliferative state. Furthermore, MTT assays revealed that knockdown of Skiv2l2 resulted in a 30% decrease in cell proliferation. Propidium iodide staining coupled with fluorescent activated cell sorting demonstrated that loss of Skiv2l2 caused an increase in the proportion of cells in G2/M phase. This result implicates Skiv2l2, a protein involved in RNA surveillance, in enhancing or regulating cell cycle progression. To further understand how Skiv2l2 mediates this effect, siRNA knockdown of PAPD5, a TRAMP complex member, was employed. Because loss of PAPD5 also enhanced differentiation, it is hypothesized that the mammalian TRAMP complex tightly regulates some RNA target to promote cell cycle progression and prevent cell differentiation. Understanding how Skiv2l2 and RNA surveillance mechanisms impact proliferative cells may have implications on developmental biology and the study of diseases associated with cell proliferation.

379 Glucocorticoid receptor-bound mRNA is rapidly degraded in a way that depends on a ligand, UPF1, and PNRC2

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Glucocorticoid receptor (GR), which was originally known to function as a nuclear hormone receptor, plays a role in rapid mRNA degradation as an RNA-binding protein. The mechanism of this process occurs remains unknown. Here we demonstrate that GR, preloaded onto the 5′ untranslated region of a target mRNA, recruits UPF1 through PNRC2 in a ligand-dependent manner, in order to induce rapid mRNA degradation. We call this process GR-mediated mRNA decay (GMD). Although GMD, nonsense-mediated mRNA decay (NMD), and staufen-mediated mRNA decay (SMD) share UPF1 and PNRC2, we find that GMD is mechanistically distinct from NMD and SMD. We also identify cellular GMD substrates using microarray analysis. Intriguingly, GMD plays role in the chemotaxis of human monocytes by targeting chemokine (C-C motif) ligand 2 (*CCL2*) mRNA. Thus, our data show molecular evidence of a posttranscriptional role of the well-characterized nuclear hormone receptor, GR, which is traditionally considered a transcription factor.

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380 Mechanistic Requirements for Targeting and Degradation of mRNA by the Nonsense-Mediated mRNA Decay Pathway

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Nonsense-mediated mRNA decay (NMD) is a conserved post-transcriptional quality control pathway responsible for the detection and destruction of transcripts which contain nonsense codons resulting in premature translation termination. The superfamily I RNA helicase Upf1 is a central component of the NMD machinery. Recent transcriptome-wide studies have shown that Upf1 associates with both nonsense-containing and normal mRNAs, suggesting that the mechanistic distinction between normal and aberrant mRNAs occurs after Upf1 associates with the transcript. To investigate the requirements for NMD at steps downstream of Upf1 association with mRNA, we applied tethered function analysis using *Saccharomyces cerevisiae* as a model. Tethering Upf1 to the 3' UTR of a reporter mRNA was sufficient to induce a >2-fold destabilization of the mRNA via a mechanism that is consistent with NMD. Destabilizing activity in the tethered system was lost when using mutant alleles of Upf1 deficient in either ATP binding or ATP hydrolysis activity. Surprisingly, mRNA destabilization was observed in the absence of required NMD factors, Upf2 and Upf3, as well as upon inhibition of translation. These results demonstrate that distinctions can be made between components of the NMD pathway which are required after Upf1 association with mRNA, and those which are required for upstream events. These new insights provide valuable information into the mechanistic details of how nonsense-containing mRNAs are distinguished from normal mRNAs.

381 Autophagy is regulated by the 5'→3' mRNA decay pathway in Saccharomyces cerevisiae

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Autophagy is a process that degrades cellular components to make nutrients bioavailable during periods of starvation or stress. To do this, cytosolic contents are sequestered into double membrane vesicles called autophagosomes that fuse with the mammalian lysosome/yeast vacuole. Those components are subsequently degraded and recycled to support continued cellular functions. We observed a subset of genes that increase ≥4-fold in abundance in a RNA-seq analysis comparing cells in nutrient rich vs. nitrogen starvation. Gene ontology analysis revealed that a number of these genes are essential for the formation of the pre-autophagosomal structure during induction of autophagy. ATG8 was the most highly upregulated autophagy gene identified in our RNA-seq experiment. Atg8p is essential for incorporating lipids into the forming autophagosome during nitrogen starvation. It shows the largest increase in mRNA and protein abundance during the induction of autophagy and is commonly measured as an indicator of the magnitude of autophagy induction. Previous studies reported that the ATG8 mRNA undergoes an 8 to 10-fold increase in abundance immediately following the onset of nitrogen starvation. However we observed only a 2-fold increase in ATG8 transcription under these conditions. Since steady-state mRNA abundance reflects a balance between the rates of mRNA synthesis and decay, we examined the effect of mRNA decay mutations on ATG8 mRNA abundance. We found that the half-life of ATG8 mRNA increases significantly following the onset of nitrogen starvation. This led us to hypothesize that ATG8 mRNA is normally destabilized during nutrient-rich conditions. We did not observe any increase in ATG8 mRNA abundance when the 3′→5′ decay pathway was disrupted with a ski7Δ mutant. However, both the steady-state abundance and half-life of ATG8 mRNA increased in a dcp2Δ strain during nutrient-rich conditions. In addition, we also observed an increase in autophagic flux during nutrient-rich conditions in the dcp2Δ strain. These results suggest that ATG8 mRNA may be preferentially degraded by the 5′→3′ mRNA decay pathway under these conditions to maintain appropriate basal levels of autophagy. We are currently testing whether the abundance of other mRNAs encoding pre-autophagosomal components identified in our RNA-seq experiment are also regulated by mRNA stability.

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382 Characterization of G-quadruplexes in the 3'-UTR of PITX1 messenger RNA

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Nucleic acids rich in guanine are able to fold into unique structures known as G-quadruplexes that are found in both RNA and DNA. They consist of four tracts of guanine bases arranged in parallel or anti-parallel strands and aligned in stacked G-tetrad planes. The tetrads are further stabilized by Hoogsteen hydrogen bonds and monovalent cations such as Na^+ or K^+ (1). G-quadruplexes play prominent roles in both transcriptional and translational regulation (2). RHAU (RNA helicase associated with AU-rich element) is a member of the ATP-dependent DExH/D family of RNA helicases that bind and resolve cellular G-quadruplexes. It contains a core helicase domain with an N-terminal extension required for quadruplex recognition and binding (3). PITX1, a member of the bicoid class of homeobox proteins is a transcription factor that plays regulatory roles with proteins such as p53 (4). RHAU interacts with PITX1 mRNA and we have identified three G-quadruplex forming regions in the 3'-UTR of PITX1 mRNA, ($PITX1_{1371\text{-}1400}$, $PITX1_{1901\text{-}1930}$, and $PITX1_{2044\text{-}2079}$). Efforts have been made to *in vitro* transcribe these RNAs for structural studies and we employed biophysical techniques including electromobility shift assays, UV-VIS spectroscopy, circular dichroism, dynamic light scattering and small angle X-ray scattering (SAXS) to characterize these RNA quadruplexes. Preliminary results show that these sequences formed stable RNA structures that interact with $RHAU_{53\text{-}105}$, a region within the N-terminal extension of RHAU.

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383 RNA/protein interactions important for structural rearrangements of large RNA

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The Saccharomyces Ty1 element belongs to a widely disseminated group of retrotransposons with pronounced structural and functional similarities to retroviruses, except Ty1 transposition is not infectious. Ty1 contains GAG and POL genes bracketed by long-terminal repeats (LTRs). The RNA genome serves as templates for reverse transcription and protein synthesis. A universal feature of retrotransposon and retrovirus propagation is a series of RNA structure dependent nucleoprotein interactions mediated by Gag or its mature products that facilitate formation and encapsidation of dimeric RNA and are critical for tRNA annealing, cyclization, strand-transfer during reverse transcription, retroviral recombination, and cDNA integration. A truncated form of Gag (p22) or a form processed by PR (p18) inhibits Ty1 transposition and mediates copy number control (CNC) by poisoning the assembly of functional VLPs (1). An internally initiated Ty1 (Ty1i) transcript encodes p22, however, two closely spaced AUG codons AUG1 and AUG2 may be utilized to initiate p22 synthesis.

Knowing the importance of secondary and tertiary structure in RNA/protein interaction we performed comprehensive analysis of the Ty1 RNA and Gag derivatives. To identify the binding sites and possible structural rearrangement of Ty1 RNA upon protein binding we performed chemical probing of ribonucleoprotein complexes (SHAPE, DMS, hydroxyl radical probing). Obtained data were used as a structural constrains for automated tertiary structure predictions with RNAcomposer (http://rnacomposer.cs.put.poznan.pl/, http://rnacomposer.ibch.poznan.pl/) (2). We analyzed also the specificity of interactions between Gag derivatives and Ty1 RNA. Using model in vitro system we characterized the translational activity of AUG1 and AUG2 and described possible modes of p22/p18 translation initiation from Ty1i transcript. Moreover, the RNA chaperone activity of the Gag derivatives was explored.

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384 Insights into the mechanism of a G-quadruplex-resolving DEAD/H-box helicase

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The unwinding of nucleic acid secondary structures within cells is crucial to maintain genomic integrity and prevent abortive transcription and translation initiation. DHX36, also known as RHAU or G4R1, is an ATP-dependent helicase highly specific for DNA and RNA G-quadruplexes (G4s). A fundamental mechanistic understanding of the interaction between helicases and their G4 substrates is of utmost importance to elucidate G4 biology and pave the way toward G4-targeted therapies. Here we analyze how the thermodynamic stability of G4 substrates affects binding and unwinding by DHX36. We modulated the stability of the G4 substrates by varying the sequence and the number of G-tetrads and by using small, G4-stabilizing molecules. We found an inverse correlation between the thermodynamic stability of the G4 substrates and rates of unwinding by DHX36. In stark contrast, the ATPase activity of the helicase was largely independent of substrate stability pointing toward a decoupling mechanism akin to what has been observed for many double-stranded DEAD/H-box RNA helicases. Our study provides the first evidence that DHX36 uses a local, non-processive mechanism to unwind G4 substrates, similar to that of eukaryotic initiation factor 4A (eIF4A).

385 Investigating the Utility of the SNAP-tag in a CRAC Protocol

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Protein-RNA interactions are ubiquitous in RNA processing. Recently, next generation sequencing methods have been used to study these interactions by cross-linking and analyses of cDNAs (CRAC). This process consists of *in vivo* UV crosslinking of the RNA bound to a protein of interest which has been fused to a protein purification tag. The cross-linked protein-RNA complex is purified extensively and the bound RNA is reverse transcribed to generate a cDNA library for deep sequencing. The resulting sequencing data reveals the RNA binding sites of the protein of interest and the relative occupancy of the protein of interest at each site.

Conventional CRAC procedures are extremely time-consuming, requiring multiple purifications steps and taking up to 8 days to complete from start to finish. We are investigating a means to simplify this procedure by employing the SNAP-tag® as the protein purification tag. The covalent nature of this purification tag may eliminate the need for time-consuming purification steps and its unique reactivity will allow for multiplexing with other CRAC purification tags. We are testing the utility of this purification tag in the context of a CRAC experiment by analyzing the RNA binding of the branchpoint binding protein (BBP) in *S. cerevisiae*, a protein whose binding to the branchpoint sequence of pre-mRNA is an integral step at the start of the splicing cycle.

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386 Synergic interplay of the La motif, RRM1 and the interdomain linker of LARP6 in the recognition of collagen mRNA expands the RNA binding repertoire of the La module

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LARP6 is a versatile protein implicated in muscle differentiation and development in vertebrates. In humans, where LARP6 is encoded by a single gene, it acts as a modulator of collagen a1(I), a2(I) and a1(III) synthesis, a role mediated by a specific interaction between LARP6 and a stem-loop structure in the 5´-UTR of these mRNAs[1]. This interaction appears to reside in the N-terminal region of LARP6 containing the 'La-module', the RNA binding unit originally identified in the La autoantigen[3] and conserved in the La-related protein (LARP) superfamily. However, despite the high sequence conservation, unexpected structural diversity and RNA binding adaptability of the La module within the LARPs has been emerging[3,4].

To uncover the molecular basis of the interaction between the La module of human LARP6 and the stem-loop structure in collagen mRNA, the Conte lab has embarked on the study of the structural and RNA binding properties of this protein. Structure-function studies thus far have shown that the LaM and RRM1 domains of human LARP6 exhibit considerable structural variation in comparison to the equivalent domains in La and reveal an unprecedented fold for the RRM1[5]. A mutagenic study guided by the structural work revealed that RNA recognition requires synergy between the LaM and RRM1 as well as the participation of the interdomain linker, probably in realizing tandem domain configurations and dynamics required for substrate selectivity. Our study highlights a considerable complexity and plasticity in the architecture of the La module within LARPs.

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387 Lessons in repair from the RNA world: enzymatic splicing of DNA 3'-phosphate and 5'-hydroxyl breaks by the RNA ligase RtcB

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Many biological scenarios generate "dirty" DNA 3'-phosphate ends that cannot be sealed by classic DNA ligases or extended by DNA polymerases. We show that the *E. coli* RNA ligase RtcB can splice these dirty DNA ends via a unique chemical mechanism, reminiscent of tRNA ligation in metazoa, archaea and bacteria. RtcB transfers GMP from a covalent RtcB-GMP intermediate to a DNA 3'-phosphate to form a 'capped' 3' end structure, DNA3'pp5'G. When a suitable DNA 5'-hydroxyl end is available, RtcB catalyzes attack of the 5'-hydroxyl on DNA3'pp5'G to form a 3'-5' phosphodiester splice junction. Our findings unveil an enzymatic capacity for DNA 3' capping and the sealing of DNA breaks with 3'-phosphate and 5'-hydroxyl termini, with implications for DNA repair and rearrangements. For instance, we show that capping protects DNA 3' ends from resection by *E. coli* exonucleases I and III and from end-healing by T4 polynucleotide 3' phosphatase. Moreover, DNA capping precludes end joining by classic DNA ligases, prevents template-independent nucleotide addition by mammalian terminal transferase, blocks exonucleolytic proofreading by *E. coli* DNA polymerase II and inhibits proofreading by *E. coli* DNA polymerase III, while permitting templated DNA synthesis from the cap guanosine 3'-hydroxyl primer by A, B, C and Y family DNA polymerases. Human DNA polymerase β (X family) extends the cap primer predominantly by a single templated addition step. Cap-primed synthesis by templated polymerases embeds a pyrophosphate-linked ribonucleotide in DNA. We find that the embedded ppG is refractory to surveillance and incision by the RNase H2 catalyzed ribonucleotide excision repair pathway. Aprataxin, an enzyme implicated in repair of A5'pp5'DNA ends formed during abortive ligation by classic ligases, is highly effective as a DNA 3' decapping enzyme, converting DNAppG to DNA 3'-phosphate and GMP. We conclude that the biochemical impact of DNA capping is to prevent resection and healing of a 3'-phosphate end, while permitting DNA synthesis, at the price of embedding a ribonucleotide and a pyrophosphate linkage in the repaired strand.

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388 The Role of AREs, HuR and p38 MAPK in the Post-transcriptional regulation of Interleukin-3

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Interleukin-3 (IL-3) is a pro-inflammatory cytokine secreted by T-cells upon stimulation. Recent results from our group have shown that the IL-3 3'UTR mediates the repression of its expression, being the Adenosine/Uridine-Rich Elements (ARE) region mainly responsible for this regulation. To understand how the AREs from the hIL-3 3'-UTR are involved in controlling translation, we conducted site-directed mutagenesis of four ARE clusters that were identified in the hIL-3 3'-UTR. Firefly luciferase reporters harboring these ARE mutations were transfected into HeLa and Jurkat cells (steady-state and activated). Results demonstrated that when the nonamer ARE is interrupted, the IL-3 3-'UTR is not able to elicit the same response as the wild type 3'-UTR. We also carried out EMSAs using protein extracts from Jurkat cells at 6, 12 and 24hrs after activation and observed that HuR binding to the IL-3 AREs is modulated upon T-cell activation. Since p38 MAPK signaling pathway is an important part of the inflammatory cytokine secretion, regulates ARE-containing mRNAs and it is also known to phosphorylate HuR, we assessed its possible role in the regulation of IL-3 expression. To mimic the immune-activation of Jurkat T-cells, we used phorbol-12-myristate-13-acetate (PMA) and ionomycin. After pre-treatment of Jurkat cells with the p38 inhibitor SB202190, IL-3 mRNA and secreted protein levels were significantly decreased upon T-cell stimulation, as measured by qRT-PCR and ELISA, respectively. We also observed changes in the polysomal distribution and phosphorylation states of HuR on activated Jurkat cells when p38 was inhibited. Furthermore, immunofluorescence analysis showed that p38 inhibition affects HuR subcellular spatial localization upon T cell stimulation. Collectively, these results suggest that p38 MAPK is involved in the post-transcriptional regulation of IL-3 by working, directly or indirectly, with HuR.

389 Analysis of DLC-1 mediated mRNA regulation in *C. elegans*

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Dynein light chain (DLC-1) was originally characterized as a cargo-binding light chain component of the dynein motor complex that is involved in retrograde transport of mRNA and protein particles or organelles. More recent studies have revealed that DLC-1 interacts with a diversity of cellular proteins and likely functions as an allosteric regulator in ribonucleoprotein complexes (mRNPs). We propose a new role for DLC-1 in translational control of developmentally important mRNAs. Specifically, DLC-1 binding to the PUF family protein FBF-2 is required for FBF-2 to localize to germ granules and function as a translational repressor in stem cells. Also, we discovered that knock-down of DLC-1 results in misregulation of *nos-2* mRNA expression at a different time during development. We hypothesize that DLC-1 mediates regulation of its mRNA targets by interacting with regulatory RNA binding proteins. In support of this hypothesis, we've been able to isolate FBF-2 target mRNAs in a complex with DLC-1. We propose that DLC-1 is involved in regulating the expression of additional mRNA targets important for developmental processes. We used affinity purification to isolate DLC-1/mRNP complexes from worms and mRNA sequencing to identify DLC-1 associated mRNAs. Together, these data provide insights into the regulatory role(s) of DLC-1 during germ cell development and identification of other important DLC-1 regulatory targets.

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390 Withdrawn

391 The Modulation of RNA Helicase Activity of RNA Helicase A

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RNA helicases impact RNA structure and metabolism from transcription through translation, in part, through protein interactions with transcription factors. However, there is limited knowledge on the role of transcription factor influence on helicase activity. RNA helicase A (RHA) encoded by DHX9 is a DExH-box RNA helicase that plays multiple roles in cellular biology. Some of RHA's functions require its activity as a helicase while others as a protein scaffold. The oncogenic transcription factor EWS-FLI1 interacts with RHA to enable oncogenesis and growth of Ewing sarcoma (ES), and a small molecule, YK-4-279 disrupts this protein complex in cells. Our current study investigates the effect of EWS-FLI1 on RHA helicase activity. We found that EWS-FLI1 reduces RHA helicase activity in a dose-dependent manner; however, the RHA kinetics indicated a complex model. EWS-FLI1 does not inhibit the intrinsic ATPase activity of RHA. The annealing activity of RHA is enhanced by EWS-FLI1. We also report a novel RNA binding property of EWS-FLI1 that was long considered as a DNA binding protein. Using separated enantiomers, only (S)-YK-4-279 reverses the EWS-FLI1 inhibition of RHA activity without changing RNA binding affinity of the respective proteins. We discover that EWS-FLI1 and RHA bound a common subset of RNA in ES cells shown by RNA immunoprecipitation (RIP). YK-4-279 inhibition of RHA binding to EWS-FLI1 alters the RNA binding profile of both proteins. We show that targeting an oncogenic protein-protein interaction by a small molecule restores wild-type RNA helicase activity. Therefore, we conclude that EWS-FLI1 modulates RNA helicase activity and the profile of bound RNA. This complex modulation of EWS-FLI1 on RHA could affect the overall transcriptome processing in ES. Further studies are needed to clarify the critical balance between the scaffolding function of a helicase and the modulation of its helicase activity, which will shed light on the process of oncogenesis while potentially identifying new therapeutic targets.

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392 Peripheral domains alter the catalytic activity and loading preference of a *S. cerevisiae* DEAD-Box protein.

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DEAD-box proteins are the largest subclass of superfamily 2 helicases and are implicated in most RNA metabolic pathways. For example, the *S. cerevisiae* ribosome biogenesis pathway requires 14 DEAD-box proteins, each with a unique and non-redundant functional role. Classically, these proteins are defined as ATP-dependent unwindases in which protein conformational changes, occurring during the catalytic cycle, facilitate RNA structure modulation. Several recent studies have shown that DEAD-box proteins are not limited to helix dissociation and can modulate RNA-protein as well as protein-protein interactions [1]. DEAD-box proteins achieve specificity by targeting protein cofactors or RNA substrates via their N-terminal and/or C-terminal peripheral domains (NTD or CTD). For example, Rok1p is a *S. cerevisiae* DEAD-box protein essential for rRNA processing. Rrp5, an RNA-binding protein, facilitates the correct association of Rok1p to the rRNA processing pathway [2]. To investigate the role that auxiliary domains play in DEAD-box mediated hydrolysis and RNA structure modulation, the degree of ATP turn-over and duplex displacement with Rok1p domain-deletion-variants were compared to full length Rok1p. Rok1p is a RNA-dependent ATPase with a k_{cat} of 19 ± 1 min^{-1} and $K_{m,ATP}$ of 0.31 ± 0.07 mM. The ATP hydrolysis rate as well as the apparent binding affinity for ATP slightly increases in the absence of the NTD or CTD. The efficiency of rRNA association is greater with the full length protein and the DEAD-box core. Protein mediated duplex dissociation was observed in an ATP-dependent fashion only with minimal substrates containing a single stranded extension. A comparative examination of various bi-substrates suggests that peripheral domains can regulate the intrinsic activity and asymmetric loading preference for the Rok1p DEAD-box protein.

[1] Putnam, A.A. and Jankowsky, E. *Biochim. Biophys.Acta.* 1829(8), 884-893 (2013)
[2] Young, C.L., et al. *Proc. Natl. Acad. Sci. U.S.A*. 110(29), E2668-E2676 (2013)

393 Transcriptome-wide redistribution of U2AF65-RNA binding by hnRNP A1

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Heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) is a global regulator of alternative splicing but its mechanisms of action are only defined at the molecular level for a handful of exons. We used individual-nucleotide resolution crosslinking immunoprecipitation (iCLIP) to determine how over expression of hnRNP A1 affects global patterns of U2 snRNA auxiliary factor 2 (U2AF2) and the serine arginine-rich protein, SRSF1. As expected, U2AF2 and SRSF1 crosslinking sites are enriched near 3' splice sites and at the edges of exons, respectively, whereas hnRNP A1 binding sites are enriched in intronic sequences. However, when hnRNP A1 is overexpressed there are significant changes in the distribution of U2AF2 crosslinking sites relative to the 3' splice sites of cassette exons but not constitutive exons. Likewise, SRSF1 crosslinking patterns relative to splice sites are independent of hnRNP A1 expression levels. Remarkably, in cells over expressing hnRNP A1 we observed a dramatic increase in U2AF2 crosslinking to intronic *Alu* elements. By contrast SRSF1 crosslinking to *Alu* elements is unaffected by hnRNP A1 over expression. Taken together, these data suggest hnRNP A1 can cause redistribution of U2AF2 on a transcriptome-wide level and that *Alu* sequences may be an important sink for these wayward splicing factors.

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394 Fam120a binds homopolymer tracts in mRNA 3'UTRs

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RNA binding proteins play essential roles in cellular physiology. The full contingent of RNA binding proteins, the identity of RNAs bound and the function of these RNA binding proteins are far from complete. While utilizing immunoprecipitation followed by mass spec to determine cytoplasmic Argonaute 2 (Ago2) protein complexes in mouse embryonic stem cells (mESCs), we identified Fam120a as an Ago2 binding protein. Although Fam120a has previously been reported to bind to RNA, in-silico analysis of the Fam120a amino acid sequence fails to recognize any traditional RNA binding domains. We have performed individual-nucleotide resolution Cross-Linking and ImmunoPrecipitation (iCLIP) against Fam120a and identified bound RNA in mESCs. Of the enriched RNA bound to Fam120a, >70% of the iCLIP tags map to 3'UTRs, corresponding to more than 2000 bound genes. MEME analysis of Fam120a bound mRNAs reveals a statistical enrichment for homopolymeric tracts including poly(G), poly(C), poly(U) and poly(A). Strikingly, comparison of Fam120a iCLIP and Ago2 iCLIP reveals that greater than one-third of mRNAs bound by Ago2 in mESCs are also bound by Fam120a (1,807 genes bound by Ago2 of which 681 are also bound by Fam120a). We have utilized the CRISPR/Cas9 system to generate Fam120a knockout mESCs and results of gene expression profiling compared to control cells will be discussed.

395 Molecular recognition of a pre-tRNA substrate by the PPR motifs in *Arabidopsis thaliana* PRORP1

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Ribonuclease P (RNase P) is an endonuclease that catalyzes the essential removal of the 5'-leader sequence on precursor tRNA (pre-tRNA). RNase P exists in all domains of life as a ribozyme with a conserved, fully catalytic RNA component. Eukaryotes also contain a protein-only RNase P (PRORP), which are independently-evolved nucleases unrelated to any component of the RNA-dependent enzyme. In humans PRORP localizes to the mitochondria and requires two other protein components for optimal catalysis. In land plants there are three PRORPs that are responsible for pre-tRNA processing in all sub-cellular compartments and appear to have replaced the ribozyme activity. Unlike the mammalian enzyme, plant PRORPs efficiently catalyze pre-tRNA cleavage as a single protein using a metal-dependent hydrolytic mechanism. Molecular recognition of the pre-tRNA substrate by PRORP remains to be clarified. *A. thaliana* PRORP1 binds *Bacillus subtilis* pre-tRNA^{Asp} at neutral pH and 250 mM salt tightly (K_d = 30 nM); the dissociation constant increases with the salt concentration, suggesting 5 phosphate contacts. PRORP1 contains tandem PPR (pentatricopeptide repeat) RNA-binding motifs that enhance the affinity for pre-tRNA by ≥30-fold‡. To identify specific interactions between pre-tRNA and PRORP1 we analyzed the effects of mutations to amino acids in the PPR domain proposed to interact with pre-tRNA: Y133, N136, Y140, N175, T180, and R184. Substitutions at these sites decrease binding up to 290-fold and, in some cases, also decrease the salt dependence. The single-turnover activity of each mutant could be rescued with the addition of excess enzyme. The sites of PRORP1-pre-tRNA contacts are being further explored using photo-crosslinking.

‡Howard, M. J., Lim, W. H., Fierke, C. A., and Koutmos, M. (2012) *PNAS U.S.A.* **109**, 16149-54.

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396 Leucyl-tRNA synthetase facilitates RNA remodeling

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Aminoacyl-tRNA synthetases (AARSs) are responsible for many essential alternative functions that are completely unrelated to their role in charging tRNAs for protein synthesis. For example, yeast mitochondrial leucyl-tRNA synthetase (LeuRS) facilitates splicing of mitochondrial group I introns (bI4 and aI4α) in an essential activity to express respiratory genes. In collaboration with the bI4 maturase, LeuRS binds directly to the group I intron to facilitate splicing of the ribozyme. Preliminary footprinting data suggested that LeuRS binds to the P4-P6 core region of the bI4 intron that is cognate to LeuRS. RNA duplexes were designed to mimic the P6 helix. We discovered that LeuRS promotes annealing of the RNA duplex mimics. Domain analysis of LeuRS determined that the C-terminal domain was critical to RNA annealing. During aminoacylation, the LeuRS C-terminal domain interacts with the corner of the L-shaped tRNA. We tested competition of tRNAs with the RNA duplex binding site, and found that tRNAs with relatively shorter variable-loops, such as yeast mitochondrial tRNALeu, competitively inhibit the annealing process. This suggested that the annealing and aminoacylation sites for RNA binding overlap. We hypothesize that LeuRS plays a key role in remodeling specific group I intron ribozymes so that they can productively self-splice.

397 Characterization of Maize Necrotic Streak Virus (MNeSV) 3' Cap Independent Translation Elements (3' CITE) Binding Mechanism in Translation Initiation

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5' m^7GpppN cap and the 3' poly adenosine (A) tail at two opposite ends of eukaryotic mRNAs are key elements for recruiting translation initiation machinery. Unlike host mRNAs, many viruses lack those elements and yet they are translated efficiently. The most striking feature is the complex structures within their untranslated regions (UTR) that allow them to bypass some cellular translation control steps. In Maize necrotic streak virus (MNeSV) 3' UTR, an I-Shape Structure CITE (ISS) has been reported to mediate the virus translation initiation progress. We applied biophysical methods to study the binding of 3' ISS with eIFs. With fluorescence quenching and anisotropy techniques, we found that eIF4A-eIF4B complex could increase binding affinity of eIF4F with 3'ISS for four times, from K_d=~208±40 nM to K_d=~52±9 nM. We also found that enhanced binding affinity was not caused by the helicase activity of the eIF4A-eIF4B complex. With stopped-flow technique, we demonstrated that the Arrhenius activation energy for binding of 3'ISS with eIF4F was 63.4±2.7 kJ/mol, which is similar with eIFiso4F-m^7GTP analog binding (63.3±1.5 kJ/mol).

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398 Expression of KSHV chemokine vCCL2 (K4) depends on posttranscriptional regulation by KSHV ORF57

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Kaposi's sarcoma-associated herpes virus (KSHV) ORF57 is a post-transcriptional regulatory factor produced at early viral infection, which plays a crucial role in the viral life cycle and virus production. ORF57promotes viral RNA splicing, stabilizes viral intronless transcripts, and enhances translation of viral RNAs. By screening genome-wide ORF57 RNA targets in KSHV-infected cells with a novel CLIP (cross-linking and immunoprecipitation) approach, we identified viral chemokine, K4, being an ORF57 target, with a 5' untranslational region (UTR) of K4 RNA in association with ORF57 protein. The interaction between K4 mRNA and ORF57 during KSHV infection was verified using anti-ORF57 CLIP and RT-PCR. Cotransfection of HEK293 cells with K4 and ORF57 expression vectors demonstrated that ORF57 promotes K4 expression both at RNA and protein levels, with K4 mRNA containing an intact 5-UTR being critical for this regulation. Moreover, BAC36 cells containing a wild-type KSHV genome express K4 protein well, but the cells containing an ORF57-null genome do not. Host cell miRNAs are also involved in regulation of KSHV K4 expression. We observed the increased level of K4 protein in $PKO^{dicer-/-}$ cells over wt PKO cells. ORF57 significantly boosts the K4 expression in the $PKO^{dicer-/-}$ cells over the wt PKO cells. Data are consistent with our previous finding that ORF57 prevents host miRNAs from their translational suppression by inhibition of RISC function. However, how ORF57 stabilizes its RNA targets from degradation remains unknown. Whether ORF57 affects the cellular RNA decay pathways are currently under active investigation.

399 Characterization of Recruitment, Unwinding and Cofactor-dependent Inhibition of the DEAD-box RNA Helicase Dbp2

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DEAD-box proteins are the largest class of enzyme in the RNA helicase family. All members of DEAD-box proteins contain a highly conserved helicase core that binds non-specifically to the RNA phosphate backbone and are ubiquitously found in all kingdoms of life. Studies have demonstrated that DEAD-box RNA helicases are involved in every aspect of RNA metabolism and many are regulated by protein binding co-factors. Previously, our laboratory demonstrated that the DEAD-box RNA helicase Dbp2 associates with transcribed chromatin in *S. cerevisiae*. This allows Dbp2 to function co-transcriptionally as a RNA chaperone to assist proper messenger ribonucleoprotein (mRNP) assembly. Furthermore, we showed that the mRNA binding protein Yra1 inhibits the unwinding activity of Dbp2. Here, we provide evidence that RNA facilitates the recruitment of Dbp2 to transcribed chromatin. In addition, we show that Dbp2 unwinds RNA duplexes in a cooperative manner, suggesting that Dbp2 functions as an oligomer. Though Yra1 does not affect the cooperativity of Dbp2, Yra1 specifically blocks the association of ATP-bound Dbp2 with single-stranded RNA *in vitro* and reduces accumulation of Dbp2 onto RNA Pol II transcripts *in vivo*. This suggests that Yra1 constrains Dbp2 to act on structured regions of RNA Pol II transcripts. We also demonstrate that the ATP hydrolysis cycle regulates the interaction of Dbp2 with both single-stranded RNA and Yra1. Specifically, the ADP-bound Dbp2 has low affinity with single-stranded RNA and Yra1. This allows the recycling of Dbp2 to act on other substrates. This constitutes a stepwise mechanism for DEAD-box helicase recruitment and regulation that may provide specificity to RNA substrates *in vivo*.

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400 Identification and characterization of Adenine/Uridine Rich Element-Binding Protein(s) in the post-transcriptional regulation of Human Interleukin-3 mRNA

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Human Interleukin-3 (hIL-3) is a cytokine that promotes myelopoiesis, differentiation of macrophages and granulocytes. Aberrant expression of this lymphokine has been associated with several hematological cancers. IL-3 3'-UTR harbors Adenosine/Uridine-Rich Elements (AREs) involved in its post-transcriptional control. These regulatory sequences are recognized by specific ARE-Binding Protein (ARE-BP) complexes. Previous results from our laboratory estimated five ARE-BP complexes from 34 to 88 kDa binding to the hIL-3 ARE using UV-crosslinking assays. Our goal is to identify and characterize the role of novel ARE-BPs that mediate the post-transcriptional regulation of hIL-3. To achieve this goal, RNA affinity purification coupled with MS/MS analysis and immunoblot analysis was performed. In order to characterize these ARE-BP(s) in the post-transcriptional regulation of hIL-3 a knockdown experiment against HuR was carried out. MS/MS results identified ~18 proteins that interact with the hIL-3 ARE. This data, in accordance with previous results from our laboratory, showed that HuR and $p32^{hnRNP\ C1/C2}$ are components of the ARE-BPs that interact with the hIL-3 ARE. Besides, we found that $p45^{AUF-1}$ and CUG-BP1 protein interacts with hIL-3 ARE via immunoblot analysis. HuR knockdown experiments suggest an effect in the IL-3 3'-UTR-mediated post-transcriptional control. Taken together, these data imply that HuR with other ARE-BP complexes can affect the IL-3 post-transcriptional regulation mediated by the ARE motif. Ultimately, elucidating the role of these ARE-BPs in IL-3 expression can provide new insights about ARE-mediated post-transcriptional control, inflammatory/autoimmune diseases and blood cancer.

401 Optimization of Antisense Drugs by Phosphorothioate Stereochemistry Control

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The most notable impact of chemistry on the antisense oligonucleotide field is the implementation of a phosphorothioate backbone. Currently, all phosphorothioate based oligonucleotide drugs under development are diastereomeric mixtures at phosphorus. Mipomersen, the first systemically delivered antisense oligonucleotide approved for treatment of homozygous familial hypercholesterolemia consists of 524,288 individual stereoisomers. Until now, there have been no means of controlling phosphorus chirality during the chemical synthesis of such oligonucleotides for pharmaceutical use. At WaVe Life Sciences, we developed a new chemistry platform for stereo-controlled synthesis of phosphorothioate oligonucleotides, which has enabled us to test these chirally pure oligonucleotides *in vivo*. Comparison of pharmacokinetic and pharmacodynamic behaviour of single stereoisomers vs. mixture will be presented here. We established that phosphorus chirality affects binding affinity to target RNA, lipophilicity, metabolic stability and RNase H1 activity. We propose a rational design based on phosphorus chirality to obtain drugs with improved therapeutic properties when compared to its diastereomeric-mixture counterparts.

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402 Critical Assessment of nucleic acids binding site predictions

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Computational prediction of nucleic acid (RNA/DNA) binding sites in proteins can help understanding protein functions. Various strategies have been proposed in the past decade with the emergence of several automated web servers. However, some criteria in the prediction are not universal and the previous methods have not been compared with large-scale tests. The state-of-the-art approaches show a great diversity in i) the definition of nucleic acid binding sites; ii) the training and test datasets; iii) the prediction strategies (machine learning or template-based search); iv) the assessment methods and v) the distribution and availability of the prediction programs.

Here, we tested 12 nucleic acid binding prediction web servers on 40 different datasets reported before to assess the current prediction achievements and bottlenecks. The four aspects of diversities were compared and analyzed.

According to the results, we find that structure-based predictors, such as aaRNA and RBscore, show better stability in the assessment of prediction relative to different datasets and binding site definition; while sequence-based predictors, e.g. RNABindRPlus and BindN+, perform better on some datasets but with larger changes in accuracies. Tests on single datasets or cross-validation cannot capture the complete view of the predictive ability of a program. Binding sites defined by different distance cutoffs can be used to assess the ability of a program for distinguishing the key binding sites from others. An ensemble of different assessment criteria can provide a better understanding of the prediction.

403 RNA aptamer inhibitors for restriction endonuclease KpnI

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Restriction endonucleases (REase) are enzymes that recognize and cleave short palindromic DNA sequences. At least in part, they are thought to protect bacterial cells against bacteriophage infection by cleaving foreign DNA. BamHI, PacI and KpnI are familiar type II REases that bind and cleave both strands of DNA. We are interested in the problem of RNA mimicry of double-stranded DNA, a concept that might be applied to inhibition of DNA-binding proteins. As a model system, we generated RNA aptamers that bind REases using systematic evolution of ligands by exponential enrichment (SELEX). After 19 rounds of selection (under different stringent binding conditions for each REase) we identified the 10 most enriched RNA aptamers for each REase. Aptamers were then screened by gel shift assay for binding and specificity, and assayed for specific REase inhibition. Interestingly, we obtained eight high-affinity (K_d~ 20-95 nM) selective competitive inhibitors (IC_{50}~ 80-300 nM) of KpnI. Aptamer secondary structures of leading anti-KpnI aptamers were probed by in-line attack assay. The inhibitors presumably are duplex DNA KpnI binding site analogs, but lack the primary consensus KpnI cleavage sequence, making their mode of DNA mimicry a fascinating puzzle. Currently approaches to REase inhibition are irreversible heat denaturation or Mg^{2+} deprivation. Neither approach is selective or practical in vivo. Anti-REase RNA aptamers could have value in studies of REase mechanism, and may give clues to a code for designing RNAs that competitively inhibit DNA binding proteins including transcription factors.

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404 DO-RIP-Seq: A novel technique that quantitates RBP-RNA interactions and distinguishes combinatorial binding transcriptome-wide

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Current techniques to identify global RNA binding sites of RNA-binding proteins rely on crosslinking, which is inefficient, has sequence biases, and does not saturate binding sites. We have developed Digestion Optimized Ribonucleoprotein ImmunoPrecipitation Sequencing or DO-RIP-Seq that can identify nearly all RNA binding sites, and thereby validate non-binding sites containing suspected motifs. Moreover, this approach improves over the use of microarrays because quantifying sequencing reads can be used to approximate relative binding strength. For example, DO-RIP-Seq was used to globally analyze binding of RBPs HuR, RBM38 and Tra2 suggesting these proteins have a subset of overlapping binding sites, and in some cases may cooperatively bind the targeted subset of mRNAs. For example, RBM38 appears to cooperate with Tra2 and with HuR, but Tra2 and HuR did not appear to cooperate with one another. Therefore, DO-RIP-Seq is capable of revealing sites at which RBPs may cooperate or compete for binding on a transcriptome-wide scale, allowing one to derive quantitative metrics of RNA targeting dynamics leading to regulatory insights via potential RNP codes and RNA regulons. This approach provides a gateway to quantitative comparison of RBP binding features within larger RNP complexes during dynamic biological regulation.

405 RNA Mango fluorescent tag for high affinity native RNA-Protein complex purification

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Recent developments in the field of RNA biology continue to demonstrate that RNA participates in a variety of complexes that regulate cellular processes. But the tools available to visualize and purify RNA complexes from cells are limiting. Recently, we developed via *in vitro* selection, an RNA tag called RNA Mango that binds to a modified thiazole orange (TO1-Biotin) fluorophore with 3.4 nM affinity and 1,100 fold fluorescent enhancement. The binding affinity of RNA Mango is unusually high and is in the same range as the RNA motifs that bind to MS2 and PP7 phage coat proteins. Since these RNA motifs are commonly inserted into RNAs to track or purify RNP complexes, we speculated that RNA Mango could be used to purify RNP complexes while simultaneously allowing the tracking of distinct complexes due to its fluorescent properties. Using 6S RNA and its well characterized binding to bacterial RNAP holoenzyme as an example, we demonstrate the purification of 6S:RNAP complex to high purity using RNA Mango.

To simply recover RNP complexes from streptavidin magnetic beads we synthesized TO1-desthiobiotin (T01-Dtb). This allows the elution of RNA Mango by the addition of free biotin. We then inserted the 29-nt long quadruplex structure of RNA Mango into a nonconserved loop sequence of the 6S RNA and expressed the construct in bacteria. This insertion had no detectable effect on either 6S or RNA Mango functionality. Extracts prepared by French press were then incubated with TO1-Dtb prior to binding to streptavidin magnetic beads. After washing, RNP complexes were eluted and applied to a size exclusion column, allowing the simple recovery of pure 6S:RNAP complex. A commonly available fluorescent microtitre plate reader (Spectramax M5) could easily detect as little as 1 pmole of Mango tagged material, suggesting that more sophisticated strategies should be able to detect 10 fmole or less. The Mango purified material can be used either for detailed biochemistry or can used as the input for biophysical characterization via mass spectrometry. RNA Mango therefore provides a versatile tool for the purification of RNP complexes in the future.

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406 Biological functions associated with potential interactions between human proteins and their own transcripts

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The presence of autoregulative control, driven by interactions with own transcripts, has been shown for a number of eukaryotic and, particularly, human proteins. The latter phenomenon exists not only for typical RNA binding proteins, but also concerns some transcription factors and metabolic enzymes, and affects different stages of the messenger RNA (mRNA) life such as splicing, export from the nucleus, nonsense-mediated decay and localization. In order to rationalize cellular functions of proteins possessing the ability to bind their own mRNA, we have performed a general computational screening of potential cognate interactions in the human proteome. Using an extended version of the previously established computational framework [1, 2] for identifying sequence-encoded potential for cognate interactions between proteins and coding regions of their cognate mRNAs, we have evaluated the interaction possibility between human full-length transcripts and their own proteins, particularly, those, which contain a substantial fraction of intrinsically disordered regions. Functional annotation of human proteins among the most specific pairs when it comes to interaction propensity with their cognate mRNAs reveals a surprisingly significant enrichment of nuclear proteins, particularly those from nucleolus and transcription factors. Our results include not only a number of proteins known to bind their own mRNA or RNA in general, but also the large fraction of proteins, whose RNA binding activity has not yet been experimentally observed. The study indicates that protein interactions with their own mRNAs may be much more common than previously thought and may be especially important in situations when the cell needs to precisely control the acting concentration of proteins (e.g. transcription factors) via regulatory feedback loops in expression.

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[2] Polyansky AA, Hlevnjak M, Zagrovic B: **Proteome-wide analysis reveals clues of complementary interactions between mRNAs and their cognate proteins as the physicochemical foundation of the genetic code.** *RNA biology* 2013, **10:**1248-1254.

407 Target selection by natural and redesigned PUF proteins

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PUF proteins are a conserved family of RNA-binding proteins (RBPs) with high sequence specificity and modularity. They have become leading platforms in the design of RBPs with new and desirable specificities. We sought to understand how designed alterations in PUF specificity perturbed the RNAs that were bound *in vivo*, with the aim of discerning on- and off-target specificities *in vivo*. To do so, we analyzed the RNA targets of a non-canonical PUF protein of *S. cerevisiae*, Puf2p, and identify binding sites of both the wild-type and re-engineered proteins *in vivo*.

Puf2p is the founding member of a clade of non-canonical PUF proteins. It binds UAAU, a different RNA sequence than other PUF proteins. It lacks several PUF repeats, and contains an RRM. We use both HITS-CLIP and PAR-CLIP methods to derive a set of 556 high confidence *in vivo* RNA targets. We developed software to identify CLIP peaks that is compatible with small datasets. Binding sites are enriched for multiple UAAU motifs.

To determine how Puf2p achieves its specificity, we performed three-hybrid assays and HITS-CLIP on Puf2p mutants. Analysis of compensatory mutants in the protein and RNA reveal that a single Puf2p monomer binds one UAAU sequence, and align the first PUF repeat with the second U of UAAU. Regions of Puf2p outside the PUF domain affect discrimination between targets; however, its prion domain does not affect RNA binding.

We then identified the targets *in vivo* of a Puf2p re-designed to bind UAAG. The redesigned protein binds UAAG *in vivo*, no longer associates with UAAU, and finds its target site in roughly 1,600 mRNAs. Using data from only the wild-type protein, we developed a model to predict PUF-RNA interactions, and then evaluated the model's performance on the UAAG-binding protein. The most frequent protein-RNA complexes of the Puf2p-like PUFs can be predicted by combining site information with mRNA abundances. We conclude that Puf2p-like PUFs most frequently associate with abundant RNAs containing multiple copies of the binding site, without requiring specific site locations.

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408 The First Step of MicroRNA Biogenesis

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In humans approximately 2,000 miRNAs help regulate an estimated 60% of all genes, but the mechanism for generating miRNA remains unclear. This work is centered on the question: how are pri-miRNA substrates recognized in the RNA-rich nucleus? Previous work has shown RNA structures called ‘junctions’ are important for pri-miRNA recognition and processing. Junctions are regions where single-stranded RNA transitions to double-stranded RNA. Pri-miRNAs contain two characteristic junctions on a pri-miRNA hairpin. We found that the DGCR8 heme binding domain also binds pri-miRNA. This RNA-binding heme domain (Rhed) is the DGCR8 moiety that recognizes junctions. Further, there are two DGCR8 dimers that bind a characteristic basal and apical junction for five different miRNAs. The Rhed is required for pri-miRNA processing in cells, and works with DGCR8s’ double-stranded RNA binding domains to accomplish high-affinity binding with the pri-miRNA substrate.

409 Structural and Functional Studies of a Novel RNA-binding Sm-like Archaeal Protein

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Sm-like Archaeal Proteins (SmAPs) are a relatively unexplored branch of the ubiquitous RNA-associated Sm protein superfamily. Sm proteins in bacteria (Hfq) and eukarya (Sm and Sm-like) have been studied extensively via their roles in RNA processing. Eukaryal Sm and Sm-like proteins are key components of large complexes involved in mRNA splicing, cell death, cell aging, and mRNA degradation. Eukaryal Sm usually act as *scaffolding*, holding the catalytic RNA components in place. Bacterial Hfq is an integral part of post-transcriptional RNA regulatory pathways that are necessary for rapid environmental response. Hfq acts as a *chaperone*, helping regulatory sRNA bind to their complementary mRNA. SmAPs have been confirmed to bind RNA *in vitro* and *in vivo* but their physiological role is still a mystery. While all Sm proteins share a common fold and overall toroidal shape, SmAPs are closer in sequence and form heptamers similar to the eukaryal Sm proteins. SmAPs appear to share many characteristics in common with the bacterial Hfq including forming stable homo-oligomers in contrast to the transient hetero-oligomeric eukaryal Sm proteins. Three putative SmAPs were bioinformatically identified in the hyperthermophillic crenarcheote *Pyrobaculum aerophilum* (*Pae*). The structures of two of them (*Pae* SmAP1 and *Pae* SmAP3) have previously been solved, confirming they are novel Sm proteins. Here we examine the third putative SmAP, *Pae* SmAP2. Biophysical characterization has indicated that *Pae* SmAP2 forms a higher order oligomer than the expected heptamer. The crystal structure has been solved in two different crystal forms, including the rare P23 spacegroup. Functional assays have confirmed *Pae* SmAP2-RNA binding, with a preference for oligo-A and oligo-U strands with separate binding sites.

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410 Computational identification and characterization of RNA binding proteins in *S. Typhimurium*

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Several studies have been carried out using experimental techniques such as cross-linking and immunoprecipitation, which have enabled the characterization of RNA-binding motifs and their regulatory mechanisms in RBPs. Unfortunately, large-scale screening for RBPs by those methods is expensive and time consuming; therefore such studies have been performed for a limited selection of organisms. Recently, computational approaches have been developed for the identification and characterization of protein-RNA interactions, but these methods have not been adapted for proteome-wide identification of RBPs.

Here, we aim to develop an efficient and cost-effective computational method for a systematic proteome-wide screening of RBPs in bacteria. We have focused on identifying functional domains in proteins that may interact with RNA and predict their regulatory roles and mechanisms. For this purpose, we assembled sequence-based approaches for the functional characterization of the proteins in an automated pipeline called APRICOT (Analysing Protein RNA Interactions by Computational Techniques). Using this pipeline, a proteome-wide prediction of RBPs was carried out in *Salmonella* Typhimurium and selected candidates are being tested by sequencing of immunoprecipitated RNA. The experimental results are recursively used to improve the computational RBP identification.

In future, APRICOT will be extended to assess the potential of a protein to interact to other molecular components in a variety of biological system.

411 Evolutionary potential and molecular speciation in RNA-Protein recognition: HIV Rev-RRE

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The binding of human immunodeficiency virus Rev protein via its arginine-rich motif (ARM) to an internal loop in the Rev Response Element region IIB (RRE IIB) is an essential step in the HIV lifecycle. Because of its small size, high specificity, induced fit, and many known variant RNAs and ARMs, Rev-RRE offers an excellent model with which to study the fine structure of how RNA-protein recognition evolves. Previously, randomized-codon libraries of Rev ARM were assayed for their ability to bind RRE IIB using a bacterial reporter system based on bacteriophage lambda N-nut antitermination. By chance and despite the essential role of Rev asparagine 40, the Rev ARM double mutant R35G-N40V was found to be functional. Rev ARM R35G-N40V binds RRE IIB with an affinity similar to wild-type Rev ARM, yet the entropy of binding is different, consistent with the use of distinct recognition strategies. To examine how RRE IIB may evolve specificities to wild-type Rev ARM and to R35G-N40V, ten RRE IIB libraries, each completely randomized in overlapping regions, were screened with wild-type Rev ARM and R35G-N40V. Wild-type Rev and R35G-N40V selections yielded similar, yet distinct, functional RNAs. Consistent with previous studies, a core element of RRE IIB did not vary and substitutions occurred at conserved residues only in the presence of other substitutions. Notably, the groove-widening, non-canonical base pair G48:G71 was mutable to U48:G71 without strong loss of binding to wild-type Rev ARM, suggesting U48:G71 performs the same role by adopting a nearly isosteric, reverse-wobble base pair (Leontis-Westhof classification: G•U trans W.C./W.C.). Originating from RRE IIB, as few as one or two substitutions are sufficient to confer specificity to wild-type Rev or Rev R35G-N40. The results support and aid the interpretation of existing Rev-RRE and future R35G-N40V-RRE IIB structures. The diversity of Rev-RRE mutants with relaxed specificity support neutral theories of evolution and illustrate paths by which viral RNA-protein interactions can evolve.

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412 Single-Molecule Kinetics of the Prototypical DEAD-box RNA Helicase eIF4A upon RNA Unwinding

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The eukaryotic translation initiation factor 4A (eIF4A) is the prototype of DEAD-box RNA helicases. It has a "dumbbell" structure consisting of two domains connected by a flexible linker. We demonstrate that eIF4A, in conjunction with the accessory protein eIF4H, bind to loop structures and repetitively unwind RNA hairpins [1]. To probe the conformational changes of eIF4A in real time using single-molecule fluorescence resonance energy transfer (FRET), we encapsulate the double-labeled eIF4A, an RNA hairpin, and eIF4H in porous lipid vesicles. We demonstrate that eIF4A/eIF4H complex can repetitively unwind RNA hairpins by transitioning between an "open" and a "closed" conformation using the energy derived from ATP hydrolysis. Our experiments directly track the conformational changes in the catalytic cycle of eIF4A and eIF4H, and this correlates precisely with the kinetics of RNA unwinding. Furthermore, we show that the small-molecule eIF4A inhibitor hippuristanol locks eIF4A in the closed conformation, thus efficiently inhibiting RNA unwinding [2]. These results indicate that the large conformational changes undertaken by eIF4A during the helicase catalytic cycle are rate limiting.

1. Y. Sun et al., Nucleic Acids Res 40 (2012), 6199-6207
2. Y. Sun et al., Structure 22 (2014), 941-948

413 Mechanism of PWI Motif Binding to Nucleic Acids

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The PWI motif is a highly conserved domain found in several proteins that are predicted or known to be involved in pre-messenger RNA processing, such as constitutive and alternative splicing and the 3'-end cleavage of transcripts. Mammalian homologs of the yeast Prp3p protein, which is associated with the U4/U6 snRNP, also contain the motif. The PWI motif is named after a nearly invariant Pro-Trp-Ile sequence, although structurally homologous PWI-like motifs that lack this trinucleotide sequence have been discovered. Three distinct classes of PWI motifs can be defined by sequence homology, the presence of an adjacent sequence rich in basic amino acids and the position of the motif in the protein. PWI motifs adopt a four-helix bundle structure and have little to no affinity for nucleic acids itself. Optimum binding of the motif to both single and double-stranded DNA and RNA requires the adjacent basic region in human SRm160, PRP3 and RBM25 proteins. The individual roles of the PWI motif and the basic region in nucleic acid binding and the mechanism by which the PWI motif containing proteins associate with nucleic acids are still not clear. We are currently using fluorescence and NMR spectroscopies to investigate the mechanism of PWI motif binding to nucleic acids, and recent results will be presented.

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414 Investigating DNA unwinding by CRISPR-Cas9 using site-directed spin labeling

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In a type II clustered, regularly interspersed, short palindromic repeats (CRISPR) system, RNAs derived from the CRISPR locus associate with the CRISPR-associated (Cas) protein Cas9 to form an RNA-guided nuclease that sequence-specifically cleaves double-stranded DNA. The CRISPR-Cas9 system has been adapted for genome engineering in a wide range of organisms, however, its mechanisms of function at the molecular level are not yet fully understood. A crucial step in recognition of a CRISPR-Cas9 DNA target involves unwinding of the DNA duplex and formation of a three-stranded R-loop structure, in which a segment of the CRISPR guide RNA is hybridized to one of the DNA strands. Here, site-directed spin labeling (SDSL) is used to gain understanding of CRISPR-Cas9 complex conformations at various stages of function, with particular focus on R-loop formation. SDSL monitors site-specifically attached stable radicals (e.g., nitroxide spin labels) using electron paramagnetic resonance (EPR) spectroscopy, and provides structural (e.g., distance constraints) and dynamic (e.g., motions at the labeling site) information on the parent molecule. Using a nucleotide-independent labeling scheme, nitroxides were attached at various locations of a target DNA with minimal impact on assembly and cleavage activity of the Cas9 complex. EPR measurements revealed lengthening of inter-strand distances in the target DNA upon recognition by the Cas9 complex, consistent with duplex unwinding and formation of the R-loop. Work is ongoing to further probe R-loop conformation, which will greatly aid in elucidating the mechanism of CRISPR-Cas9 function.

415 *Discovery of protein-RNA networks*

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RNA-binding proteins regulate a number of cellular processes, including synthesis, folding, translocation, assembly and clearance of RNAs. Recent studies have reported that an unexpectedly large number of proteins are able to interact with RNA, but the partners of many RNA-binding proteins are still uncharacterized.

Using a theoretical approach, we are studying ribonucleoprotein interactions linked to inherited intellectual disability, amyotrophic lateral sclerosis, Creutzfeuld-Jakob, Alzheimer's, and Parkinson's diseases. We previously investigated RNA interactions with fragile X mental retardation protein FMRP, self-regulatory associations between proteins and their own transcripts as well as formation of ribonucleoprotein granules. Our results are in striking agreement with previous experimental evidence and provide new insights that we are currently testing in our wet lab.

We recently found that co-expressed protein and RNA molecules have a high propensity to interact, which allows us to screen ribonucleoprotein networks and select candidates amenable for experimental validation. The integration of in silico and ex vivo data unraveled two major types of protein-RNA interactions, with positively correlated patterns related to cell cycle control and negatively correlated patterns related to survival, growth and differentiation.

More discoveries made on the way will be presented.

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416 Investigation of the importance of pre-tRNA folding status in the binding of the RNA chaperone La

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La is a highly abundant RNA binding protein that associates with several RNA substrates, such as pre-tRNAs, snRNAs and mRNAs. The best understood substrates of La are primary polymerase III transcripts that contain a poly-uridylate sequence at their 3' end, which La engages to protect these from 3'exonucleases. Human La as well as *S. pombe* La (Sla1p) have also been identified as RNA chaperones, with the capacity to assist with the folding and structural stability of pre-tRNA substrates. Consistent with this, La deletion in budding yeast is synthetically lethal when combined with the loss of tRNA modifications predicted to contribute to the native pre-tRNA fold, such as the N2, N2-dimethylation of G26 by the methyltransferase Trm1p[1,2], suggesting that La functions redundantly with elements that stabilize tRNA structure. The interaction between La and pre-tRNAs has been studied to determine which domains of La are required for pre-tRNA contacts[3], and La binding has been shown to alter the structure of tRNA anticodon stems predicted to misfold due to mutation[4]. However, whether La preferentially binds misfolded pre-tRNAs in order to specifically assist these to attain their native state, or whether La engages all pre-tRNA substrates irrespective of their folding status, remains poorly understood. Using northern blots, we have identified which G26 containing pre-tRNAs are depleted in *trm1*$^-$, *sla1*$^-$ and *trm1*$^-$/*sla1*$^-$ strains in order to identify which tRNAs rely most on Sla1p in the absence of G26 modification. We have tested whether La preferentially associates with such pre-tRNAs in a Trm1p dependent fashion by pull-down of endogenous, native Sla1p/pre-tRNA complexes from *trm1*$^+$ vs *trm1*$^-$ strains as well as EMSA of pre-tRNAs modified by Trm1p *in vitro*. Our findings address the capacity of La to survey pre-tRNA folding status as a determinant of pre-tRNA binding, and may have implications on the capacity of RNA chaperones to discriminate their substrates more generally.

1. Copela et al., 2006. *RNA* 12(4):644-54
2. Steinberg and Cedergren, 1995 *RNA* 1(9):886-91
3. Bayfield and Maraia, 2009 *NSMB* 16(4):430-7
4. Chakshusmathi et al., 2003 *EMBOJ* 22(24):6562-72

417 Characterization of human DEAD-box protein DDX5

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RNA is a central player in the regulation of gene expression. RNAs largely exist in cells as ribonucleoprotein complexes (RNPs), the composition and structures of which are not static and need to be tightly regulated. DEAD-box proteins are a class of RNA helicases that function as RNA chaperones and RNP remodeling enzymes. Previous studies in the Tran lab have revealed that the DEAD-box protein Dbp2 in *Saccharomyces cerevisiae* functions in gene regulation by promoting co-transcriptional mRNP assembly and by facilitating long non-coding RNA (lncRNA) activity. Moreover, Dbp2 is an active RNA helicase and ATPase *in vitro*, suggesting that Dbp2 can modulate RNA secondary structures and remodel RNPs *in vivo*.

The mammalian ortholog of Dbp2, DDX5, is an essential protein in mammals that plays roles in tissue differentiation and carcinogenesis. DDX5 is involved in multiple steps of RNA metabolism, as well as transcription regulation. However, the precise RNA targets and mechanisms of action remain unknown. Recently, DDX5 has been linked to long non-coding RNA (lncRNA) function *in vivo*, suggesting that the role of DDX5 in transcription might be through modulation of lncRNAs and/or lncRNP complexes. To ask this question, we set up to assay if DDX5 and Dbp2 are biochemically and functionally conserved. Using recombinant protein, we show that DDX5 is a *bona fide* helicase with a higher *in vitro* helicase activity than Dbp2. Moreover, the C-terminus of DDX5 is required for the high enzymatic activity of DDX5 in higher eukaryotes. Finally, we find that DDX5 expression rescues *dbp2Δ* phenotypes in *Saccharomyces cerevisiae.*

Future work will determine the precise biochemical role of DDX5 *in vivo* with an emphasis on its role in gene regulation through lncRNA. This is an important step to characterize DDX5's RNA-dependent function *in vivo*.

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418 Induction of CPEB1 expression and host 3' UTR shortening during human cytomegalovirus infection

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Human herpesviruses persistently infect the majority of the population, significantly impacting global health. The extent to which one member of this family, human cytomegalovirus (HCMV), influences host RNA processing during infection is not well understood. We have performed comprehensive transcriptome analysis of three HCMV-infected primary cell types, and report here the identification of thousands of infection-altered host splicing and polyadenylation events. Notably the majority of the alternative polyadenylation events favored shorter 3′-untranslated regions (3′UTRs). Our comparative analysis of cell type-independent host gene expression changes revealed significant induction of cytoplasmic polyadenylation element binding protein 1 (CPEB1), an RNA binding protein with roles in alternative splicing and 3′UTR processing. We further tested exogenous delivery of CPEB1 to non-infected cells and observed similarly widespread effects on both alternative splicing and shortening of alternative 3′UTRs, impacting a set of targets that largely overlap with the infection-altered events. Prevalent 3′UTR shortening was also observed in herpes simplex virus (HSV)-infected cells, demonstrating that herpesvirus infections broadly impact host RNA 3′-end formation.

419 Identification of the functional binding domains of eIF4G with 3' Cap-independent translation element of Barley Yellow Dwarf Virus

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Unlike from canonical translation initiation, some plant viral RNAs lack a 5'cap (7-methyl guanosine). They utilize a cap independent translation element (CITE) to efficiently start translation. Barley yellow dwarf virus (BYDV) has a translation element (BTE) located in its mRNA 3'UTR . BTE binds with eukaryotic initiation factor eIF4G and then recruits other initiation factors for starting viral protein synthesis. The details of BTE initiated translation are still unclear. Here we identified one truncated fragment of eIF4G –eIF4G$_{601\text{-}1196}$ as the core domain of eIF4G. This domain includes eIF4E, eIF4A, eIF4B, PAPB binding sites and possible the BTE binding region. We found that eIF4G$_{601\text{-}1196}$ is competent for rescue of BTE mediated translation in eIF4F depleted wheat germ extract. Adding eIF4E slightly increased the translation; while the translation is further increased by supplement with eIF4A-4B-ATP helicase complex. We measured the fluorescein labeled BTE binding affinity with eIF4G by its fluorescence anisotropy changes. The results shows eIF4G$_{601\text{-}1196}$ has tighter binding (K_D=40±4nM) with BTE than full length protein (K_D =177±10nM). This binding can be strengthened by adding eIF4E or/and helicase complex. Therefore, BTE only needs a short region of eIF4G for binding and recruiting other translation initiation factors.

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420 Role of SR proteins and their splicing targets in breast cancer initiation and metastasis

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Alternative splicing (AS) is a key control point in gene expression, whose misregulation contributes to cancer malignancy, including breast cancer. Although certain splicing factors (SFs) and their targets are altered in cancer, the functional significance of these alterations remains unclear. We previously showed that the splicing factor SRSF1 is upregulated in human breast tumors and promotes transformation in vivo and in vitro. SRSF1 is a prototypical member of the SR protein family, composed of 12 structurally related proteins. However, little is known about differences and redundancies in their splicing targets and biological functions. Here, we demonstrate that additional SFs also promote breast cancer, using transformation models that mimic the relevant biological context, and investigate the overlap in splicing regulation using next-generation RNA sequencing (RNA-seq).

To model SFs alterations detected in human breast tumors, we used SF-overexpressing human mammary epithelial MCF-10A cells grown in organotypic 3-D culture. These cells form polarized growth-arrested acinar structures, similar to mammary ducts; various oncogenes are known to disrupt the acinar growth and/or architecture. Interestingly, only a subset of SFs were oncogenic in this context, differentially affecting cell proliferation, apoptosis, and/or acinar organization, suggesting non-redundant functions. Furthermore, specific SFs promoted cell invasion and metastasis in vivo and in vitro. We then defined the global repertoire of SF-regulated AS events in 3-D culture using the SpliceTrap/SpliceDuo pipeline to quantify splicing variation in RNA-seq data. To gain a better understanding of SFs regulatory mechanisms, we performed de-novo discovery of SFs binding motifs, and constructed a Bayesian model predicting the positional effects of SFs binding on cassette exons. We then compared the target specificities of various SFs. Strikingly, SFs that promoted similar phenotypic changes regulate a set of overlapping AS events, suggesting that they target common pathways in breast cancer. Finally, we identified AS targets regulated by oncogenic SFs both in human breast tumors as well as in 3-D culture, which are thus likely to play a relevant role in SF-mediated transformation.

In summary, we gained new insights into the regulatory mechanisms of SR proteins and identified novel oncogenic SF-regulated AS events that represent potential targets for therapeutics development.

421 Tepatocyte-specific deletion of a splicing regulatory protein causes spontaneous and severe Nonalcoholic Steatohepatitis in mice

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Non-Alcoholic Steatohepatitis (NASH) is emerging as one of the most common liver disease in the American population. It is a metabolic disorder in which fat accumulation within the liver (steatosis) is associated with inflammation, hepatic injury and cirrhosis without significant consumption of alcohol. Despite affecting 2-5% of the American population, there are currently no effective therapeutic treatments for NASH. Current knowledge of this disease is limited because early stages (simple steatosis) are asymptomatic and difficult to detect. Furthermore, development of effective therapeutics against NASH pathology has been slow due to lack of a feasible and robust model system. Here I describe that hepatocyte-specific ablation of a splicing regulatory protein, SRSF1, triggers severe and early onset of NASH phenotype as early as five weeks of age.

SRSF1 is a highly conserved pre-mRNA splicing factor, which plays numerous roles in both constitutive and alternative splicing. Although the structural and functional roles of SRSF1 in splicing have been extensively characterized, its role in tissue physiology is not well understood. To study its function in liver we created a hepatocyte-specific SRSF1 knockout (HKO) using the Cre-loxP technology. Histological and serum analyses of these mice revealed spontaneous and progressive liver injury accompanied by steatosis, inflammation, and fibrosis. Strikingly, these pathologies strongly correlate with pathology observed in patients affected with NASH. We hypothesized that lack of SRSF1 in hepatocytes results in misregulated splicing and expression of specific transcripts leading to the development of NASH. In order to identify the underlying transcriptome defects, we performed a high-resolution RNA-Seq on livers of five-week old wildtype and SRSF1 HKO mice. Computational analysis of the data revealed hundreds of genes with altered splicing and expression many of which are related to fatty acid metabolism, lipid peroxidation and inflammation. We have successfully validated several of these findings using gene- and splice isoform-specific RT-PCR assays. Taken together our results demonstrate that SRSF1 is essential for maintaining transcriptome integrity of the hepatocytes and that impairment of its activity induces spontaneous and accelerated NASH phenotype in mice.

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422 Genome-wide mRNA expression profiling in HIV-1 and HIV-2 infected Peripheral Blood Mononuclear Cells (PBMCs)

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Genetic variation across human individuals has impact on the activity and function of the genes, either directly or via complex regulatory networks. In turn, these variations of gene expression condition the ability of the immune system to protect against pathogens. Here, our focus was to determine the differential expression of host genes in response to HIV-1/HIV-2 infection.

To achieve this, we analyzed the effects of HIV-1 (MN) and HIV-2 (ROD) infection on the expression of host factors in PBMCs at the RNA level using the Agilent Whole Human Genome Oligo Microarray platform as well as validate major genes identified using the qRT-PCR technique. RNA was isolated from PBMC of 5 independent donors infected with either HIV-1 or HIV-2 collected at 7 day post-infection (dpi) and analyzed by real-time PCR. In a similar manner, RNA from Jurkat cells infected with HIV-1 and HIV-2 in 3 independent experiments was used to validate the microarray results. In addition, to determine whether the differential gene expression observed at 7 dpi was consistent over a period of time, we also analyzed RNA from PBMC that were cultured for 15 dpi. However, there were considerable variations in the expression pattern of differentially expressed genes among the RNA samples isolated from different donors. This could be due to inherent differences in infectivity observed among different donors. Real-time PCR results from RNA isolated from Jurkat cells and PBMC infected with HIV-1 or HIV-2 on 7 dpi showed a more consistent pattern of differential gene expression between the two viruses thereby validating the microarray results. In addition, differential gene expression observed at 15 dpi demonstrated a consistent pattern of gene up-regulation of PHGDH and PSAT1 and down-regulation of APOC1, FADS2, FXYD2, and MMP12 over a period of time in HIV-1 infected PBMC. In HIV-2 infected PBMC only the HSPA6 gene was up-regulated on 15 dpi, consistent with the microarray and day 7 real-time PCR results. These genes identified warrant additional investigations as they may offer new insights into pathogenic differences between HIV-1 and HIV-2 infection and be likely candidate biomarkers that differentiate infection due to the viruses.

423 *TERT* promoter mutations, telomerase reactivation and patient mortality in urothelial cancer.

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Reactivation of telomerase, the chromosome end-replicating enzyme, drives human cell immortality and cancer. Point mutations in the *telomerase reverse transcriptase* (*TERT*) gene promoter occur at high frequency in multiple cancers, including urothelial cancer (UC), but their impact on telomerase function has been unclear. In a study of 23 human UC cell lines, we show that these promoter mutations correlate with higher levels of TERT mRNA, TERT protein, telomerase enzymatic activity and telomere length. Although previous studies found no relationship between *TERT* promoter mutations and UC patient outcome, we find that elevated TERT mRNA expression strongly correlates with reduced disease-specific survival in two independent patient cohorts (n = 35; n = 87). These results suggest that high telomerase activity may be a better marker of aggressive UC tumors than *TERT* promoter mutations alone.

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424 *SRSF2* mutations alter exon recognition to promote myelodysplasia

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Mutations affecting spliceosomal proteins are the most common class of mutations in patients with myelodysplastic syndromes (MDS), yet their role in MDS pathogenesis has not been delineated. Therefore, we created an accurate murine model of these mutations by introducing the most common *SRSF2* mutation into its endogenous locus, finding that this mutation impairs hematopoietic differentiation *in vivo*, which is not due to loss of SRSF2 function. In contrast, *SRSF2* mutations alter SRSF2's normal sequence-specific RNA binding activity, such that C-rich variants of the normal SSNG motif are preferentially recognized versus their G-rich equivalents. The corresponding alterations in SRSF2-dependent recognition of exonic splicing enhancers (ESEs) are readily visible in the global transcriptomes of our murine model, isogenic human cells, and primary leukemic samples carrying *SRSF2* mutations.

Altered ESE preferences drive widespread mis-splicing of genes previously implicated in myelodysplasia, including *EZH2*, which is commonly subject to loss-of-function mutations in MDS. We show that mutant SRSF2 promotes ESE-dependent inclusion of a "poison" cassette exon of *EZH2*, triggering mRNA degradation via nonsense-mediated decay. *SRSF2*-mutant cells correspondingly exhibited reduced levels of EZH2 protein and H3K27me3. Restoration of correctly spliced EZH2 mRNA partially rescued the hematopoietic differentiation defect of *SRSF2*-mutant cells, providing a concrete link between SRSF2 mutation-dependent splicing changes and impaired hematopoiesis.

In summary, our data describe the biological and mechanistic consequences of a common spliceosomal mutation, and connect ESE-dependent alterations in splicing of a key hematopoietic regulator to impaired hematopoiesis.

425 IFNL3 mRNA structure is remodeled by a functional non-coding polymorphism associated with hepatitis C virus clearance

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Polymorphisms near the interferon lambda 3 (IFNL3) gene strongly predict clearance of hepatitis C virus (HCV) infection. We analyzed a variant (rs4803217 G/T) located within the IFNL3 mRNA 3′ untranslated region (UTR); the G allele is associated with elevated therapeutic HCV clearance. We show that the IFNL3 3′ UTR represses mRNA translation and the rs4803217 allele modulates the extent of translational regulation. We analyzed the structures of IFNL3 variant mRNAs at nucleotide resolution by SHAPE-MaP. The rs4803217 G allele mRNA forms well-defined 3′ UTR structure while the T allele mRNA is more dynamic. The observed differences between alleles are among the largest possible RNA structural alterations that can be induced by a single nucleotide change and transform the UTR from a single well-defined conformation to one with multiple dynamic interconverting structures. These data illustrate that non-coding genetic variants can have significant functional effects by impacting RNA structure.

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426 PGRN Network-wide Project: Transcriptome Analysis of Pharmacogenes in Human Tissues

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Gene expression variation is crucial to the etiologies of common disorders and the molecular underpinnings of pharmacologic traits; however, the nature and extent of this variation remains poorly understood. The NIH Pharmacogenomics Research Network (PGRN) Network-wide RNA-seq project aims to create a community resource containing quantitative information on annotated and novel isoforms of genes involved in therapeutic and adverse drug response (pharmacogenes).

Using 18 samples from each of 5 tissues of pharmacologic importance (liver, kidney, adipose, heart, and lymphoblastoid cell lines [LCLs]), we performed transcriptome profiling by RNA-Seq with the goal of determining differences in expression of pharmacogenes across tissues and between individuals. The data were analyzed for expression quantification, and we used the JuncBASE tool developed by members of our consortium to identify and quantify splicing events

In each of the tissues and LCLs, 11,223-15,416 genes were expressed at a substantial level. In pairwise comparisons of tissues, 105-211 pharmacogenes were differentially expressed (≥2-fold difference, FDR<0.1). For example, as expected, the CYP enzymes CYP2C19 and CYP2D6 were 10-fold and 100-fold more highly expressed in the liver than in other tissues. Other important drug metabolizing enzymes such as DPYD and TPMT showed more balanced gene expression patterns. In general, pharmacogenes were among the most variably expressed between individuals.

We also observed that 72-93% of pharmacogenes are alternatively spliced within each tissue. There was substantial variation in both annotated and novel splicing events both between tissues and between individuals. For example in SLC22A7, a gene encoding a transporter for various drugs, we found evidence of a novel alternative last exon that is variably spliced between individuals. LCLs are important pre-clinical models for human genetic studies, but they highly express less than half of pharmacogenes as compared with the 66-83% expressed at a substantial level in each of the physiological tissues. However, a number of genes like BRCA2 and SLC6A4 are much higher in LCLs than the tissues, as are alternative splice events of many genes.

These studies demonstrate that important pharmacogenes are variably expressed across tissues of pharmacologic relevance, and across different individuals, and that the vast majority is alternatively spliced.

427 Spliceosomal Mutations Associated with Myelodysplastic Syndromes in Humans Change the Fidelity of Branchsite Selection in Yeast

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Eukaryotic pre-mRNAs are transcribed from the genome containing intervening, non-coding sequences that must be removed by the spliceosome prior to translation by the ribosome. Recent studies have shown that mutations in the core splicing machinery are associated with human diseases such as myelodysplasia (MDS) and related blood disorders and cancers. Prominent among these mutations are those found in the essential protein, SF3B1. SF3B1 encodes a protein involved in the recruitment of the spliceosomal U2 snRNP to the branch point. The MDS mutations in SF3B1 occur in regions of high phylogenic conservation between humans and the *S. cerevisiae* homolog, Hsh155p. We have constructed a genetic system in *S. cerevisiae* to interrogate the effect of these mutations on pre-mRNA splicing and cell proliferation. None of the thirteen mutations associated with MDS that we tested are directly limiting for growth. However, a subset of these mutations either increase or decrease the pre-mRNA splicing of substrates containing nonconsensus UACcAAC or UACUcAC branchsites. These results suggest that these mutations impact the fidelity of branchsite recognition by the U2 snRNP. To further understand the effect of the MDS mutations in Hsh155p on the splicing of endogenous transcripts, we are utilizing RT-PCR to compare pre-mRNA and mRNA levels of specific genes. The results will be critical for understanding the role of SF3B1 in MDS and in pre-mRNA splicing.

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428 Antisense and sense RNA foci derived from hexanucleotide repeat expansions of *C9ORF72* have similar protein-interactions but distinct neuronal expression patterns

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Introduction: GGGGCC-repeat expansion of *C9ORF72* represents the most common genetic variant of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). It is suggested that toxicity may be caused directly by RNA foci transcribed from the repeat sequence or indirectly via repeat associated non-ATG translation of dipeptide repeat proteins (DPRs). RNA foci are formed by sense and antisense transcription; we aimed to determine whether the location and behaviour of these species are distinct.

Methodology: Pathological material was obtained from the Sheffield Brain Tissue Bank. Sense and antisense RNA foci were visualized by RNA fluorescence *in-situ* hybridization (FISH). Interaction with proposed foci binding partners and with TDP-43 was examined by immunohistochemistry (IHC). DPRs were also examined by IHC. Direct binding to the sense and antisense repeat sequences was examined by UV-crosslinking.

Results: *C9ORF72*-ALS is associated with pathology of motor and non-motor areas. In the cerebellum the cellular distribution of sense and antisense RNA foci are relatively distinct: sense foci are more abundant in the granule neurons ($p<0.05$) whereas antisense foci are more abundant in the Purkinje cells ($p<0.05$). In the motor neurons of the ventral horn, which are the primary target for pathology in ALS, antisense foci are present at a higher frequency ($p<0.05$). The presence of antisense ($chi^2, p<0.05$) but not sense ($chi^2, p=0.75$) RNA foci correlates with nuclear loss of TDP-43 in motor neurons. Moreover, sense-RNA derived DPRs are present at a higher frequency than antisense-RNA derived DPRs within neuronal inclusions located in cerebellar granule cells, but the opposite is true in motor neurons ($p<0.01$). Protein interactions were not different between sense and antisense foci.

Discussion: Our data suggests that if sequestration of protein binding partners is important to disease pathogenesis then sense and antisense RNA foci should be equally toxic. However nuclear loss of TDP-43 in motor neurons, which correlates directly with neurodegeneration, is associated with the presence of antisense but not sense RNA foci. This suggests the increased frequency of antisense foci in motor neurons may be key to pathogenesis. Factors determining antisense transcription of the repeat expansion are unknown but may represent a novel therapeutic target.

429 The *in vivo* role of *Borrelia burgdorferi* 6S RNA in the tick-mouse model of Lyme disease

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The Lyme disease bacterium *Borrelia burgdorferi* is transmitted to a vertebrate host via the bite of a tick. Successful transmission and infection requires the induction of an arsenal of genes regulated by the alternative sigma factor RpoS. The molecular mechanisms controlling the RpoS program of gene expression are complex and not fully defined, but we hypothesize that *B. burgdorferi* 6S RNA (Bb6S RNA) has a role in activating the RpoS regulon during transmission and infection. In *Escherichia coli*, 6S RNA binds to and down-regulates RpoD-RNA polymerase in stationary phase, allowing up-regulation of RpoS-dependent gene expression. Bb6S binds *purified* RNA polymerase holoenzyme from both *E. coli* and *Bacillus subtilis* (with RpoD and SigA, respectively). We are currently investigating the binding of Bb6S RNA to *B. burgdorferi* RNA polymerase. To examine the role of Bb6S RNA in the tick-mouse model of Lyme disease, cells lacking Bb6S RNA were constructed by replacing the chromosomal *ssrS* locus with an antibiotic resistance cassette (Δ*ssrS*). *ΔssrS* cells are defective for mouse infectivity by needle inoculation. Inoculated mice can seroconvert but live Δ*ssrS* cells cannot be recovered from mice, suggesting that the initial infection persists long enough to stimulate a humoral response before clearance. Bb6S RNA accumulates in wild-type *B. burgdorferi* infected ticks as they molt from larvae to nymphs. Surprisingly, Δ*ssrS* mutants only have a modest survival defect in ticks. No *in vitro* growth conditions, including cell density, temperature and pH, have been identified that modulate Bb6S RNA expression.

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430 The mRNP-associated TTF complex provides a molecular link between schizophrenia and fragile X syndrome

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The fragile X mental retardation protein (FMRP) affected in fragile X syndrome is a key regulator of eukaryotic mRNA translation. However, how it is recruited into target mRNA-protein complexes (mRNPs) remains enigmatic. Here we show that FMRP forms a heterotrimeric complex together with the adaptor protein TDRD3 and the topoisomerase TOP3β. This complex is integrated into mRNPs that are engaged in the pioneer round of translation via an interaction of TDRD3 with the exon junction complex. TOP3β, so far implicated only in DNA unwinding, is shown to be a nucleo-cytoplasmic shuttling protein that is in direct contact with mRNA. Interestingly, we also provide evidence that the TOP3β -gene is associated with intellectual disability and schizophrenia. Hence, our data uncover a novel mechanism for the recruitment of FMRP into mRNPs that is independent of RNA cis-elements. Furthermore, they provide a molecular link between two major neuropsychiatric disorders.

431 The role of polyadenylation in the induction of inflammatory genes

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Cordycepin (3' deoxyadenosine) is a nucleoside analogue derived from caterpillar fungi prized in Far Eastern traditional medicine for the treatment of many conditions, including inflammation. This nucleoside analogue is a chain terminator of mRNA polyadenylation, but not transcription.

We have shown that cordycepin selectively inhibits inflammatory gene expression in human airway smooth muscle cells. Here we show the same is true in LPS-stimulated mouse-macrophage-like cells (RAW 264.7 cell line), with repressed genes including Tnf and Il1b, while housekeeping mRNA levels are unchanged. A microarray carried out in these cells with subsequent gene ontology analysis confirmed inflammatory genes clustered in the most strongly repressed genes by cordycepin.

To investigate if cordycepin is working as a polyadenylation inhibitor, we optimised the methods for poly(A) analysis. The RNA ligation mediated poly(A) test (LM-PAT) was improved and we developed a standardised gel scanning procedure to compare tail lengths. This allows us to quantitatively model poly(A) tail changes. Our investigations indicate that some persistent larger products on PAT gels are artefactual multimers formed by imperfect base pairing of different sized poly(A) and poly(T) stretches. We are currently optimising a high throughput method of determining poly(A) tail sizes.

Analysis of poly(A) tail lengths has thus far shown that induction of inflammatory mRNAs is accompanied by an elongation of the poly(A) tail. Cordycepin causes shortening of the poly(A) tail for Tnf but not for a housekeeping mRNA. This suggests a possible posttranscriptional level of control of these genes, namely through polyadenylation. However, unspliced qPCR data demonstrate that the effects of cordycepin occur at least in part through effects on transcription, suggesting effects on transcription termination may be more important.

Tnf and Il1b, implicated in the pathogenesis of osteoarthritis, are both strongly repressed by cordycepin in RAW 264.7 cells. In a rat model of this disease, cordycepin altered pain behaviour, indicating that it may interfere with the production of such pro-inflammatory molecules and reduce joint pathology in vivo.

These data suggest that poly(A) tail metabolism plays an important role in the inflammatory response and that polyadenylation inhibitors such as cordycepin may become a novel class of anti-inflammatory drugs.

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432 Transcriptomic signatures of disease in *Drosophila* models of Spinal Muscular Atrophy

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Spinal Muscular Atrophy (SMA) is caused by deletion or mutation of the *Survival Motor Neuron 1* gene (*SMN1*), but connecting this loss to disease phenotypes has proven to be difficult. SMN functions in the assembly of small nuclear ribonucleoproteins (snRNPs), which catalyze pre-mRNA splicing. However, it is not clear how disruptions to this ubiquitous process result in neuromuscular disease. SMN has been implicated in other tissue-specific pathways, but the molecular role it plays in these pathways is unknown. *Drosophila* is a simple but powerful genetic system for sorting out complex biological problems, and SMA model flies recapitulate the full range of phenotypic severity seen in SMA patients. Here, we used transcriptome profiling of mutants with disruptions to different snRNP biogenesis factors, *SMN*, *Phosphorylated adaptor of RNA export* (*Phax*) and *Arsenite-resistance 2* (*Ars2*), to identify mRNA changes that are snRNP-associated and those that are specific to loss of SMN. Each of the mutants exhibited decreased steady state levels of snRNAs and comparable developmental delays. They also exhibited numerous small (< two-fold) but few large alternative-splicing changes, relative to wild-type controls. By manipulating minimum cutoffs for gene expression and relative degree of splicing change, our analysis pipeline enriched for visually identifiable alternative splicing differences. Using these more stringent criteria, we identified a few overlapping, likely snRNP-dependent, changes in pre-mRNA alternative splicing between *SMN* mutants and the other snRNP biogenesis mutants, but these changes did not correlate with disease severity in the distinct *SMN* transgenic lines. Additionally, all of our snRNP biogenesis mutants displayed a trend toward shorter transcripts, but most of these changes were small (< two-fold) differences from wild-type controls. Lastly, comparison of differentially expressed transcripts between *SMN* and the other snRNP biogenesis mutants revealed that activation of an innate immune stress response in *SMN* animals is independent of snRNP levels. In contrast to identified snRNP-dependent splicing changes, this activation of stress signaling correlated with disease severity in respective *SMN* transgenic lines. We conclude that activation of innate immune stress signaling is a conserved feature of SMA models. Future studies will address how this signaling contributes to SMA pathology.

433 Defects in tRNA anticodon loop 2'-*O*-methylation are implicated in intellectual disability

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Post-transcriptional tRNA modifications are crucial for efficient and accurate protein synthesis, and defects in modification are often associated with disease. In the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, 2'-*O*-methylation of residues N_{32} (Nm_{32}) and N_{34} (Nm_{34}) requires the Trm7-Trm732 protein pair and the Trm7-Trm734 protein pair, respectively. *S. cerevisiae* and *S. pombe* mutants lacking Trm7 (the methyltransferase subunit) are sick due to lack of Cm_{32} and Gm_{34} on $tRNA^{Phe}$, and lack of these modifications also results in loss yW_{37} formation from m^1G_{37} on $tRNA^{Phe}$ (Guy MP. et al., (2012) RNA. 10:1921-33; Guy MP. and Phizicky EM (2015) RNA. 1:61-74). In humans, *FTSJ1* is the likely *TRM7* homolog, and *ftsj1* mutations are strongly associated with non-syndromic X-linked intellectual disability (NSXLID) (e.g. Freude K. et al., (2004) Am J Hum Genet. 2:305-309), although the molecular cause is not clear.

Here we report that $tRNA^{Phe}$ from lymphoblastoid cells derived from an NSXLID patient bearing an *FTSJ1* deletion lacks Cm_{32}, Gm_{34}, and has severely reduced levels of $O2yW_{37}$. We also find that $tRNA^{Phe}$ from an NSXLID patient bearing a missense *ftsj1* allele has normal levels of Cm_{32} and $O2yW_{37}$, whereas Gm_{34} is nearly absent. To further examine the defect in this FTSJ1 variant protein, we constructed the corresponding *S. cerevisiae* Trm7 variant and find that it forms Cm_{32}, but not Gm_{34} on $tRNA^{Phe}$ in yeast. The inability of the Trm7 variant to form Gm_{34} appears to be due a catalytic defect, since the variant only has minor defects in expression and Trm734 binding, each of which is compensated by overexpression of the variant, but overexpression only leads to a minor increase in Gm_{34} modification. A genetic analysis of other *TRM7* variants indicates that some amino acid substitutions in the same region cause a similar defect in Gm_{34} formation, but not Cm_{32} formation on $tRNA^{Phe}$. Thus, we have strong evidence directly linking defective 2'-*O*-methylation of the tRNA anticodon loop to *ftsj1* lesions, suggesting that lack of these modifications is one of the causes of NSXLID, and have further demonstrated the high conservation of the mechanism of Trm7-dependent 2'-*O*-methylation of the $tRNA^{Phe}$ anticodon loop in eukaryotes.

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434 Effects of the IVSI-110 Mutation on human β-globin mRNA

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Almost 300 alleles associated with β-thalassemia have now been documented in the database, including point mutations in both coding and non-coding regions of the β-globin mRNA. The IVSI-110 (intervening sequence I-110) mutation is a single base change located in the first intron of the human β-globin gene, inducing an alternative 3' splice (acceptor) site and resulting in a marked decrease in synthesis of wild type β-globin. We have investigated the effects of the IVSI-110 mutation on expression of β-globin gene in murine erythroleukemia (MEL) cells transduced by lentivirus containing the human β-globin gene. We detected two mRNAs of different lengths from the β-globin gene with the IVSI-110 mutation by RT-PCR (~80% alternatively spliced and 20% wild type) and only one mRNA from the wild type, and a 60% decrease in expression of wild type β-globin quantified by HPLC. Interestingly, the IVSI-110 mutation resulted in accumulation of un-spliced pre-mRNA in the nuclei, suggesting the possibility of conformational change in folded mutant pre-mRNA that inhibited splicing. The rate of degradation of β-globin mRNA containing the IVSI-110 mutation was not significantly different from that of the wild type. However, the steady-state level of the IVSI-110 mutant mRNA was much lower than that of the wild type, suggesting that the mutation resulted in greatly decreased efficiency of both transcription and splicing.

435 Probing the Accessibility of Repeat Expansion RNA with Molecular Beacons

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Expansions of simple repeat sequences in the human genome are known to cause a wide range of neurological disorders. For the majority of these diseases, an RNA containing the repeat expansion sequence is expressed. Repeat expansion RNAs are potential targets for both therapeutic molecules and diagnostic assays.

Repeat expansion RNAs are typically GC-rich and form secondary and tertiary structures that prevent effective nucleic acid hybridization. This in turn limits nucleic acid therapeutic and diagnostic applications by making targeting less predictable. As repeat expansion size grows, RNA structure becomes more challenging to investigate. This is evident in the repeat sequences responsible for Huntington's disease (HD) and spinocerebellar ataxia-3 (SCA3). These diseases are both characterized by a repeated CAG sequence. However, they are located in different places in their respective genes and are flanked by differing sequences. Our research has shown that targeting these CAG repeats can result in inhibition of HD gene, but not the SCA3 gene.

To improve the targeting and detection of repeat expansion RNA accessibility and structure, we are exploring the use of fluorescent nucleic acids known as molecular beacons. Molecular beacons have the potential to sense repeat expansion RNA structure and provide a sensitive readout for accessibility to nucleic acid hybridization.

Here we present results from targeting CAG repeat expansions associated with Huntington's disease (HD). Using synthetic RNAs and RNA patient-derived cells, we observe expansion-specific detection. Molecular beacons targeting repeat expansion RNA can differentiate between normal repeat sizes and repeat expansions in the pathogenic range. We are currently optimizing molecular beacon activity.

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436 Mechanisms of RAN translation at CGG repeats in the Fragile X gene, *FMR1*.

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CGG trinucleotide repeat expansions in the 5' UTR of the Fragile X gene, *FMR1,* trigger a progressive neurodegenerative condition known as Fragile X-associated Tremor Ataxia Syndrome (FXTAS). FXTAS is characterized pathologically by the formation of ubiquitinated aggregates in patient brains. Previously, we discovered an unconventional form of translational initiation within the *FMR1* 5'UTR that produces cryptic poly-glycine (FMRpolyG, +1 ORF) and poly-alanine (FMRpolyA, +2 ORF) proteins, respectively, by translating through the repeat in the absence of an AUG start codon. This process, known as repeat-associated non-AUG (RAN) translation, occurs in multiple repeat-elicited neurodegenerative disorders, including ALS and Frontotemporal Dementia. In FXTAS, the poly-glycine containing product, FMRpolyG, triggers inclusion formation and elicits neurodegeneration in model systems. Here we delineate the molecular mechanisms by which upstream AUG-independent open reading frames (uORFs) in the *FMR1* 5' UTR initiate translation through the use of a series of new *in vitro* and *in vivo* CGG repeat RAN translation-specific luciferase reporters. Translation of both +1 and +2 *FMR1* uORFs are m^7G-cap and eIF4E-dependent and require the canonical eIF4A helicase for initiation. Mutational analysis of predicted near-AUG start codons identified the use of two specific near-AUG codons (ACG and GUG) just 5' to the repeat sequence as sites of initiation for the +1 uORF, and initiation at these sites occurred even in the absence of the CGG repeat. In contrast, translation of the +2 uORF required the presence of the repeat and appeared to initiate within the repeat itself. These new models provide useful tools to study both RAN translation and non-AUG initiated upstream open reading frame translation in normal and disease states and suggest that repeat frame and sequence context strongly influence unconventional initiation dynamics.

437 Crystal structure and properties of the CCUG repeats related to myotonic dystrophy type 2.

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Expanded CCUG repeats are associated with myotonic dystrophy type 2 (DM2). Crystal structures of two CCUG-containing RNA oligomers show the RNA strands associating into slipped duplexes. They contain non-canonical C-U pairs whose crystal structure indicates that they have undergone tautomeric transition resulting in a Watson-Crick-like pairing. The overhanging ends of the molecules interact and form U-U pairs which also show tautomerism. The molecules are aligned end-to-end forming supercoiled semi-infinite helices. The duplexes show deviations from the standard A-RNA, such as closing of the major groove and varying width of the helix at the C-U pairs. Thermodynamic measurements show that duplexes consisting of CCUG repeats are less stable than CNG repeats and that introducing LNA residues increases their stability and raises the melting temperature of the studied oligomers by approx. 10°C.

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438 Defining Motor Neuron Enriched miRNAs in ALS: Implications for Motor Neuron Disease

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One of the greatest limitations to developing effective therapeutics for Amyotrophic Lateral Sclerosis (ALS) is an incomplete understanding of the selective vulnerability of motor neurons (MN) to ALS disease pathology. Recent studies have shown that other central nervous system (CNS) cell-types contribute to ALS disease progression, but it is MN loss and pathology that defines the disease onset. Therefore, it is critical to elucidate the unique expression patterns of MNs, which likely contribute to their selective vulnerability. microRNAs (miRNAs) are highly conserved, 20-22 nt RNAs that regulate nearly every cellular process from development to senescence. miRNAs are also vital mediators of stress signaling, such as the unfolded protein response. Consequently, miRNA expression is often dysregulated in neurodegenerative disorders; defining *in vivo* MN-enriched miRNA expression is necessary to delineate miRNA contributions to MN susceptibility but also challenging, as miRNA expression in the other CNS cell-types must first be established. To generate high-throughput and physiologically relevant data sets of miRNA expression within the CNS, we isolated miRNA from distinct cell populations in the brainstem and spinal cord through immunoprecipitation of a tagged miRNA binding protein expressed in genetically-labeled mice. We generated large datasets of well-validated miRNA expression data for neurons, motor neurons, microglia, and astrocytes. After discriminating MN-enriched miRNA expression, we then selectively probed the dysregulation of these miRNAs in MNs during disease timecourse in ALS-model $SOD1^{G93A}$ mice. Overall, these data explore a new means of studying MN-specific expression and its relation to MN disease.

439 Biochemical characterization of pathogenic mutations in human mitochondrial RNase Z (ELAC2)

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Human mitochondrial RNase Z, encoded by ELAC2, is an endonuclease responsible for the endonucleolytic cleavage at the 3' end of mitochondrial (mt-) tRNAs in the primary, polycistronic transcript. Recently, we showed that compound heterozygous or homozygous mutations within the nuclear gene encoding the ELAC2 protein reduced mtRNA endonucleolytic processing and were associated with hypertrophic cardiomyopathy (Haack et al., AJHG 93:211, 2013). A total of five individuals were identified across three independent families, harboring a combination of the following alleles: F154L, L423F and T520I. Despite the increased utility of genetic testing, establishing pathogenicity of novel variants remains challenging and functional studies should remain an integral part of the evaluation. With this in mind, mutated ELAC2 proteins harbouring each of these variants were expressed using the baculovirus system and the effects of the substitutions on pre-tRNA processing reaction relative to wild-type enzyme were determined. The mutant proteins were stable, suggesting that none of them are grossly misfolded or subject to severe intracellular degradation. The in vitro mt-tRNA processing assay revealed that the mutations impair efficiency of tRNA processing and correlated well with the severity of mtRNA processing defect observed in patient samples. Our results are consistent with a suggested molecular role of ELAC2 in mitochondrial disease.

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440 Long non-coding RNA genes are direct disease causal candidates in human metabolic disorders: insights from the GWAS-transcriptome interface.

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The ENCODE (Encyclopedia of DNA Elements) Consortium has revealed that two-thirds of human genes do not encode proteins, and has pinpointed long non-coding RNA (lncRNA) genes as the largest ncRNA gene class. Diverse roles have been identified for a growing number of lncRNAs, but an integrated functional understanding of the nearly 20,000 such genes in the human genome is still lacking. More than 95% of significant disease-associated single-nucleotide polymorphisms (SNPs) from genomewide association studies (GWAS) are outside of protein-coding regions. The functional meaning of these SNPs remains poorly understood. We posited that significant disease-associated GWAS SNPs may be incorrectly annotated in the current literature, where a SNP's nearest protein-coding gene is often listed as the disease candidate gene, while lncRNA genes closer to, or directly encompassing, the SNP are ignored. Intersecting ENCODE lncRNA annotations and the curated NHGRI catalogue of significant disease-associated SNPs pinpointed 71 formerly "intergenic" or "intronic" SNPs in lncRNA exons. One hit, in the lncRNA LOC157273, was independently included in 19 independent GWAS studies of type 2 diabetes (T2D), obesity, cardiovascular function, and related metabolic phenotypes. Our trans-ethnic mapping of T2D GWAS within the CHARGE (Cohorts for Heart and Aging Research in Genomic Epidemiology) Consortium identified recombination breakpoints separating LOC157273 from PPP1R3B, the nearby protein-coding gene previously thought to be the disease candidate. Therefore, the lncRNA LOC157273, revealed by single-molecule HeliScope cDNA-CAGE to be restricted to human hepatocytes, emerges as the sole T2D candidate gene in this region. Re-assessing significant "intergenic" and "intronic" CHARGE T2D SNPs outside of lncRNA exons, we found rs651007 in the first intron of ABO (a T2D candidate gene) as residing near the promoter of an ABO exon 1 antisense lncRNA expressed exclusively in pancreatic beta cells and hence relevant to diabetogenesis. Our results challenge the incumbent paradigm of non-coding disease SNPs as either passenger bystanders in long linkage-disequilibrium blocks or markers pointing to protein-coding candidate genes, and suggest that disease-causing lncRNAs can be highlighted by the union of GWAS and expression datasets. {This work is supported by a National Institutes of Health (NIH) Director's New Innovator Award, 1DP2-CA196375, 2014-2019, sole PI: Lipovich.}

441 Anti-sense Oligonucleotides Can Re-direct SmgGDS RNA Splicing and Decrease Breast Cancer Cell Proliferation

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Small GTPases have important roles in the progression and metastasis of breast cancer. Activation of small GTPases requires a prenylation reaction that increases protein localization to the plasma membrane where effector proteins are engaged. SmgGDS (small G-protein guanine nucleotide dissociation stimulator 1; Rap1GDS1) is expressed as two alternative splice variants that harbor or skip exon 5. Exon 5-containing SmgGDS-607 protein (607 amino acids) binds un-prenylated small GTPases and regulates their entry into the prenylation pathway while SmgGDS-558, which lacks exon 5, binds prenylated GTPases and facilitates their trafficking to the plasma membrane. SmgGDS is overexpressed in breast cancers, and siRNA knockdown of SmgGDS-558 alone is sufficient to reduce breast cancer cell proliferation in vitro and in a mouse xenograft model. These observations highlight SmgGDS-558 as an attractive therapeutic target whose reduction in cells may have benefits for cancer.

As a beginning to therapeutically targeting SmgGDS, we sought to determine if anti-sense oligonucleotides could promote exon 5 inclusion (redirect splicing away from the 558 isoform) and, similar to siRNAs, reduce proliferation of cultured breast cancer cells. In an initial ASO walk, ASOs spanning exon 5 and near intron sequences were transfected into breast cancer cell lines. Many ASOs promoted exon skipping, but three targeted to the downstream intron decreased 558 mRNA and protein as efficiently as siRNA. A microwalk centered on the most active ASO revealed one with increased activity. ASOs that reduced the 558 isoform also decreased breast cancer cell proliferation to a similar degree as siRNA. Surprisingly, an ASO that substantially increased 558 was most efficient at inhibiting proliferation. These observations show that SmgGDS isoform levels can be manipulated by ASOs and support the therapeutic potential of ASOs in the treatment of breast cancer.

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442 The RNA Binding Protein LARP1 promotes cell survival and tumourigenesis by stabilizing trascnripts encoding pro-oncogenic proteins.

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There is growing evidence that mRNA-binding proteins (RBPs) can be post-transcriptional drivers of cancer progression. LARP1 is a conserved RBP that interacts with poly-A binding protein (PABP) and has been shown to regulate 5'TOP mRNA stability and translation. To identify the pool of mRNAs that interact with LARP1 we performed RNA immunoprecipitation coupled with exon microarray analysis. We found 3000 transcripts that were significantly enriched in the LARP1-bound fraction and included TOP and non-TOP mRNAs. Functional analysis revealed that the LARP1-interactome mRNAs were enriched for pathways involved in cancer development and progression. Transcriptomic deep sequencing following LARP1 knockdown revealed alterations in levels of mRNAs linked to processes such as cell survival and tumour growth and suggest that LARP1 could act to both promote and inhibit transcript stability. We confirmed that LARP1 is a component of mTOR, BCL2- and BIK-containing messenger ribonucleoprotein (mRNP) complexes and recognises sequences within the 3'-untranslated region of its target transcripts. Studies using cancer cell lines have shown that LARP1 promotes cancer cell invasion, clonogenicity and the maintenance of cancer stem-cell population with a prominent role in promoting cell survival. Moreover LARP1 depletion using RNAi increases cisplatin sensitivity in chemotherapy-resistant ovarian cancer cell lines. *In vivo* studies showed that LARP1 is required for tumorigenesis and tumour growth and expression of LARP1 in tumour tissues correlates with progression of disease and adverse prognosis. We conclude that the aberrant expression of LARP1 in tumour tissue contributes to the post-transcriptional regulation of gene expression by modulating the stability of a subset of pro-survival mRNAs. Given the fundamental role played in cell survival and chemotherapy resistance LARP1 could prove to be a target for therapeutic intervention.

443 Chronic low levels of SMN causes perturbations in the transcriptome of testis of a murine model of spinal muscular atrophy

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Spinal muscular atrophy (SMA) is caused by low levels of survival motor neuron (SMN) protein. Studies in mouse models have established that motor neurons and muscle are preferentially susceptible to reduced SMN. Evidence of abnormalities in heart, intestine, liver, pancreas and lung have also been reported in SMA mice. Experiments in *Drosophila* reveal requirement of SMN for the maintenance of male germline. However, consequences of reduced SMN on growth and development of sex organs of vertebrates is not known. Here we utilized the allele C mouse, a mild SMA model, to examine the impact of low levels of SMN on growth and development of reproductive organs. We observed a marked decrease in size and mass of testes of allele C mice compared to the wild type (WT) littermates. Histological analysis revealed abnormalities in seminiferous tubules of allele C mice. Consistently, we recorded a substantial reduction in sperm count. While SMN was only slightly reduced in whole testis lysates, we observed a significant reduction in Gemin2, an SMN-interacting protein essential for most SMN functions. Although there was little difference in the splicing pattern of several genes that are alternatively spliced during the first wave of spermatogenesis, several differences emerged by 6 weeks of age when spermatogenesis is complete. High throughput RNA sequencing and subsequent validation by quantitative real-time PCR revealed significant perturbations in the transcriptome of allele C testis. These drastic changes were largely consistent with the altered cell composition of testes between WT and allele C mice. For example, genes known to be highly expressed in late spermatocytes and spermatids were found to be strongly downregulated. The differentially expressed genes corresponded to a variety of biological pathways such as apoptosis, including both pro- (several caspases, Bax, calpains, and Aifm1) and anti- (Bcl3, Akt) apoptotic genes. Confirming enhanced apoptosis, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay showed increased DNA fragmentation in seminiferous tubules of allele C males. Our results underscore that low levels of SMN has an adverse effect on male reproductive organ development. These findings are instructive for clinical studies geared for SMA patients who survive beyond puberty.

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444 Characterizing and Antagonizing Dux4 mRNA 3'end formation

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The importance of accurate and efficient processing of the 3'end of the mRNA is evident in several diseases, one of which is Facioscapulohumeral Dystrophy (FSHD). FSHD is an autosomal dominant muscle dystrophy attributed to the inappropriate expression of Double homeobox 4 (Dux4) in muscle cells. Dux4 is generally suppressed in somatic tissue and its pathogenic expression has been shown to promote apoptosis contributing to muscle atrophy. The inappropriate expression of Dux4 in FSHD patient skeletal muscle cells is facilitated by the creation of a nonconsensus polyadenylation signal (PAS) due to a single nucleotide polymorphism occurring in a transcriptionally derepressed environment. The Dux4 PAS is surprisingly active despite utilizing a suboptimal cleavage site and the lack of any well-defined DSE. Taken together, Dux4 cleavage and polyadenylation represents an intriguing biological context to investigate *cis* regulatory elements of cleavage and polyadenylation.

To interrogate the elements that contribute to Dux4mRNA cleavage and polyadenylation we designed a reporter system in which the Dux4 3′ end processing elements are removed from their epigenetic context. The reporter system utilizes transcriptional read-through as a measure of cleavage and polyadenylation. We have validated our reporters using the well-studied SV40PAS in HEK293T and have demonstrated sensitivity of our reporter to mutations within *cis* elements mediating cleavage and polyadenylation. We demonstrate that, not only as previously shown, is the PAS necessary for the stabilization of the transcript, we identify additional *cis* regulatory elements that do not appear to match a G/GU-rich DSE regulating the Dux4 mRNA 3′ end processing. Using the data acquired from the characterization of the Dux4 PAS we designed antisense oligonucleotides (ASO) to impair 3′ end processing. As a proof of principle we designed ASOs targeting the SV40PAS. We observed that both steric blocking ASOs and RNAse H sensitive ASOs targeting the SV40 polyadenylation signal can impair reporter protein expression.

445 A Potential Role for snoRNAs in PKR Activation during Metabolic Stress

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Protein Kinase RNA-activated (PKR) is an interferon-inducible kinase that responds to viral double stranded RNA (dsRNA) as part of the innate immune response. PKR is also activated by metabolic stress providing a potential mechanistic link to the observed chronic inflammation in disorders like obesity and type II diabetes.[1]Experiments using mouse embryonic fibroblast cells (MEFs) reveal that PKR activation in response to palmitic acid (PA), which induces metabolic stress, is dependent on a functional dsRNA-binding domain (dsRBD).[1]Our lab pursued the identification of endogenous RNA activators of PKR during metabolic stress by utilizing RNA immunoprecipitation followed by high-throughput sequencing (RIP-Seq). Results from our RIP-Seq analyses revealed that the major class of RNA that associates with PKR in a dsRBD- and PA-dependent manner is snoRNA. To determine if snoRNAs could directly activate PKR, we employed an in vitro autophosphorylation assay. Each snoRNA tested efficiently activated wild type PKR (PKR_{WT}), even those not identified by RIP-Seq. Importantly, PKR with a point mutation in each motif of the dsRBD was not activated by perfectly duplexed dsRNA nor tested snoRNAs. PKR_{WT} activation by snoRNA followed the canonical bell-shaped curve, requiring approximately 100-fold molar excess of RNA to observe substrate inhibition. For some snoRNAs efficient activation required a 5'-triphosphate and did not occur with a 5'-monophosphate or hydroxyl. This difference likely reflects the double-stranded nature of each snoRNA, with more rod-liked RNAs displaying less dependence on the presence of a 5'-triphosphate. We have also demonstrated that snoRNAs transfected into MEFs can activate PKR_{WT} and display a similar 5'-triphosphate dependence. Our results suggest an intriguing model where snoRNAs represent a novel class of RNA capable of activating PKR during metabolic stress.

1 Nakamura, T. *et al.* Double-Stranded RNA-Dependent Protein Kinase Links Pathogen Sensing with Stress and Metabolic Homeostasis. *Cell* **140**, 338-348, doi:http://dx.doi.org/10.1016/j.cell.2010.01.001 (2010).

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446 Splicing factor hnRNP A2 activates the Ras-MAPK-ERK pathway by controlling A-Raf splicing in Hepatocellular carcinoma development

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In recent years it has become clear that splicing factors play a direct role in cancer development. We showed previously that splicing factors SRSF1, SRSF6 and hnRNP A2/B1 are up-regulated in several cancers and can act as oncogenes when up-regulated. Here we examined the role of splicing factors hnRNP A1/A1b and hnRNP A2/B1 in hepatocellular carcinoma (HCC). We show that the splicing factors hnRNP A1 and hnRNP A2 are up-regulated in HCC tumors derived from inflammation-induced liver cancer mouse model. Overexpression of hnRNP A1 or hnRNP A2, but not the splicing isoform hnRNP B1, induced tumor formation of immortalized liver progenitor cells, while knockdown of these proteins inhibited anchorage-independent growth and tumor growth of human liver cancer cell lines. In addition, we found that cells overexpressing hnRNP A2 showed constitutive activation of the Ras-MAPK-ERK pathway. In contrast, knockdown of hnRNP A2 inhibited the Ras-MAPK-ERK pathway and prevented ERK1/2 activation by EGF. Moreover, we found that hnRNP A2 regulates the splicing of A-RAF, reducing the production of a short dominant-negative isoform of A-Raf and elevating the full-length A-RAF transcript. Taken together, our data suggest that hnRNP A2 up-regulation in HCC induces an alternative splicing switch that down-regulates a dominant-negative isoform of A-Raf leading to activation of the Raf-MEK-ERK pathway and cellular transformation.

447 Characterization of MDS-specific point mutations in serine/arginine-rich Splicing Factor SRSF2

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SRSF2 is a member of the SR protein family of splicing regulators. The structure of SRSF2 consists of an RNA recognition motif (RRM) and an arginine-serine rich domain (RS) involved in protein-protein interactions. These regions are separated by a hinge region of approximately 12 amino acids. In addition, there is a nuclear retention signal (NRS) in the C-terminal end of the protein that prevents SRSF2 from nucleus/cytoplasm shuttling that many other SR proteins do. In addition to SRSF2's function in constitutive and alternative splicing, SRSF2 is also required for an RNA-dependent regulation of transcriptional elongation at many highly regulated gene promoters.

A proline residue (P95) within the hinge region of SRSF2 has been found to be mutated to histidine, leucine, or arginine in 10-15% of patients with myelodysplastic syndrome (MDS). The narrow and specific nature of these mutations, along with their presence in the earliest stages and persistence throughout the disease, suggest an important, yet unidentified, role in MDS pathogenesis.

We have developed stable, inducible cell lines in TF-1 erythroleukemia cells that express wildtype SRSF2 ($SRSF2^{WT}$), $SRSF2^{P95H}$, $SRSF2^{P95L}$, or $SRSF2^{P95R}$, or SRSF2 with one of its four major domains deleted ($SRSF2^{\Delta RRM}$, $SRSF2^{\Delta hinge}$, $SRSF2^{\Delta RS}$, $SRSF2^{\Delta NRS}$). Analysis of these cell lines has shown that while overexpression of the point mutants and $SRSF2^{\Delta NRS}$ mimic $SRSF2^{WT}$ in the alternative splicing of the endogenous SRSF2 3'UTR, $SRSF2^{\Delta RRM}$, $SRSF2^{\Delta hinge}$, and $SRSF^{\Delta RS}$ do not. This shows that the point mutations do not cause a loss of function for the hinge region. Interestingly, the point mutants, and not the wildtype or deletion mutants promote an increase in alternative splicing of CDC25C to a shorter, coding transcript, suggesting a possible gain of function. Cell lines were also assayed for apoptosis, which occurs at a higher rate in MDS patient bone marrow than healthy bone marrow. Overexpression of each of the point mutants and deletion mutants results in an increase in apoptosis compared to overexpression of $SRSF2^{WT}$. Changes in protein-protein and protein-RNA interactions that occur upon point mutation or deletion mutation of SRSF2 are currently being investigated.

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448 Disease Causing Coding Variation Frequently Affect Pre-mRNA Splicing

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Understanding the mechanism of disease causing variants is a high priority for medical research. We re-analyzed 4,964 published disease causing coding variations for defects in splicing. Approximately 10% of coding variants that cause disease also disrupt splicing both in vivo and in vitro. In 80% of these cases the mutant spliced significantly less efficiently than the wildtype (FDR 5%, 1.5 fold). Certain features like high conservation and short exon length were associated with coding variants that altered splicing. Finally, this panel of 4,964 loci with single point mutation/wildtype controls in splicing and spliceosomal assembly assays provided a powerful tool to explore the sequence determinants and disease mechanisms of splicing mutations. Most (73%) coding variants that disrupted splicing were preferentially retained in the A complex. Comparing the differences in allelic matches to RNA binding protein recognition motifs to allelic differences in splicing activity allowed for the assignment of activator and repressor functions. In the context of disease causing mutations, 12 factors were found to function as repressors and 18 as activators.

449 Alternative splicing shapes the phenotype of a mutation in BBS8 to cause nonsyndromic Retinitis Pigmentosa

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Bardet-Biedl syndrome is a genetic disorder affecting multiple systems and organs in the body. Several mutations in genes associated with Bardet-Biedl syndrome (BBS) affect only photoreceptor cells and cause nonsyndromic Retinitis Pigmentosa (RP), raising the question why certain mutations manifest as a systemic disorder, while other changes in the same gene affect only a specific cell type. We show that cell type specific alternative splicing is responsible for confining the phenotype of the IVS1-2A>G mutation in the BBS8 gene to photoreceptor cells. The IVS1-2A>G mutation leads to mis-splicing of BBS8 exon 2A producing a frameshift in the BBS8 reading frame and thus eliminating the protein specifically in photoreceptor cells. Cell types other than photoreceptors skip exon 2A from the mature BBS8 transcripts, which renders them immune to the mutation. We also show that the splicing of Bbs8 exon 2A in photoreceptors is directed exclusively by redundant splicing enhancers located in the adjacent introns. These intronic sequences are sufficient for photoreceptor cell-specific splicing of heterologous exons, including an exon with randomized sequence. Genome wide profiling of alternative splicing in photoreceptor cells revealed that Bbs8 exon 2A is a part of a characteristic splicing program that primarily targets the cytoskeleton.

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450 Investigating unsolved Mendelian disorders with Spliceosaurus, a software for detection and prediction of mutations that affect pre-mRNA processing

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The effects of synonymous and intronic variation are difficult to detect and quantify, but these types of variation are known to alter sequence elements recognized by the splicing machinery. This variation can alter existing splice sites, generate cryptic splice sites and create or destroy cis-acting regulatory elements, all of which can result in aberrant splicing events. These usually result is in either a non-functional coding sequence or a transcript that is targeted by nonsense-mediated decay. We report the development of Spliceosaurus, a lightweight C++11x codebase written using the Standard Template Library (STL) and open standards. This software permits the rapid analysis of constitutive splicing signals and the effects of sequence variation in splice site prediction. Potentially deleterious splicing events are interrogated by predicting new exon boundaries and the effects on the aberrant transcript as a whole. Unlike existing splicing analyses suites, the input and output for Spliceosaurus is a standard VCF file allowing seamless integration into existing exome and genome-wide data analysis pipelines.

We deployed Spliceosaurus on Mendelian disorders of diverse phenotypes. These cases, were previously taken in by the UW Center for Mendelian Genomics and subjected to hybridization capture/2nd generation sequencing to generate high quality exome data. For unsolved cases where standard analyses did not identify a deleterious mutation in a coding sequence or splice site dinucleotide, Spliceosaurus was used to search for variants that might alter splice site strength or create new splice sites. We present results from several cases where the algorithym identifies a strong hit in a gene associated with the phenotype exhibited in that Mendelian disorder.

451 The LARP6 protein is required for pollen tube guidance and male fertility in *Arabidopsis thaliana*

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Successful sexual reproduction in animals and plants requires communication between male and female gametes. Flowering plants' pollen grain consists in a large cell which cytoplasm embeds the decondensed vegetative nucleus and two sperm cells. Desiccated pollen grains can be transported over long distances by wind or animals. When they land on a stigma, they undergoe adhesion and hydration, before initiating the germination of pollen tubes which will grow along the pistil, protect and deliver the sperm cells to the ovule. The whole fertilization process requires an extensive communication between the male and female tissues, allowing the maternal tissue to support pollen tube growth and guidance until fertilization of the ovule. The plant male-female communication process involves the secretion of female- but seemingly also male-emitted peptides, small proteins , amino acids but also hormones , nitric oxide, etc.

LA and **R**elated **P**roteins (**LA** and **LARP**s) share the structured LA-Motif (LAM) and constitute a superfamily of RNA binding factors with several hundred members scattered in protists, fungi, plants and animals. They classify into five evolutionarily distinct subfamilies: the genuine La and LARP1, 4, 6 and 7. Most LA and Related Proteins have a bipartite La-Module comprising the LAM and a subfamily specific RRM1. LARP6 proteins also share a specific C-terminally located domain denoted LSA (LA and S1-Associated). In Human, LARP6 binds the type-I collagen a1 and a2 mRNAs at their 5'UTRs, and coordinates their subcellular localization and translation. In higher plants, the LARP6 proteins further evolved into three subclusters with members of clusters b and c displaying a PAM2 peptide which mediates binding to the Poly(A) Binding Protein. The Arabidopsis genome codes for three LARP6 proteins, one of each subcluster a, b and c which we proposed, fulfil distinct functions likely as mRNA binding proteins. We found that AtLARP6c is a male specific protein dispensable for pollen maturation, pollen tube germination and growth but necessary for pollen tube guidance to the ovule. Our data support that AtLARP6c is involved in the post-transcriptional regulation of genes involved in the transport and/or secretion processes.

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452 Structural modeling of human Splicing Factor 3b using PyRy3D software

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One of the major challenges in structural biology is to determine the structures of macromolecular complexes and to understand their function and mechanism of action. However, structural characterization of macromolecular assemblies is very difficult. For this reason a hybrid computational approach is used to incorporate spatial information from a variety of experimental methods into modeling procedure.

We developed PyRy3D (see http://www.genesilico.pl/pyry3d) - a computational tool that applies hybrid approach in order to build low-resolution models of large macromolecular complexes. The model building procedure applies a Monte Carlo approach to sample the space of solutions. Spatial restraints are used to define components interacting with each other, and a simple scoring function is applied to pack them tightly into contours of the entire complex (e.g. cryoEM density maps).

Splicing Factor 3b (SF3b) is a protein complex responsible for the recognition of the intron's branch site in U2- and U12-dependent introns. Human SF3b complex consists of seven proteins: SF3b155, SF3b145, SF3b130, SF3b49, SF3b14a, SF3b14b and SF3b10. However, high-resolution structures have been determined experimentally only for a few SF3b components or their fragments. A structure of the whole complex has been determined by cryoelectron microscopy at 9.7 Å resolution [1]. However, despite intensive research on SF3b, its complete structure and mechanism of action remain unknown.

Thus far, we modeled the spatial structures of all spliceosomal proteins, including SF3b components [2]. Now, we applied the hybrid modeling approach implemented in PyRy3D software in order to build ensembles of structural models of the human SF3b complex that agree with currently available experimental and theoretical data. Our model identified likely positions of all proteins that form the SF3b complex, with SF3b155 serving as a scaffold, and interacting with most of the SF3b proteins. SF3b14a is located in the center of the complex, surrounded tightly by SF3b155. We predict a mechanism that includes a movement of SF3b155 and SF3b145, which allows for revealing of SF3b14a and its interaction with pre-mRNA at the initial steps of splicing.

453 A retrovirus packages nascent host noncoding RNAs from a novel RNA surveillance pathway

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A remarkable property of retroviruses is their propensity to non-randomly package host cell RNAs during viral assembly. Although up to one-half of the total RNA mass of a retrovirus is host-encoded, the spectrum of packaged RNAs and the mechanisms by which they are recruited have been largely unknown. We used high-throughput sequencing to obtain a comprehensive description of the RNAs packaged by a model retrovirus, murine leukemia virus (MLV). Surprisingly, although MLV assembles in the cytoplasm, we discovered that many newly synthesized "nuclear" ncRNAs are enriched in virions, such as pre-tRNAs, U6 snRNA, and precursors to specific small nucleolar RNAs (snoRNAs). Consistent with their cytoplasmic recruitment, packaging of both pre-tRNAs and U6 snRNA requires the nuclear export receptor Exportin-5. Since cytoplasmic forms of these RNAs could escape detection if they were degraded after export, we tested if depleting one or more ribonucleases increased their levels in virions, as would be expected if MLV packages these RNAs from a RNA decay pathway. Remarkably, adenylated and uridylated forms of these RNAs accumulate in cells and virions when the cytoplasmic exoribonuclease DIS3L2 and subunits of the RNA exosome are depleted. Similar extended forms of U6 snRNA accumulate in the cytoplasm of uninfected mouse cells when these ribonucleases are depleted. Together, our data reveals that MLV recruits at least some host RNAs from a previously undetected pathway in which unprocessed and unneeded ncRNAs are exported to the cytoplasm for degradation.

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454 Structural basis for 7SK RNA recognition by the C-terminal RRM domain of the La-related protein LARP7

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The 7SK ribonucleoprotein (RNP) complex binds to and inactivates P-TEFb, an essential eukaryotic transcription factor required for the transcription of all mRNA. The 7SK RNP is a dynamic complex, with several protein subunits coming on and off of an RNA scaffold; however, the La-related protein group 7 (LARP7) family protein, named LARP7, is constitutively bound to the 7SK RNA at the 3' terminus. LARP7 is required for 7SK RNA stability and P-TEFb sequestration. LARP7 contains a La module (La and RRM1 domains) that binds the 7SK RNA 3' single-stranded polyuridine tail and a predicted C-terminal RRM2 domain with no identified RNA binding partner. This C-terminal domain is essential for LARP7 specificity to 7SK RNA and is necessary for the 7SK RNP to sequester P-TEFb. Deletion of the C-terminal domain is linked to gastric cancer and primordial dwarfism, suggesting a crucial functional role. To investigate the structural basis of its function, we determined the solution NMR structure of the human LARP7 C-terminal domain. The C-terminal domain contains an atypical RRM with b1-a1-b2-b3-a2-b4-a3 topology and a disordered C-terminal tail. Using a combination of NMR, isothermal calorimetry, and electrophoretic gel shift mobility assays, we find that the LARP7 RRM2 binds the 7SK stem-loop 4 (SL4) RNA with high affinity in a sequence-specific manner. NMR chemical shift perturbation and mutagenesis studies identify the requirements for high-affinity binding. The LARP7 RRM2 topology, sequence homology, and RNA binding data show that this domain is an xRRM, first identified in the LARP7 family protein p65, a component of *Tetrahymena* telomerase. Together, these studies shed light on the structural basis for the important role of the LARP7 RRM2 in the 7SK RNP.

455 *Trypanosoma cruzi*: mRNP Dynamics upon stress conditions.

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Gene expression regulation in trypanosomatids is mainly posttranscriptional. Specific proteins associate to mRNAs to form mRNP complexes which will ultimately determine mRNA fate in the cell. The differentiation of non-infective to infective forms in the parasite *Trypanosoma cruz*i is triggered by a nutritional stress. Analysis of mRNP complexes isolated from polysomal and post-polysomal fractions under normal and stress conditions allowed to identify several mRNA associated proteins. The characterization of TcZC3H39, a zinc finger protein, showed that there is a shift on the bound targets according to the physiological conditions of the cell. Under stress conditions the TcZC3H39-mRNP acts sequestering highly expressed mRNAs slowing translation activity. Accordingly, the number of target mRNAs was higher in stressed as compared to non-stressed parasites. Interestingly, under stress conditions the TcZC3H39-mRNP was enriched in targets highly expressed under non-stress conditions, such as ribosomal proteins and cytochrome c complex. Proteins without a canonical RNA binding domain were also observed associated to mRNA in mRNP complexes. This was the case of eukaryotic elongation factor 1 (EF1-a). A specific subset of mRNAs was associated to EF1-a mRNP in unstressed and stressed parasites. Some targets were common to both physiological conditions whereas there was a set of targets specific to each condition. The data indicated the functional association of some transcripts bound to EF1-a and to TcZC3H9, supporting the RNA regulon theory, according to which mRNAs encoding proteins with related functions are associated with specific proteins in mRNP complexes, controlling their fate in the cell.

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456 Protein localization to the U2 snRNP: a study in RNA/protein allostery

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The U2 snRNP contains the U2B″ and U2A′ proteins in vertebrates, or SNF and U2A′ in most other metazoans. U2B″ and SNF bind directly to Stemloop IV (SLIV) of U2 snRNA, using their N-terminal RNA Recognition Motif (RRM). SNF is also a protein component of the U1 snRNP, where it binds to SLII of U1 snRNA. Our in vitro RNA binding data show that U2B″ can also bind U1 snRNA SLII. Only in the U2 snRNP do SNF and U2B″ use their RRM to bind to both SLIV and to the protein U2A′.

Here, we address two outstanding questions in snRNP assembly:

First, in organisms that use SNF, how is U2A′ excluded from the U1 snRNP?

Second, in organisms that use U2B″, why is the U2B″/U2A′ complex found only in the U2 snRNP?

We look for answers to these questions using thermodynamics, x-ray crystallography, and NMR.

457 Withdrawn

458 Structural Analysis of a Salt Stable Spliceosome Core

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The spliceosome is a large multi-subunit ribonucleoprotein (RNP) complex responsible for intron removal and sequential exon ligation. This operation, also known as pre-mRNA splicing, is an essential eukaryotic RNA processing event. Assembly of the spliceosome from a large number of protein and RNA components is dynamic. Structural models of the spliceosome from electron microscopy (EM) are limited in resolution, and the three-dimensional position of components within the complex is unknown. To characterize the interactions between components of catalytic spliceosomes we challenged C complex spliceosomes, which are arrested before second step chemistry, with lithium acetate. Splicing reactions separated via glycerol gradient centrifugation in the presence of 2M lithium show a shift in the sedimentation of the splicing intermediates, suggesting the presence of a salt stable complex that has lost components. After purification of the salt stable complex we still detected all of the RNA components including U2, U5 and U6 snRNAs. Preliminary mass spectrometry analysis revealed the identity of the proteins that are retained within the salt stable spliceosome. EM analysis shows the salt stable spliceosome shares similar architecture to that of C complex purified under normal salt conditions but with specific regions of lost density. Additionally, to further characterize spliceosome structure we are using chemical probing to differentiate between solvent-exposed and buried surfaces of spliceosome proteins. This method is also being used to probe the differences between the surfaces of salt stable spliceosome and C complex. By constraining relative positions of components within the structure, our data will serve as a starting point for generating a more detailed model of the spliceosome.

459 NMR structure of the full length U1-A reveals two RRMs that interact unexpectedly together

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U1snRNP is critically involved in early stages of spliceosome assembly since it is required for the recognition of the 5' splice site. U1snRNP is composed of the U-rich U1snRNA, seven Sm proteins forming a ring around the Sm site and three U1 specific proteins. The low-resolution crystal structure of U1snRNP has explained the atomic organization of the particle (1) even though several parts were not visible, including the 100 amino-acids long linker and the C-Terminal RRM (RRM2) of U1-A. It is well known how the N-Terminal RRM of U1A (RRM1) binds to U1snRNA-SL2 but there is no structural data showing the full length protein free or bound to U1snRNA-SL2. Moreover, the U1-A RRM2 function remains unknown and we cannot describe its arrangement in the context of U1snRNP. We aim to gain insights into the binding mechanism of the full length U1-A to U1snRNA by solving the structure of the protein both free and bound to U1snRNA-SL2.

We have solved the solution structure of the full length U1-A in its free state using NMR spectroscopy. The structure reveals quite unexpectedly that both RRMs interact together in an anti-parallel fashion. This interaction is mediated by helix α1 and the loop between helix-α2 and strand-β4 in RRM1 and the two α-helices in RRM2. The structure also shows that the β-sheet surface of RRM2 is fully exposed to the solvent whereas the β-sheet surface of RRM1 is partially masked by its N-Ter helix and possibly other parts of the interdomain linker. This is the first time than such an interaction between the two RRMs of U1-A has been shown. Comparison with other tandem RRM structures as well as to the structure of the individual domains will be presented. The implication of the structure for U1A-RNA interaction will also be discussed.

1. Pomeranz Krummel DA et al., Nature 2009

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460 Retinitis pigmentosa mutations of human Prp8 cause defects in spliceosome assembly and reduce splicing efficiency

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The Prp8 protein is a highly conserved pre-mRNA splicing factor and a component of the U5 small ribonucleoprotein particle (U5 snRNP). Mutations in human Prp8 (hPrp8) cause autosomal dominant retinal disorder called retinitis pigmentosa (RP). Here, we prepared eight RP mutations of hPrp8, tagged the mutants with GFP and expressed them stably in human cell culture. We show that all the eight mutations reduced splicing efficiency with effects ranging from strong to mild. We further demonstrate that six out of eight tested RP mutations inhibited various steps of U5 and tri-snRNP formation. We also provide evidence that RP-hPrp8 mutant proteins are less stable and undergo faster degradation compared to the wild-type protein. Finally, we tested the effect of RP-Prp8 mutations in Drosophila melanogaster Interestingly, tissue specific overexpression of RP-Prp8 mutants in resulted in developmental arrest, delayed pupation or no phenotype depending on the specific mutation. Importantly, the severity of the phenotypes in Drosophila correlated with the effect of mutations on splicing efficiency and snRNP assembly in human cell culture. In conclusion, our data suggest that defects in spliceosome assembly and pre-mRNA splicing are reasons underlying RP.

461 Large-scale proteomics and sequencing approaches systematically identify stress granule protein and RNA components in human neuronal cells

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Stress granules are ribonucleoprotein aggregates in mammalian cells that assemble in response to cellular stresses such as oxidation or heat shock. Stress granules are thought to be sites of mRNA "triage", where mRNAs can be temporarily sequestered from translation until the cell recovers. Recently, stress granules have been implicated in the pathogenesis of some neurodegenerative diseases, potentially by facilitating the formation of toxic aggregates of RNA binding proteins that are frequently mutated in diseases such as Amyotrophic Lateral Sclerosis. Despite this key role in cellular control of translation and potentially human disease, we know very little about the protein and RNA components within stress granules. To address these deficits, we have utilized CRISPR/Cas9 genome editing, ascorbate peroxidase-mediated biotinylation and mass spectrometry to build the first systematic and unbiased inventory of stress granule proteins in human iPSC-derived neural progenitor cells. In parallel, we have developed a modified immunoprecipitation followed by sequencing procedure to identify genome-wide RNA targets of stress granule associated RNA binding proteins in neural progenitor cells and motor neurons. Not only are these cell types directly relevant to human neurodegenerative disease, but importantly the genome editing approach also eliminates the need for exogenous overexpression of stress granule components. We are currently expanding this combined proteomic and RNA-centric approach to other known stress granule components. Our results enhance our understanding of molecular interactions within stress granules and guide our efforts to modulate stress granule dynamics in the context of human disease

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462 Structural studies on the yeast and human U6 snRNPs

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U6 is the shortest of the five spliceosomal RNAs, but has key functions in pre-mRNA splicing, such as binding of the intron 5′ splice site and coordination of two catalytic magnesium ions. Thus, precise control of the conformation of U6 snRNA is crucial for spliceosome function. We seek to determine the structure of U6 snRNA and its interaction partners throughout the splicing cycle, beginning with the U6 snRNP, which comprises U6 snRNA, the RNA chaperone Prp24, and the Lsm2-8 heteroheptameric protein ring.

Yeast Prp24 contains four RNA Recognition Motifs (RRMs) and a conserved C-terminus that interacts with the Lsm2-8 ring. We recently determined the structure of most of yeast U6 snRNA bound to all four RRMs of yeast Prp24, revealing a unique interlocked protein-RNA architecture that sequesters the 5′ splice site-binding region in U6 snRNA and generates a tri-RRM electropositive groove on the surface of Prp24 that likely functions in U4/U6 annealing. We now seek to determine the structure of the complete U6 snRNP, containing full-length U6, Prp24 and the Lsm2-8 ring. To this end, we have reconstituted milligram quantities of homogenous U6 snRNP particles for structural studies, which are expected to reveal the physical basis for cooperative binding of Prp24 and the Lsm2-8 ring to U6, and how Lsm2-8 enhances Prp24-mediated annealing of U4/U6.

The human ortholog of Prp24 contains only two apparent RRMs, and seven Half A Tetratricopeptide (HAT) repeats. Interestingly, *S. cerevisiae* Prp24 has no HAT repeats and *S. pombe* Prp24 has 4 RRMs and at least 3 HAT repeats. To clarify structural and mechanistic similarities between U6 snRNPs separated by more than 1 billion years of evolution, we generated an isotopically-labeled di-RRM construct of human Prp24 that yields multi-dimensional NMR spectra of outstanding quality. In addition, we isolated a protease-resistant fragment of human Prp24 that contains all of the HAT motifs and is crystallizable. Structure determination of human Prp24 will help to explain how the two RRMs correspond to the tetra-RRM architecture in yeast, and provide a basis for understanding the function of the variable HAT motifs.

463 Investigating snRNP biogenesis using single RNA imaging in human cells

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Intracellular single-molecule, high-resolution localization and counting (iSHiRLoC) is a technique developed by Nils Walter's lab that combines RNA microinjection with highly inclined laminar optical sheet (HILO) microscopy to enable single-particle tracking, localization, and photobleaching analysis of singly-labeled RNAs in human cells. We have begun to use iSHiRLoC to study the processes of snRNA maturation and snRNP biogenesis in human U2OS cells.

We are investigating the role of the U1-A protein in the nuclear export of the U1 pre-snRNP. U1-A protein binds directly to the second stem loop (SLII) of the U1 snRNA. Using nucleotide substitutions within SLII which disrupt this interaction we are studying the effect of loss of U1-A protein binding on the nuclear export kinetics of U1 snRNAs that have been microinjected into human U2OS cells. Microinjection provides us with a high degree of control over the amount of RNA introduced into the cell and the time and site of introduction. In combination with HILO microscopy, we can image single U1 snRNA molecules with a resolution well beyond the diffraction limit and determine the transport kinetics and cellular distribution of the U1 snRNAs post microinjection. The newest results from this approach will be presented.

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464 Genome-wide data reveal molecular functions of mRNA-binding proteins

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Cotranscriptional packaging of mRNA into an mRNP by RNA-binding proteins is an essential step of gene expression. In S. cerevisiae, the conserved TREX complex couples transcription to mRNA export and binds cotranscriptionally to the mRNA. It consists of the subcomplex THO, a heteropentameric complex, the nuclear mRNA export factors Sub2 and Yra1 and the SR-like proteins Gbp2 and Hrb1. Further proteins cotranscriptionally binding to the mRNA are the SR-like proteins Nab2 and Npl3. Genome-wide chromatin immunoprecipitation (ChIP) experiments of these proteins revealed that the occupancy at genes of THO and the TREX components Sub2 and Yra1 increases from the 5'- to the 3'-end of the gene. This pattern is similar to the one of phosphorylation of serine 2 (S2) of the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (RNAPII), a platform for the recruitment of mRNA processing and packaging proteins. Biochemical experiments revealed that the increase in TREX components is mediated by its direct binding to the S2-S5-phosphorylated CTD. Furthermore, this 5' to 3' increase is important for the expression of long mRNAs. In addition, by this genome-wide approach we discovered that Nab2 occupies genes transcribed by a different RNA polymerase, RNAPIII. Biochemical data showing that Nab2 is important for efficient RNAPIII transcription will be presented.

465 Scaffolding by B complex-specific proteins during spliceosome catalytic activation

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Pre-mRNA splicing is catalyzed by a large and highly dynamic RNA-protein (RNP) molecular machine, the spliceosome, which is composed of five small nuclear (sn) RNPs and numerous non-snRNP proteins. For each round of splicing, a spliceosome is assembled, catalytically activated and, after splicing catalysis, disassembled in a stepwise fashion. Transitions between functional stages in this reaction pathway are characterized by the ordered recruitment and release of snRNPs and non-snRNP proteins. While the functions of the snRNPs and many of their components have been studied extensively, comparatively little is known about the functions and mechanisms of action of the non-snRNP proteins. In human, a group of seven non-snRNP proteins, the B-specific proteins, joins the spliceosome during B complex formation and leaves immediately afterwards during the B-to-B^{act} transition. Their temporally restricted appearance during a phase of the splicing cycle that is characterized by major RNP remodeling is reminiscent of the action of many spliceosomal RNA helicases, but the B-specific proteins do not include a helicase activity. Using *in vitro* assembly and splicing assays, we show that the three B-specific proteins, Prp38, MFAP1 and Snu23, are each required for the B-to-B^{act} transition during spliceosome activation and that MFAP1 mediates recruitment of Prp38 to the spliceosome. Binding analyses demonstrate that MFAP1 and Snu23 interact with Prp38 but not with each other. Bioinformatics and biophysical studies reveal that large parts of MFAP1 and Snu23 belong to a special subgroup of intrinsically disordered proteins, the charged single α-helical (CSAH) proteins, and crystal structure analyses of binary and ternary complexes show that they maintain small interfaces with Prp38 *via* helical interaction motifs. Quantitative binding studies demonstrate that these interactions, nevertheless, can be very tight. Besides these short, stable α-helical interaction modules, other parts of the B-specific CSAH proteins appear to be more dynamic and might undergo disorder-to-order transitions upon binding of other partners. We suggest that MFAP1 and Snu23 act as contractile scaffolds that bring building blocks of the B complex into the correct spatial arrangement to trigger the transition to the B^{act} stage and subsequent catalytic activation of the spliceosome.

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466 The capacity of target silencing by *Drosophila* PIWI and piRNAs

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Although Piwi proteins and Piwi-interacting RNAs (piRNAs) actively silence transposable elements (TEs) at the transcriptional level, it is unclear how the highly diverse piRNA populations direct Piwi proteins to silence TE targets without silencing the entire transcriptome. To determine the capacity of PIWI/piRNA-mediated silencing, we introduced piRNA-targeted reporter genes into *Drosophila* OSS cells, containing a simplified version of the Piwi pathway. These reporters show that a bulk threshold of piRNAs are required to target the reporter's transcripts to engage PIWI-mediated silencing. Despite this bulk requirement of piRNA targeting, our data also indicates a differential silencing capacity between distinct piRNA populations in our OSS cells. These results suggest that the extent of PIWI/piRNA-mediated silencing may be dependent upon both the piRNA sequences themselves, as well as their abundance. Subsequent experiments using this reporter platform will help facilitate future dissections of the PIWI-targeting mechanism.

467 Small RNA Pathways as Guardians of Germline Gene Expression

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Small RNA pathways have emerged as key players in maintaining a balance between silencing the expression of exogenous or deleterious (nonself) nucleic acid and licensing the expression of endogenous (self) genes in various species. These functions are especially critical in the germline, which is an immortal cell lineage in organisms such as the nematode *C. elegans*. In the *C. elegans* germline, the piRNA-mediated surveillance system encodes tens of thousands of unique 21-nucleotide piRNAs, which set off a cascade of events to silence a variety of deleterious nucleic acids. If left unchecked, the piRNA pathway would have the potential to recognize and silence nearly the entire *C. elegans* germline transcriptome, thus counter measures are also in place to promote germline gene expression.

My lab recently demonstrated that the Argonaute CSR-1 is guided by its 22G-RNA partners to nascent transcripts, where it promotes germline transcription to counteract piRNA pathway silencing. We are now using a variety of approaches to dissect the molecular mechanisms by which CSR-1 modulates chromatin at its targets to license transcription. First, we have characterized the complexion of euchromatic histone modifications present at the germline genes targeted by CSR-1 and demonstrate that several of these histone modifications are altered in response to loss of *csr-1*. Concurrently, other regions of the genome (not targeted by CSR-1) also become mis-regulated upon loss of *csr-1*, pointing to a key role for CSR-1 in genome-wide chromatin organization. Second, through a series of screens and candidate approaches, we have identified conserved chromatin modifying factors, including a histone chaperone and a histone methylase, that physically interact with CSR-1 and are likely to execute several of the chromatin-directed functions of this pathway. Together, these histone modifications and small RNAs are transmitted from one generation to the next to maintain an epigenetic memory of germline gene expression and promote fertility. Finally, our recent comparative genomic studies of *Caenorhabditis* nematodes indicate that the conserved gene targets of the CSR-1 pathway are evolving more slowly than other conserved germline genes, indicating that CSR-1 may also play a role in the evolutionary preservation of its target genes.

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468 microRNAs as biomarkers of drug-induced liver and heart injury

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MicroRNAs (miRNAs) are small non-coding regulatory RNAs found in all multicellular organisms. Since their discovery in 2001, there has been impressive progress in miRNA research, and a great deal is now known about the biosynthesis of miRNAs and their regulatory function in messenger RNA degradation and translation. Furthermore, the potential roles of microRNAs in cellular responses to xenobiotic stress, the development of pathophysiological changes and other toxicological phenomenon such as susceptibility/resistance to xenobiotics are currently the focus of active research. In particular, we have investigated the temporal sequence of molecular and pathological perturbations at early stages of phenobarbital (PB) mediated liver tumor promotion in vivo. Molecular profiling (mRNA, miRNA, Histone modification, DNA methylation, and proteins) of mouse liver during 13 weeks of PB treatment revealed progressive increases in hepatic expression of long noncoding RNAs and miRNAs originating from the Dlk1-Dio3 imprinted gene cluster, a locus that has recently been associated with stem cell pluripotency in mice and various neoplasms in humans. Our data identify Dlk1-Dio3 ncRNAs as novel candidate early biomarkers for mouse liver tumor promotion and provide new opportunities for assessing the carcinogenic potential of novel compounds.

MicroRNAs have also important functions in cardiac physiology and pathologies and have also been implicated in mechanisms of drug-induced cardiac tissue injury. We have profiled microRNAs from 8 different heart structures across species by using a miRNA-seq approach to build a microRNA heart atlas. The resultant microRNA signatures were used to discriminate between different heart tissue substructures. Potential microRNA-mRNA interactions were explored using anticorrelation expression analysis and microRNA target prediction algorithms. A subset of microRNA/mRNA interactions were further characterized and validated using *in vitro* reporter gene assays. Moreover, we have explored the potential correlation between drugs known to trigger cardiac valve liabilities in rats and elevated levels of cardiac valve-enriched microRNAs in plasma/serum.

Our data show that the profiling of miRNAs in the context of drug safety assessment can add value by providing 1) novel insight into molecular mechanisms of toxicity and 2) novel safety biomarkers, especially when measured in body fluids such as plasma.

469 MiR-200 family and HuR play a new game in the regulation of the proto-oncogene c-Jun

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MiR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) is a cluster of miRNAs highly correlated with epithelial-mesenchymal transition (EMT). A biphasic role of miR-200 family is supported by a lot of studies that show that primary cancer cells downregulate miR-200 expression at the invasive front where they undergo EMT and upregulate miR-200s in the resulting metastasis where mesenchymal to epithelial transition (MET) facilitates colonization of a distant tissue . Therefore, the miR-200 family seems to have a dynamic role in tumor progression, following a still unclear mechanism.

In order to clarify this mechanism, in this work we describe the regulation of c-Jun and Dual-specificity phosphatases 1 (DUSP1) mRNA mediated by two members of the miR-200 family: miR-200a and miR-200b. C-Jun is the main component of the AP-1 transcription factor that play an essential role in almost all areas of eukaryotic cellular behavior, DUSP1 is one of many phosphatases coded by the mammalian genome, its role is to dephosphorylate and therefore inactivate the MAP kinases such as ERKs and JNKs.

By bioinformatics analysis we identified putative binding sites for both miR-200a and miR-200b in 3'UTR of mRNA of c-Jun and DUSP1 . Surprisingly, our reporter assay revealed an opposite action of the two miRNAs on this 3'UTRs: overexpression of miR-200b reduces luciferase activity whereas miR-200a increase it.

Interestingly, miR-200a target site is into the well-described ARE for both mRNAs, and this ARE is bounded by the HuR RBP. So we wondered whether this RBP could play a role in this non canonical regulation. After transfection of the siRNA against HuR, we lost the stabilizing effect of miR-200a, suggesting the presence of a potential interaction between the RBP and the miR-200a on the mRNAs 3'-UTR.

These results suggest a new regulatory mechanism for microRNA in cooperation with HuR and highlight a new potential role for miR-200a in tumor progression.

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470 De novo design of primary microRNAs

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MicroRNAs (miRNAs) are small regulatory RNAs that are processed from stem-loop regions of primary transcripts (pri-miRNAs), with the choice of specific stem-loops for initial processing being the most selective step of the miRNA biogenesis pathway. Previous high-throughput approaches for determining the pri-miRNA features that influence this choice were limited to examining the loop and flanking regions of pri-miRNA hairpins, because examining the stem region was more technically challenging. Here, we developed a bar-coding approach that overcame this challenge, and applied this approach to generate >50,000 stem-region variants of three pri-miRNA stem-loops and determine the extent to which each variant retained function. Apart from a mismatch motif in the basal portion of the stem, which can enhance pri-miRNA processing, pairing was preferred throughout the stem region, with a narrow tolerance observed for stem length. Satisfying only these structural constraints was sufficient for the design of artificial pri-miRNAs that were processed nearly as well as natural miRNAs. Adding known primary-sequence elements increased processing such that it exceeded that of natural miRNAs. Using these insights, we were then able to generate functional pri-miRNAs resembling those of humans but designed without reference to any natural sequence. This ability to reliably design pri-miRNAs de novo demonstrates improved understanding of critical features of miRNA genes.

471 A Novel Role for Symplekin in the Biogenesis of Endogenous Small Interfering RNAs

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Small RNAs (siRNAs) participate in numerous cellular processes to control gene expression and consequent protein production within the cell. Errors in metabolism of these small RNAs can lead to developmental defects and disease, including cancer. Because siRNAs are ubiquitously involved in overall cellular function, it is important to study their maturation. siRNAs are derived from both exogenous (exo-siRNA) and endogenous (endo-siRNA) sources. Exo-siRNAs have been extensively studied in *Drosophila* since the late 1990s, however, endo-siRNAs have only recently been discovered. Endo-siRNAs are found in *Drosophila* and mice, and silence both mobile elements and protein coding genes. Maturation of these endo-siRNAs from their precursor dsRNAs requires specific proteins such as Dicer-2 (Dcr-2) and Loquacious (Loq), however other factors involved remain uncharacterized. Recent data from our lab suggests that one of these uncharacterized factors may be the scaffold protein Symplekin. Symplekin binds several other proteins (such as CPSF73 and CPSF100) forming a core cleavage complex responsible for correct processing of the 3' end of both histone and canonical mRNAs. An immunoprecipitation assay (IP) using an antibody specific to Symplekin followed by mass spectroscopy revealed not only the suspected binding partners of Symplekin, but also novel proteins such as Dcr-2. The Symplekin-Dcr2 interaction was then confirmed via co-immunoprecipitation (co-IP) followed by western blotting. Additionally, Dcr-2 was found to bind other members of the core cleavage complex (CPSF73 and CPSF100) as well. To investigate the potential role of Symplekin in endo-siRNA biogenesis, northern blots probing for a specific endo-siRNA (esi2.1) were performed. This experiment revealed that when Symplekin is RNAi-depleted, levels of esi2.1 also decrease, suggesting a role for Symplekin in its production. This result was further confirmed by RT-qPCR. To further investigate this novel role of Symplekin, our lab performed High Throughput Sequencing (HTS) on small RNA libraries that have been RNAi-depleted of Symplekin. Preliminary results show global decreases in small RNA levels, specifically with respect to endo-siRNAs. These data suggest that in addition to the role of Symplekin in 3' end processing of mRNA, it may also have a role in the biogenesis of endo-siRNAs.

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472 Reducing Bias in Small RNA-Sequencing

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Next generation sequencing is an ideal technology for the study of small RNAs, as it allows precise measurement of closely related small RNAs and novel small RNAs that hybridization-based methods like microarray and qPCR cannot achieve. Unfortunately, NGS approaches for small RNA analysis are not without their own challenges. Several studies have now shown entire datasets, including those in miRBase, to contain severe sequence bias; specifically, small RNA expression that is not accurately represented by sRNA-seq. Significant effort has gone into identifying the cause of this misrepresentation, and it is now generally accepted that bias in sRNA-seq libraries is primarily introduced during the adapter ligation steps in library preparation. Specifically, RNA ligases show sequence-specific preferences toward certain adapter-small RNA pairs, resulting in preferential inclusion of some small RNAs in sRNA-seq libraries, at the expense of others. Simply using two different adapter sequences during ligation can result in up to 30-fold differential expression for some microRNAs.

Studies have shown that ligation bias can be greatly reduced by using adapters with 2-4 random nucleotides at the ligation junctions. Thus, our approach to overcoming ligation bias in sRNA-seq libraries involves using a pool of adapters with 4 random bases at the ligation sites. Using our randomized adapter strategy, small RNA libraries were prepared and sequenced using both synthetic small RNAs and total RNA isolated from various human tissues as starting material. The results clearly demonstrate the vastly reduced bias achieved through the use of adapters with randomized ends, and show that this kit is effective in preparing small RNA libraries using total RNA from various tissues. These results demonstrate that our new streamlined small RNA-seq protocol is ideal for those needing to accurately assess small RNA abundance in diverse sample types.

473 Apple miRNAs and their role in Fire Blight resistance

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Micro RNAs (miRNAs) are small, single stranded RNA molecules, which are key players in multiple biological processes in plants and animals. To date, it has been reported that plant miRNAs, by targeting many regulatory genes, play an important role in such processes as: plant development, hormone signaling or biotic and abiotic stress response. In apple (Malus domestica), 200 microRNAs are known, which most probably represent only a fraction of miRNAome diversity. As a result, more effort is required to better annotate miRNAs and their functions in this economically important species.

In the first part of our research we characterized miRNAs, which are specific for Gala apple scions grafted on four different rootstocks: B.9, G.30, M.27 and M.111, presenting diverse Fire Blight resistance. In order to identify miRNA species, as well as their expression levels in selected trees, SOLiD sequencing of small RNAs has been performed. All the reads have been mapped to the apple genome (http://www.rosaceae.org/projects/apple_genome) and searched for conserved and apple-specific miRNAs. Performed analyses allowed us to extend the apple miRNA repertoire by 38 conserved and 78 novel, apple specific, miRNA as well as verify 143 miRNAs from previous studies. We confirmed five of new miRNAs using qPCR or RT-PCR. We also identified miRNAs with significantly changed expression among analyzed rootstocks, which might be potentially involved in Fire Blight resistance. Performed analyses let us define four apple miRNAs potentially involved in fire blight resistance in apple trees: mdm-miR169a, mdm-miR160e, mdm-miR167b-g, and mdm-miR168a,b. These miRNAs are known to be involved in response to stresses across other plant species, usually by targeting stress response proteins. Our data suggests that apple microRNAs might be considered as regulators and markers of fire blight resistance.

The second part of our research is focused on the apple response to E. amylovora infection. Using Illumina sequencing method to identify miRNAs and their expression levels in apple leaves inoculated with bacteria, we identified 234 novel miRNA candidates. A few of them seem to be differentially expressed in response to inoculation or just leaf cutting with no bacteria, which may suggest their role in bacterial stress response in apple trees.

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474 Multidrug resistance-specific small RNAs in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa (PA) is a Gram-negative bacterium of the family *Pseudomonadaceae* and can switch from being an environmental isolate to human pathogen. One of the major characteristics of this bacterium is its resistance to multiple antibiotics which is due to the highly coordinated and complexity of regulatory networks, whereas small RNA (sRNA) is one of the key players in post-transcriptional regulation of immediate responses to stresses. The majority of the sRNA-induced events commonly required the bacterial Sm-like protein Hfq, which interacts with regulatory sRNAs and mRNAs to facilitate the generally short and imperfect complementary base pairing interactions.

In this study, we sequenced the sRNA repertoires and transcriptomes of six representative clinical isolates that included three drug-susceptible and three multidrug-resistance (MDR) strains based on their susceptibilities to different antibiotics, and identified numerous sRNAs that are specifically down-regulated in MDR strains. Moreover, overexpression of these sRNAs in the MDR strain reduces the minimal inhibitory concentration (MIC) of certain antibiotics which suggests the regulatory role of these sRNAs contributed to the MDR of this bacterium. After correlating the expression patterns of all genes to the sRNAs, we identified and validated several sRNA-regulated genes that involve directly in the resistance mechanisms of multiple antibiotics. In summary, we identified numerous novel sRNAs in PA by RNA sequencing and characterized the intrinsic role of two MDR-specific sRNAs as well as their mechanisms to MDR. These findings provide us potential genomic determinants of MDR in PA which can be used as new targets for therapeutic as well as the design of diagnostic techniques in the future.

475 The structure and sequence determinants of sRNA competition for binding to Hfq

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Small bacterial RNAs (sRNAs) are involved in the response to stress conditions, participate in the maintenance of cellular homeostasis, and affect the virulence of pathogenic strains. sRNAs regulate translation by binding to partly complementary sequences in selected mRNAs. The homohexameric, Sm-like protein Hfq is often required for efficient sRNA binding to their targets. Recent data showed that there is a hierarchy among sRNAs in the competition for access to Hfq, which could be important for the tuning of this regulation. Hfq contains three independent RNA binding sites on both faces of its ring and on the outer rim. It has been proposed that the distal face binds adenosine-rich sequences in mRNAs, while the proximal one binds the uridine-rich sequences in sRNAs.

Here, competition properties of several structurally different sRNAs were compared using a filter-based assay. Moreover, their binding to Hfq mutants was compared to test their specificity towards the RNA-binding sites on Hfq. The data showed the important role of the proximal and rim sites of Hfq for the binding of 6 out of 7 sRNAs. However, ChiX sRNA bound Hfq in a unique way using the opposite, distal and proximal, faces of this ring-shaped protein. The data suggested that this unique mode of binding was enabled by separate adenosine-rich and uridine-rich sequences in the long, single-stranded region of ChiX. Finally, several other sRNA molecules were compared in competition against oligoriboadenylates to test if the distal face binding is also used by other sRNAs.

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476 Comparison of ovary- and testis-derived piRNAs reveals gender-specific piRNA clusters in Syrian hamster

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PIWI proteins associate with PIWI-interacting RNAs (piRNAs), 23-32 nucleotides in length, and mainly repress transposons in germ cells. In mice, piRNAs are most abundantly expressed in male germ cells, especially during spermatogenesis, and are loaded onto the three PIWI proteins (PIWIL1, PIWIL2 and PIWIL4). Depletion of individual PIWI genes causes male-specific sterility because of severe defects in sperm formation, indicating their nonredundant functions of PIWI genes in the testes. In contrast, PIWI proteins in *Drosophila* are required for both male and female germ cell formation, indicating the essential role of piRNAs regardless of genders in the fruit fly. We have characterized PIWIs and piRNAs in Syrian hamster. Unlike mouse PIWIL1, Syrian hamster PIWIL1 is highly expressed in ovarian germ cells where it associates with piRNAs, implicating that PIWIL1-piRNA complexes may have a role in ovarian development. Characterization of PIWIL1-associated piRNAs derived from male or female gonad revealed PIWIL1-associated piRNA precursors are transcribed from gender-specific piRNA clusters. In this meeting, we will discuss potential functions of PIWIL1 in ovarian development in Syrian hamster.

477 Hfq C-terminus affects RNA binding and annealing

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Hfq is a bacterial RNA chaperone which post-transcriptionally regulates mRNAs involved in many cellular processes including metabolism, stress responses and virulence. Hfq binds to small RNAs (sRNAs) and mRNA leader sequences, increasing the rate of base pairing and stabilizing the sRNA-mRNA complex. Positively charged, arginine-rich patches on the rim of the Hfq hexamer are essential for its annealing activity. Access to these positively charged patches may be controlled in order to decrease the propensity of Hfq to bind RNA non-specifically, limiting its chaperone action to true substrates. In many different genera, Hfq possesses an intrinsically disordered C-terminal tail. The role of these tails is debated and there is conflicting data for their importance in riboregulation *in vivo*. We truncated *E.coli* Hfq, removing the entire disordered tail. The remaining 65 residue Sm-core (Hfq_{65}) was less active in annealing of sRNAs to actively transcribed mRNAs *in vitro*, yet more active than full-length Hfq on unstructured "toy" RNA lacking a strong, specific Hfq binding site. Overall, our results suggest that the Hfq C-terminus obstructs the arginine-rich active sites of Hfq. This auto-inhibitory function of the Hfq C-terminal domain enforces the formation of annealing-competent complexes of Hfq with long and structured RNAs.

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478 Essential roles of a Tudor domain-containing protein, Krimper, in piRNA biogenesis in Drosophila ovaries

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Transposable elements (TEs) potentially cause harmful genomic mutations; hence, TE silencing is essential for living organisms. TE silencing in the germline involves PIWI-interacting RNAs (piRNAs). piRNAs are not accumulated in *krimper* (*krimp*) mutant ovaries, suggesting its requirement in piRNA biogenesis. Krimp contains a Tudor domain, a module known to be associated with protein partners through their symmetrical dimethylarginines (sDMAs). In this study, we found that Krimp associates with a Drosophila PIWI protein Ago3, but not with remaining PIWI proteins Piwi and Aurbergine (Aub), in an sDMA-insensitive manner and that Ago3 within the Krimp complex is devoid of piRNAs. In *krimp* mutants, Ago3 is not dimethylated and fails to localize to the nuage, a perinuclear region considered to be the site for the ping-pong cycle, resulting in deterioration of heterotypic ping-pong between Aub and Ago3. Instead, the signature for homotypic ping-pong by Aub alone was observed; yet, it failed to supply piRNAs sufficient for TE silencing. Loss of Aub caused Krimp mislocalized in distinct cytoplasmic foci, where Ago3 also accumulated. Studies using OSC line showed that Ago3 has capability to be associated with primary piRNAs. Thus, sequestration of Ago3 to Krimp-positive foci is highly likely to avoid homotypic ping-pong by Ago3. Based on these observations, we suggest that Krimp has dual functions to control the ping-pong cycle; by promoting Ago3's sDMA modification to guarantee heterotypic ping-pong between Ago3 and Aub, and by sequestering Ago3 to specific cytoplasmic foci to avoid primary piRNA loading into Ago3.

479 Multiplex STTMs for functional interaction of microRNAs: A comparison between poly-cis STTMs and poly-ribozyme-cleaved STTMs

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MicroRNAs (miRNAs) are small non-coding RNAs, containing 21~24 nucleotides, which function in post-transcriptional regulation of gene expression. miRNAs play key roles in controlling plant development and defense against biotic and abiotic stresses. Recently, Short Tandem Target Mimic (STTM) technology, that can target specific miRNAs of interest for destruction without affecting other miRNAs, has been developed. The utilization of STTM makes it easier to understand the functions of different miRNAs. Based on the STTM technology, we invent two multiplex STTMs methods: Poly-cis STTMs and Poly-ribozyme-processed STTMs to investigate the functional interactions between different miRNAs. Poly-cis STTMs link different STTMs together by the linker which functions as a complex after being transformed to plants. While Poly-ribozyme-processed STTMs function independently after been transformed to plants. We use STTM165/166, STTM156/157 and STTM172, which all have different strong phenotypes in Arabidopsis, to form the two kinds of multiplex STTMs. Both the multiplex STTMs have the same characteristics with those three STTMs. Functional characterizations and comparison of the two types of multiplex STTMs are under way.

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480 Competition between Spliceosome, Microprocessor and Polyadenylation Machineries in the biogenesis of miRNAs located within the first intron of host genes in *Arabidopsis thaliana*

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MicroRNAs are small non-coding RNAs (21 nt), which act by regulating expression of many genes. In plants, miRNAs are encoded mostly by independent transcription units, but in *Arabidopsis* at least 29 plant miRNAs are embedded within introns of other genes.

Is there a crosstalk between spliceosome, microprocessor and polyadenylation machinery during plant intronic microRNA biogenesis?

For our analysis we selected miR402 located within the first intron of a protein-coding gene - At1g77230. We found upregulation of miR402 level in heat-stress conditions which correlated with the inhibition of splicing of miR402-carrying intron. Additionally, within this intron we identified alternative proximal polyadenylation site. Its activation was strongly associated with splicing inhibition and miR402 accumulation. Similar results were obtained in the case of other abiotic stress conditions. It suggests that miR402 is not processed from an intron, but rather from shorter transcript after selection of proximal polyA site within this intron.

To evaluate the exact role of splicing machinery during miR402 processing we generated constructs containing MIR402 host gene with mutated splice sites and transiently expressed them in N. benthamiana. The strong accumulation of the miRNA was observed in each construct carrying mutated 5'SS, which correlated with the splicing inhibition and activation of proximal polyadenylation site. Additionally, we incubated *Arabidopsis* seedlings with Gex-1A to generally inhibit splicing events and we confirmed that selection of proximal polyadenylation site within retained intron is crucial during miR402 processing.

We also shifted pre-miR402 from the intron to the first exon of MIR402 gene and we observed that the active 5'SS plays an opposite role in microRNA maturation depending on its position relative to this splice site - after splicing inhibition of the first intron, the level of miR402 produced from exon was downregulated despite the activation of proximal polyA site. Moreover, shifting the pre-miR402 to the second intron abolished miRNA production.

Performed experiments point to the strong competition between spliceosome and plant microprocessor complexes in the case of miRNA production which are located downstream of the U1snRNP binding site. We also revealed that the accessibility of polyA sites plays an important role in plant intronic microRNA biogenesis.

481 Application of Short Tandem Target Mimics (STTMs) For Deciphering Functions and Regulatory Networks of microRNAs in Crop Plants

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microRNAs are small non-coding RNAs that suppress the expression of their complementary target genes at the post-transcriptional level through either cleavage and/or translational inhibition. miRNAs play key roles in controlling plant development and defense against biotic and abiotic stresses. Hundreds of miRNAs have been identified from different plant species and are awaiting functional analysis. Despite underlying importance of regulatory roles of miRNAs, a robust methodical way to study miRNA functions has not been devised due to established redundancy among different members of a miRNA family and lack of tools to effectively knock down miRNA levels. Recently, Short Tandem Target Mimic (STTM) technology, that can target specific miRNAs of interest for destruction without affecting other miRNAs, has been developed. In this study, we have applied STTM technology in a few major crops and generated a large population of STTM miRNA-knockdown transgenic collections both as a resource and materials for functional genomics studies of crop miRNAs. STTM transgenic plants of rice, tomato, soybean and Arabidopsis, targeting some conserved and functionally important miRNAs, are characterized and presented in detail. One such study found that STTM transgenic plants targeting miR165/166 exhibit quite similar phenotype across different species, which includes deformed and curled leaves, short stature and reduced seed set. Deep sequencing analyses of the above STTM transgenic plants targeting different miRNAs is being done. Functional conservation and diversification of miRNA-regulated networks across different species are discussed.

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482 Can pha-siRNAs integrate into large scale regulatory networks?

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The discovery of RNA silencing has revolutionized our understanding of gene regulation and expression in eukaryotic cells. Investigations of how RNA silencing operates in cells are the focus of the new field of small RNA (sRNA) biology. Several sRNA pathways have been identified, and the biochemical machinery has been described. Thus far, one class of sRNAs has only been observed in plants, the phased small interfering RNAs (pha-siRNA). Selection in plants of an adaptive immune response analogous to that found in animal systems may have driven the evolution of this pathway, first as a defense response and later adopted more broadly for modulation of gene regulation. Pha-siRNAs have the potential of amplifying an initial signal delivered by microRNAs (miRNA) into a cascade affecting the expression of multiple genes. Several cascades have been reported to be involved in regulation of major cellular processes, including cell development, metabolism and defense responses to abiotic and biotic stress. Considering in silico analyses that reveal a large number of potential pha-siRNA interactions and cascades, I hypothesize the existence of a novel mechanism for broad scale gene regulation based on the integration of siRNA cascades into complex pha-siRNA networks. I am developing an experimental model in which I can demonstrate how these networks operate to induce and alter gene expression during infection by a pathogen.

The ability of viruses to alter host gene expression and to interfere with sRNA metabolism provides a suitable model system to investigate the development of pha-siRNA networks and profile resulting altered gene expression. To investigate the mechanism of gene regulation by pha-siRNA networks, an Arabidopsis thaliana-Cucumber mosaic virus (CMV) model system will be employed. Using the Arabidopsis-CMV model system, my objectives are: 1) to determine if pha-siRNA mediated networks function in gene regulation during a plant response to virus infection, and 2) to determine how a virus interferes with the establishment of pha-siRNA networks.

483 Crystal structure of the MA3 domain of CWC22 and studying its functional role in splicing

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CWC22 is a splicing factor that is recruited to the spliceosome before the first catalytic step. The *S. cerevisiae* orthologue of CWC22 is important for positioning or regulating the splicing DExH-box helicase Prp2. The mammalian orthologue of CWC22 is required for the assembly of the exon junction complex (EJC), an assembly that plays crucial roles in translation and surveillance of spliced mRNPs. CWC22 binds a central component of the EJC, the DEAD-box protein eIF4AIII, and recruits it to the splicing machinery (Barbosa et al., 2012; Steckelberg et al., 2012, Alexandrov et al., 2012).

CWC22 contains two conserved domains, an N-terminal MIF4G domain and the following MA3 domain. We have previously shown how the MIF4G domain of CWC22 binds eIF4AIII, restraining it in an inactive, open conformation (Buchwald et al., 2013). We have now determined the crystal structure of the CWC22 MA3 domain, showing an unusual HEAT-repeat architecture. We have engineered structure-based mutants and analyzed their effects in *in vitro* splicing reactions. Hence, we identified mutants that do not affect eIF4AIII binding to CWC22 but prevent the recruitment of the heterodimer by active spliceosomes. These results indicate that the MA3 domain of CWC22 is necessary for coupling EJC assembly to splicing.

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484 SF3B1 cancer mutations select a cryptic 3' splice site that lead to downregulation of cancer relevant genes

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Heterozygous mutations in SF3B1, a component of the U2 complex involved in the recognition of 3' splice sites (ss), have been identified in various malignancies including in myelodysplastic syndrome (MDS), chronic lymphocytic leukemia (CLL) and solid tumors. To study the impact of SF3B1 mutations on splicing, RNAseq data obtained from breast cancer, melanoma, CLL and MDS patients with mutant ($SF3B1^{MUT}$) or wild-type SF3B1 ($SF3B1^{WT}$) were compared. Aberrant splice junctions were observed in SF3B1MUT samples and usage of an alternative 3'ss was the most frequent abnormality, suggesting a neomorphic function of the $SF3b^{MUT}$ complex. Several aberrant junctions were common across all hotspot mutations and diseases; however, a unique aberrant splicing profile was found for each disease suggesting lineage and disease specific effects. Next, motif analysis revealed that the cryptic AG used by $SF3B1^{MUT}$ was located most commonly 15 to 24 nucleotides upstream of the canonical AG and it was associated with a shorter and weaker polypyrimidine tract (PPT, sequence important for the recruitment of $U2AF^{65}$). By minigene assay, we confirmed the intron sequences and cryptic AG necessary to observe aberrant splicing and that a healthy $U2AF^{65}$ binding site was also required. These results suggest that the canonical AG is identified by the U2AF proteins whereas $SF3B1^{MUT}$ complex is involved in the recognition of the cryptic AG during step II of splicing.

To evaluate the fate of the aberrantly spliced genes we tested the hypothesis that the alternative 3' ss junction introduced a premature termination codon leading to nonsense mediated decay (NMD) and downregulation of the canonical gene level. Computational analysis predicted approximately 50% of the aberrantly spliced genes would be NMD-candidates. This finding was confirmed by treating $SF3B1^{MUT}$ isogenic lines with cycloheximide to inhibit NMD. In addition, SILAC proteomic analysis using the same isogenic lines confirmed the downregulation of several canonical proteins associated with aberrantly spliced genes. These data suggest that $SF3B1^{MUT}$ induces aberrant splicing resulting in downregulation of fundamental genes that are involved in malignancies. Currently, efforts are ongoing to validate potential driver genes that are affected and their mechanistic contribution to cancer pathways.

485 Global suppression of non-canonical 3'-splice site usage by Prp18

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Accurate splice site selection is essential for optimal gene expression. After the first step of splicing and subsequent ATP-dependent remodeling of the spliceosome, Slu7, Prp18, and Prp22 are recruited to facilitate the second step. In this study, we analyze the splicing factor Prp18, which interacts with Slu7 to promote efficient catalysis by stabilizing base pairing between the exon ends and the U5 snRNP invariant loop. Prp18 is not essential for splicing at normal temperatures. However, its role in stabilizing the U5-exon 1 and U5-exon2 interactions suggests that, in addition to promoting splicing efficiency, it may also contribute to the fidelity of 3'-splice site selection. Accordingly, we previously reported a novel role of Prp18 in the suppression of splicing at a non-canonical AUG 3'-splice site in the *GCR1* pre-mRNA. In this study we expand upon these findings by globally analyzing splice-junctions in cells lacking Prp18, and demonstrate that the loss of Prp18 results in the activation of non-AG 3'-splice sites at more than one third of all intron-containing genes. Many of these alternatively spliced species are degraded by nonsense-mediated decay (NMD), as shown by their increased accumulation after inactivation of the NMD helicase component Upf1. Strikingly, relative to the average 3' splice site-branch point distance of ~37 nucleotides for annotated splice sites, the non-AG 3'-splice sites in *prp18*Δ*upf1*Δ exhibit an average distance of ~11 nucleotides. We validate cases of alternative splicing by RT-PCR and demonstrate that these branchpoint-proximal alternative 3' splice sites are specifically activated in *prp18*Δ, and do not occur in the absence of the second step-splicing factor Prp17. These results illustrate a previously unappreciated role for Prp18 in the suppression of branchpoint-proximal non-AG 3'-splice sites throughout the yeast transcriptome.

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486 Reconstitution and Single Molecule Characterization of Yeast Commitment Complex

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Splicing in *Saccharomyces cerivisiae* is often initiated by the formation of the commitment complex on the intron of precursor messenger RNA (pre-mRNA), with U1 snRNP at the 5′ splice site (5′SS) and Msl5•Mud2 at the branchpoint sequence. Biochemical and genetic evidence suggest that U1 snRNP recognizes the 5′SS by RNA basepairing, but is stabilized by interactions with the other commitment complex components. Here we describe the isolation and analysis of a fluorescently labeled yeast U1 snRNP complex for use in biochemical and single molecule experiments. The complex is intact and functional in splicing extracts. Single molecule colocalization experiments are being used to determine the sequence-dependent binding interactions between the 5′SS and yeast U1 snRNP as well as a protein-free U1 mimic. In addition, cross-linking mass spectrometry is being used to analyze the protein connectivity of U1 snRNP and how it changes in response to RNA binding or commitment complex formation. These studies establish a new avenue for studying the properties which guide early assembly of the spliceosome and present a starting point for the reconstitution of the entire yeast commitment complex from purified components.

487 RNA and protein interactions of the DEAD-box RNA helicase DDX41 in the spliceosome core and its role in myeloid neoplasms

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Members of the DEAD-box RNA helicase family have prominent roles throughout RNA metabolism. In addition to unwinding RNA duplexes, these proteins can remodel RNA-protein complexes and serve as scaffolds on which ribonucleoprotein complexes assemble. Members of this protein family play a prominent role in the splicing of pre-mRNAs where they serve as key components of the spliceosome. DDX41 is a metazoan-specific helicase that has been proteomically identified as a component of spliceosomal C complexes. Sequencing of myeloid neoplastic tumors from patients has revealed mutations (both germline and tumor-specific) in DDX41. The most common germline mutation causes a frame shift in the protein, likely resulting in a nonfunctional polypeptide and reduced protein expression. The most common tumor-specific mutation, R525H, affects the ATP-binding site and abrogates the ATPase activity of the protein. While DDX41 appears essential for cell growth, little is known of its function in the spliceosome. RNA-seq analysis of mutant patient tumors has revealed a number of genes with altered spliced isoform ratios. Knockdown of DDX41 in cells results in the inhibition of splicing of a subset of introns. RNA CLIP-seq analysis of tagged DDX41 revealed preferential crosslinking to both U2 and U12 snRNAs was well as to 5' and 3' splice site regions of pre-mRNAs. Proteomic analysis of proteins co-precipitated using tagged DDX41 revealed numerous splicing factors particularly from spliceosomal C complexes. Similar analyses using the R525H mutant protein showed reduced association with several key factors including PRPF8 and SF3B1, which are also mutated in myeloid neoplasms. These data support our hypothesis that DDX41 is a core spliceosome component whose altered expression can affect the splicing of key tumor suppressor genes.

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488 Characterization of yeast strains that modulate the function of C-rich exon motifs

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To better understand how the substrate is selected and positioned at the catalytic center of the spliceosome, we identified several classes of exon sequence motifs that compensate for a suboptimal 5' splice site in yeast. C-rich exon motifs strongly suppress defects of a 5'SS-G5a mutation, which destabilizes 5'SS interactions with both U1 and U6 snRNAs, inhibiting spliceosome assembly and the first step. Whereas the C-rich motifs improve splicing of G5a introns by improving the first step, they inhibit splicing of other intron mutants limiting for the second step - U2a, A3c, BS and 3'SS mutants. Thus, these motifs generally act by improving the first step but inhibiting the second.

To identify alleles that modulate the function of C-rich exons, a *CUP1*Δ strain carrying an *ACT1-CUP1* reporter with a suboptimal C-rich exon upstream of the G5a intron was UV irradiated, and mutants with an improved copper tolerance were selected. The selected strains were subjected to genomic DNA sequencing, and in several cases the identified mutations were further confirmed in appropriate deletion strains.

The screen yielded one clear pair of mutant genes, *npl3* and *mtr10*, that dominated the results. Mtr10 and Npl3 proteins are functionally linked, as Mtr10 is a nuclear importin (karyopherin) that mediates nuclear localization of Npl3 - a nucleus-cytoplasm shuttling yeast SR-like mRNA binding protein.

As predicted, both *mtr10* and *npl3* alleles result in the accumulation of Npl3 in the cytoplasm. Furthermore, using a strain with deletion of *npl3* and appropriate *npl3*-carrying plasmids, we show that two mutations identified in the selected *npl3* mutant strain, *npl3*-L219S and a 49 aa deletion in the RGG box, contribute to the suppression of splicing defects of reporters with G5a introns and suboptimal C-rich exons. The outcome of the screen provides new insights into regulation of pre-mRNA biogenesis, and Npl3 emerges as a factor that modulates splicing fidelity.

489 Role of conformational dynamics and the domain linker for Py tract RNA binding by U2AF65

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Pre-mRNA splicing is an essential mechanism in eukaryotic mRNA processing and greatly contributes to proteome diversity by alternative splicing. An essential early step involves defining the exon/intron boundaries in the pre-mRNA transcripts. In the early spliceosomal E complex, the 3'-splice site is defined by binding of SF1 and the large (65) /small (35) subunits of the heterodimeric U2 auxiliary factor (U2AF) to the branch-point sequence (BPS), polypyrimidine (Py) tract and 3'-AG dinucleotide, respectively. Recent structural studies have revealed the molecular details of these interactions. We have demonstrated that RNA binding involves a conformational shift between open and closed arrangements of the tandem RNA-binding domains (RRM1-RRM2=RRM1,2) of U2AF65. These studies imply an important role of conformational dynamics for Py tract RNA recognition. However, the detailed structural mechanisms of how U2AF recognizes Py tract RNAs of different "strength", i.e. binding affinity, are not fully understood.

We have studied the role of the flexible linker, that connects RRM1 and RRM2, for RNA recognition and discovered that it weakly interacts with the RNA-binding interface of RRM2. This suggest an auto-inhibitory role of this interaction for RNA binding. Indeed, upon replacing the linker by a stretch of Gly-Gly-Ser repeats, we have observed noticeable increases in RNA binding affinity. Intriguingly, while the RNA binding for a strong Py tract is only slightly enhanced, a substantial increase in affinity is observed for the binding of RRM1,2 with a GS linker to weak Py tracts, as monitored by NMR and ITC experiments. This suggests a potential role of auto-inhibitory activity of the RRM1,2-linker in selecting/defining correct splice sites as a proofreading mechanism. We analyze the structure of wildtype U2AF65 RRM12 to unravel the linker/RRM2 interaction using NMR and SAXS and characterize the functional activity of wild type and GS-linker variants in spliceosome assembly assays.

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490 Functional significance of miRNA-snRNA interactions on levels of snRNAs and snRNA variants

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Although snRNAs are highly and ubiquitously expressed, snRNA expression levels and their regulation are not well understood. Moreover, there are many snRNA variants (80 U2s, >1000 U6s) annotated in the human genome with varying degrees of sequence conservation to the most highly abundant snRNAs. These snRNA variants have not been fully characterized. It is unknown which are expressed, assemble into mature snRNPs, or participate in splicing as part of the spliceosome. In spinal muscular atrophy (SMA), the SMN complex required for snRNP biogenesis is mutated, resulting in tissue-specific alterations in snRNA levels and aberrant splicing [1]. Therefore, we hypothesize that changes in snRNA levels and the expression of variants could be a general mechanism of splicing regulation. Here we will describe the results of a bioinformatics analysis of snRNA variants using published RNA-seq datasets and ongoing work to confirm our findings and hypotheses. We have identified snRNA variants that are transcribed [2], bound by Sm [3], and associated with argonaute proteins of the RISC complex [4]. This association with RISC provides a potential mechanism to regulate snRNA expression in a developmental and tissue-specific manner. We hypothesize that these miRNA-snRNA interactions regulate snRNA levels through modulation of competition between binding of argonaute proteins and binding of the Sm particle during snRNA biogenesis. In addition, these interactions provide a potential mechanism for a quality control pathway that could ensure proper snRNP assembly. Lastly, because snRNAs are so highly expressed, miRNA-snRNA interactions could regulate the abundance and availability of miRNAs to target other transcripts. This could provide an example of the competing endogenous (ce)RNA hypothesis.

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491 Factors contributing to U1 snRNP-5'SS association during commitment complex formation revealed by single molecule colocalization spectroscopy

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Commitment complex (CC) is often the earliest stage of spliceosome assembly and is composed of pre-mRNA, the U1 small nuclear ribonucleoprotein (snRNP), the branchpoint bridging protein (BBP)/Mud2 heterodimer, and the cap binding complex (CBC). During CC formation U1 recognizes a short conserved sequence that identifies the 5' end of the intron known the 5' splice site (5'SS) through snRNA-RNA and RNA-protein interactions. Here we use Colocalization Single Molecule Spectroscopy (CoSMoS) to monitor the interaction between fluorescently labeled U1 and pre-mRNA. By directly observing U1/pre-mRNA binding and selectively disrupting individual CC interactions we have been able to quantify their contributions. Our results show that the U1 snRNP can have both short (t_1≈20s) and long-lived (t_2≈180s) interactions with a pre-mRNA substrate containing canonical splice sites. Prevention of U1 snRNA/pre-mRNA basepairing by mutation of the 5'SS or oligonucleotide directed RNase H digestion of the 5' end of the U1 snRNA eliminates long-lived U1 binding events suggesting that basepairing is required for stable U1 association. In agreement with this result, extension of U1 basepairing to the pre-mRNA from 6 to 10 basepairs increases the long-lived lifetime (t_2≈210s). Mutations to the U1C protein (e.g., L13F) can eliminate the need for ATP-dependent exchange of U1 for U6 at the 5'SS, presumably by destabilizing U1 interactions. Surprisingly, we have found that incorporation of the L13F mutation only reduces the long-lived lifetime by 32% (t_2≈121s). This suggests that kinetic hurdles between ATP-dependent vs. ATP-independent U1/U6 exchange are modest at best. In contrast to U1C mutations, elimination of interactions to other CC components has a more dramatic impact. Long-lived U1/5'SS associations are sensitive to the presence of CBC or BBP/Mud2 and lifetimes are reduced by 64% in their absence with a concomitant reduction in the number of binding events. These features are only observed upon removal of both CBC and BBP/Mud2 occupancy on the pre-mRNA. This is in agreement with a "buffering" model for stabilizing U1/pre-mRNA interactions in CC obtained using yeast genetics by Schwer *et al*. Our data provides a more detailed kinetic description how individual CC components contribute to the stability of the U1 snRNP/pre-mRNA interaction.

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492 High throughput identification of novel factors involved in the regulation of splicing of multi-intronic transcripts in S. pombe.

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Many eukaryotic protein-coding transcripts are interrupted by introns, which must be excised before the transcript can be translated. Pre-mRNA splicing is catalyzed by the spliceosome, a macromolecular machine that assembles, excises the intron, and disassembles for every intron that is to be spliced. This splicing cycle involves the stepwise assembly of components of the spliceosome on the transcript at sequences within the intron. It has been demonstrated many times over that spliceosome assembly is largely a co-transcriptional process, with the spliceosomal snRNPs being added in a step-wise fashion to the nascent transcript. In higher eukaryotes, many transcripts contain more than one intron, and the mechanisms by which spliceosomes are recruited to and assembled on downstream introns remains largely unclear and is an important question in the field. Here, we describe a high throughput method that we have designed to examine this problem, specifically by identifying and characterizing novel and known factors that are involved in regulating the splicing of downstream introns. Our work takes advantage of the highly tractable genetics in the fission yeast S. pombe. Splicing in S. pombe in many ways resembles that in higher eukaryotes, particularly in that splice site sequences in S. pombe introns are highly degenerate, and more than half of the intron-containing genes in S. pombe contain two or more introns. With the use of robotic methods that were designed in our lab, we measured the in vivo levels of each intron in a multi-intronic gene in the background of thousands of randomly mutagenized yeast strains. Importantly, we identified several mutants for which splicing of the first intron is unaffected, but splicing of the downstream introns is significantly impaired. Interestingly, these mutants also demonstrate similar defects in other multi-intronic genes. Backcrossing and genome sequencing is being performed to identify the causative mutations in these mutant strains. Further global analysis will elucidate how widespread the difference is between regulation of splicing of first introns compared to downstream introns. Identification of these mutants and further biochemical analyses will provide insight into the mechanisms of spliceosome assembly in downstream introns.

493 Prp8 RP alleles decrease the efficiency of multiple steps of the splicing cycle

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Splicing is an essential cellular process that must proceed with high fidelity. While fidelity has largely been ascribed to spliceosomal helicases, other components including Prp8, the ‘master regulator' of splicing, are also implicated. Prp8's Jab1/MPN domain regulates snRNA unwinding by spliceosomal helicase Brr2. Recent crystallographic evidence implicate a C-terminal extension of the Prp8 Jab1/MPN domain thought to control Brr2 activity by inserting itself into to RNA-binding pocket of the Brr2 helicase. Consistent with this, in Retinitis Pigmentosa (RP), a common form of heritable blindness, a subset of autosomal dominant RP alleles map to the Prp8 Jab1/MPN domain, many specifically to a "hinge" in the C-terminal extension that could disrupt the movements of the regulatory tail. This has led to the hypothesis that RP pathogenesis arises from a defect in the regulation of Brr2 throughout the splicing cycle. However, how these defects manifest and what their consequences on splicing fidelity are remain unknown. Using the ACT-CUP splicing reporter system, we show that Prp8 RP alleles have decreased copper tolerance specifically in response to altered pre-mRNA branch site and/or 3′ splice-site mutants, suggesting alterations in the regulation of Brr2 activity by Prp8 during splicing catalysis. We show an overall decrease of splicing efficiency at multiple genes in yeast mutants carrying homologous RP mutations by qRT-PCR. In vitro bimolecular splicing assays show that this decrease in efficiency comes from a reduction in the rates of both the first and second steps of splicing. We further demonstrate negative genetic interactions between Prp8 mutants and multiple first and second step splicing factors, including Prp2, Prp16, Slu7, and Prp18. Together these data implicate decreased splicing efficiency in RP pathogenesis, as well as implicate Prp8 Jab1/MPN domain control of Brr2 unwinding activity at multiple points in the splicing cycle.

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494 Reversibly constraining the human U1 snRNP and the spliceosome to a pre-mRNA via an engineered site-specific disulfide bond

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Pre-messenger RNA splicing of protein-coding RNA transcripts governs metazoan development. Pre-mRNA splicing is catalyzed by the dynamic mega-dalton spliceosome, formed by an ordered assembly of five U snRNPs (U1, U2, U4, U5 and U6 snRNPs) and non-U snRNP proteins onto a pre-mRNA substrate. The U snRNPs bind to and separate from the pre-messenger RNA at key stages in the pre-catalytic assembly of the spliceosome. The spliceosome assembly cycle is initiated with the recognition of the pre-mRNA substrate 5' splice site by the U1 snRNP which remains associated at this site during the recruitment of other U snRNPs. The U1 snRNP as well as the U4 snRNP and several other factors are subsequently displaced from their pre-mRNA binding sites during the formation of a catalytic spliceosome. The mechanism, order, and importance of these displacement events is poorly understood, in part due to the challenges presented by the spliceosome's large size, dynamics, and crude source of material. In order to investigate the pre-catalytic to catalytic transition that the spliceosome undergoes, we have engineered a disulfide bond between a rationally designed U1-C cysteine residue and a site-specific thiol-modified backbone of a pre-mRNA substrate, allowing us to establish a reversible disulfide crosslink between a pre-mRNA and the U1-C protein alone as well as in the context of the U1 snRNP. This strategy will allow for a mechanistic elucidation of the critical pre-catalytic to catalytic transition in spliceosome assembly, including examination of a possible sequential displacement of pre-catalytic complexes in the activation of the spliceosome.

495 Analysis of U6-Cwc2 interactions within the yeast spliceosome

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We have previously identified a class of C-rich, U6-like exon motifs that influence splicing, presumably by altering interactions within the spliceosome. U6 snRNA, one of the central components of the catalytic center, was shown to extensively contact Cwc2, an NTC component. One hypothesis holds that C-rich exons act by competing with U6 for binding to Cwc2 at the catalytic center. To better understand substrate positioning during the first step of splicing, we are interested in Cwc2 and its interactions with U6.

The *cwc2*-W37A allele, shown to suppress cold sensitivity of *prp16-302*, is synthetically lethal with U6 helix Ia mutant U6-A53U (Hogg et al., 2014). Our genetic analysis indicates that the *ts* phenotype of *cwc2*-W37A allele can be rescued by U6 mutations in the telestem region. Furthermore, *cwc2*-W37A allele rescues cold sensitivity of a U6 mutant in the ISL loop, U6-C67A. Previously, we have identified a large number of U6 mutants that similarly rescue U6-C67A allele; the distribution of these intragenic suppressors closely resembles the map of Cwc2-U6 physical interactions (Rashe et al., 2012). Together, these results are consistent with the proposed involvement of Cwc2 in stabilizing U6 interactions at the catalytic center and form a basis for genetic screens for additional *cwc2* and U6 alleles; these studies aim at understanding the delicate balance of dynamic interactions involving U6, Cwc2, and the substrate.

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496 Analysis of pH-activity measurements using molecular dynamics simulations at constant pH

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The twister ribozyme is a recently discovered self-cleaving ribozyme that undergoes acid-base catalysis via a phosphoryl transfer reaction. The importance of understanding the reaction rate as a function of pH is essential when investigating the mechanism of general acid-base reactions. Such rates can be largely influenced by the pKa values of the residues directly implicated in the catalytic reaction. In order to gain insight into the catalytic mechanism of twister ribozyme, we study the active site dynamics and compute directly the conditional probability of the general acid and base corresponding to being in the catalytically active form, as well as the pKas for the proposed general acid and base, A7 and G45, respectively. Previously, constant pH molecular dynamics simulations in explicit solvent (CpHMD) with replica exchange in the pH-dimension (pH-REMD) has been applied as a tool to enhance sampling of important protonation states over a range of pH values and to predict complex pH-rate relationships for protein catalysts. To our knowledge, this is the first time this methodology has been extended to a nucleic acid catalytic system. Preliminary results are consistent with A7, at the N3 position, acting as the general acid since an upshift in pKa is observed. Additionally, G45 stabilizes the transition state by hydrogen bonding to the non-bridging oxygens of the scissile phosphate. Based on our results, we predict that an N3 deaza modification at the G45 position will decrease the reaction rate while an N7 deaza substitution at the A7 position will increase the reaction rate.

497 Comparison of two Small Molecule Modulators of pre-mRNA Splicing

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Pre-mRNA splicing is an important step in gene expression. However, in contrast to other steps in gene expression, including transcription and translation, few well characterized chemical inhibitors are available with which to study the splicing process, particularly *in vivo*. Therefore, the identification of specific and selective splicing inhibitors/modifiers would not only be extremely valuable for research purposes as biotools, but also potentially useful for therapeutic applications.

To date only a few natural compounds and their synthetic derivatives have been identified as general splicing inhibitors. In addition, several other natural compounds, derived either from extracts of plants, or microbes, have been reported either to inhibit splicing *in vitro,* or to change splicing of certain transcripts in cells.

From a high throughput screen of 71,504 small, drug-like chemical compounds we have identified several small molecule splicing modulators that both inhibit splicing *in vitro* and modulate splicing in cells. Two of these compounds, DDD00107587, also termed 'madrasin' and DDD00040800, were investigated in more detail. Madrasin stalls spliceosome assembly after A complex formation, whereas in the presence of DDD00040800 only the H complex is formed. Treatment of human cell lines with madrasin led to cell cycle arrest in S and G2/M phase and treatment with DDD00040800 to a cell cycle arrest in G2/M phase. Both compounds induced a specific reorganization of the subnuclear localisation of Cajal body components. Preliminary deep RNA sequencing results indicate that treatment with either madrasin, or DDD00040800, for 24h can change >2,000 pre-mRNA splicing events in both HeLa and HEK293 cells

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498 Splicing without U1: the surprisingly reduced spliceosome of C. merolae

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The chemical steps of pre-mRNA splicing are so simple that RNA molecules are able to self-splice without outside assistance. Nevertheless, the human complex responsible for this reaction - the spliceosome - consists of over 200 proteins and five small, nuclear RNAs, raising the question of which of these factors contribute to splicing efficiency, and which play a more peripheral or modulatory role. Given the estimate that over 60% of genetic diseases exert their effects through deficiencies in splicing, there is good reason to try to better understand how the spliceosome functions.

Here, we describe the surprisingly reduced spliceosome of Cyanidioschyzon merolae, an acidophilic, freshwater red alga. C. merolae is notable for haboring only 27 annotated introns in its genome. We used bioinformatic and biochemical methods to assess the composition of C. merolae's spliceosome. While the U2, U4, U5, and U6 snRNAs appear largely canonical, we found no evidence for the U1 snRNA, either computationally or in an anti-TMG pulldown followed by deep sequencing. To test the apparent absence of U1, we searched computationally for all known splicing proteins. We found ~70 total, of which ~40 are predicted to be core snRNP-associated or splicing proteins, but none of the known U1 proteins. In addition, C. merolae has no detectable homologue of Prp28, which has been shown to be responsible for the switch of U6 for U1 at the 5' splice site (5'ss), again supporting the inference that the U1 snRNP is absent. We have confirmed our bioinformatic predictions with 2'OMe oligo pulldowns of the snRNAs followed by mass spectrometric protein identification.

The apparent absence of the U1 snRNP raises the issue of how the 5' splice site might be recognized. Intriguingly, the U5 snRNA has large 5' and 3' extensions relative to other organisms, and its extreme 5' end is complementary to the 5'ss. We are currently testing this possibility, and anticipate that C. merolae will provide a useful system for studying the core features of the splicing mechanism.

499 EWS-FLI1 is a network hub that regulates alternative splicing and alters ARID1A function.

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Identifying aspects of cancer cells that differentiate them from non-cancer cells remains an ongoing challenge. In cancer, many genes are alternatively spliced such that specific isoforms may be associated with tumor progression or resistance to therapy. We used the oncogenic fusion protein EWS-FLI1 as a model to study alternative splicing in Ewing Sarcoma (ES). Our unbiased proteomic screen identified that EWS-FLI1 is highly connected to spliceosome through direct protein interactions. Using data from both exon arrays and RNA-seq experiments *ARID1A*, a member of the SWI/SNF complex, was both highly expressed and alternatively spliced in ES. These changes were modulated by EWS-FLI1 reduction and also occurred when ES cells were treated with a small molecule inhibitor of EWS-FLI1, YK-4-279. At least one isoform of ARID1A, that includes the HIC1 (Hypermethylated in Cancer 1) binding site, was lost when EWS-FLI1 was reduced. In addition, loss of full length ARID1A significantly reduced cell proliferation. Re-expression of the ARID1A long isoform restored ES cell proliferation while the short isoform, lacking the HIC1 site, did not restore growth. Expression of several downstream targets of ARID1A including CDKN1A and SMAD3 were increased when EWS-FLI1 was reduced that may explain the phenotypic growth suppression. ARID1A binds to EWS-FLI1 and this complex is disrupted by YK-4-279. In addition, treatment of YK-4-279 mimics the ARID1A isoform pattern seen with the loss of EWS-FLI1 and increased CDKN1A expression. Analysis of ES patient samples shows a similar exon expression pattern compared to ES cell line models, supporting the clinical relevance of our findings. These results demonstrate a functional role of EWS-FLI1 in modulating the alternative splicing mechanism of ARID1A. Future work will be focus on connecting ARID1A function to EWS-FLI1 with regards to chromatin modification or epigenetic reprogramming.

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500 Investigating the role of spliceosomal components SF3B3 and SF3B5 within SAGA transcriptional co-activator and histone modifying complex

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Proper splicing is an important regulator of gene expression and splicing misregulation can lead to major dysfunction and disease. The SF3b component of the U2 small nuclear ribonucleoprotein (snRNP) is an essential component of the catalytically active spliceosome. We have identified spliceosomal U2 snRNP subunits SF3B3 and SF3B5 as subunits of the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex in *Drosophila melanogaster*. SAGA is a modular complex which has many activities that regulate transcription initiation and elongation. Interestingly, we generally see a decrease in SAGA occupancy on transcribed regions of intron-containing genes as compared to intronless genes. In addition, preliminary results show an increase in ubiquitinated histone 2B (H2Bub) in $sf3b5^{EY12579}$ mutants globally. This is not due to the indirect transcriptional effects of the $sf3b5^{EY12579}$ mutation, as transcription of the deubiquitinase module of SAGA is not changed in this mutant as compared to wild type. In addition, we are testing the complex integrity of SAGA within these mutants, however it is likely unaltered as there is no global change in histone acetylation levels. Therefore, we hypothesize that the spliceosomal subunits SF3B3 and SF3B5 incorporate into SAGA to enhance SAGA's occupancy on transcribed regions and allowing for its deubiquitinase activity. Current work is being done to look at SAGA occupancy in these mutants to determine if SF3b5 and SF3b3 plays a direct role in SAGA recruitment to gene bodies. This recruitment could feed back to dictate transcriptional speed and splice site choice, providing novel insight into the direct mechanistic link between splicing and transcription.

501 Effects of splicing inhibitors after altering the branch point sequence and polypyrimidine tract length

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The spliceosome is the macromolecular machine responsible for intron removal to produce a mature mRNA during eukaryotic gene expression. One component of the spliceosome is the protein SF3B1, which is involved in branch point recognition and is the target of a class of small molecule inhibitors. Two important features for intron recognition are the branch point sequence and the polypyrimidine tract. In cells SF3B1 inhibitors have differential effects on splicing events that putatively correlate with branch point sequence strength. This observation led us to hypothesize that splicing substrates with a weak branch point sequence will be more sensitive to SF3B1 inhibitors relative to those with a strong branch point sequence. We tested this hypothesis in an *in vitro* splicing assay using substrates with varying branch point sequence strength and polypyrimidine tract length. Although these substrates are spliced with different overall splicing efficiencies, they surprisingly showed similar sensitivity to SF3B1 inhibitors. Our results demonstrate that in nuclear extract substrates with different branch point sequences or polypyrimidine tract length have the same dependence on SF3B1 activity. The results also indicate that splicing sequence strength alone does not confer increased sensitivity to SF3B1 inhibitors, and suggests that in cells other factors and or sequence context play a role in mediating the differential splicing changes observed for transcripts with SF3B1 inhibitor treatment.

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502 U11-regulated alternative splicing is mediated by exon definition interactions

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We have previously described an autoregulatory feedback loop for U11-48K and U11/U12-65K mRNAs, which code for specific protein components of the U12-type spliceosome (1). The feedback loop is based on an atypical exonic splicing enhancer called USSE (U11 snRNP-binding splicing enhancer), consisting of two U12-type consensus 5' splice site (5'ss) motifs. Binding of U11/U12 di-snRNP to the USSE activates an upstream U2-type 3'ss and promotes alternative mRNA isoform formation that leads either to mRNA degradation by the NMD pathway (48K), or mRNA retention in the nucleus (65K).

Phylogenetic analysis of almost 200 USSE elements in metazoan and plant U11-48K and U11/U12-65K genes revealed that the distance between the USSE element and the upstream 3'ss is tightly constrained, particularly in mammals, where the average distances are 44 nt (48K) and 63 nt (65K). Shortening of this distance within a reporter construct leads to a progressive loss of splicing activity. mRNA tethering experiments show that the U11-35K protein, a functional analog of U1-70K, is sufficient for splice site activation. Furthermore, the activation is dependent on the presence of an RS domain. Splice site activation is consistent with the classical exon definition model where shortening of the exon size below 50 nt would lead to a steric clash with spliceosomal complexes binding to the 3' and 5' ends of the exon. This suggests that the exon definition interactions between the major and minor spliceosome components are similar to those bridging the interactions between components of the major spliceosome. Through manipulation of the distance between the two U12-type 5'ss within the USSE element, we provide evidence that simultaneous binding of two U11/U12 di-snRNPs to the USSE element is necessary for upstream 3'ss splice site activation. Thus, in USSE mediated alternative splicing, two U11/U12 di-snRNPs are stabilized by mutual interaction and this complex uses exon definition interactions to activate the upstream 3'ss.[VJ1] [VJ2]

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503 Exon circularization through back-splicing is regulated by canonical splicing factors

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A large number of circular RNAs (circRNAs) have been identified in eukaryotes by analyzing high-throughput RNA-sequencing data. However, the biogenesis and functions of these circRNAs are largely undetermined. The majority of exonic circRNAs are believed to be generated through back-splicing, a specialized splicing reaction in which the upstream 3' splice site (accepter site) is joined to the downstream 5' splice site (donor site). We developed a series of back-splicing minigenes containing split fluorescence proteins encoded by exons at reversed order. The back-splicing of these exons can produce circular RNAs that serve as translational template of GFP. We found that back-splicing can be promoted by complementary pairing, but such pairing is not necessary. Moreover, back-splicing can be regulated by many general splicing factors and cis-elements, but the regulatory rules are different from canonical splicing. Translation from circular mRNA are also controlled by different set of cis-regulatory elements. We further analyzed the difference between the regulation of back-splicing and canonical splicing using a dual-colored reporter system, and found that back-splicing can happen in comparable efficiency to the linear splicing. We also use this system to refine the regulatory rules of back-splicing, and found a large number of RNA binding proteins can control backsplicing in diverse fashion.

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504 Brr2 plays a role in spliceosomal activation in addition to U4/U6 unwinding.

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Brr2 is a DExD/H-box RNA helicase that is responsible for U4/U6 unwinding, a critical step in spliceosomal activation. Brr2 is a large protein (~250kD) that consists of an N-terminal domain (~500 residues) with unknown function and two Hel308-like modules that are responsible for RNA unwinding. We demonstrate that removal of the entire N-terminal domain is lethal to *S. cerevisiae* and deletion of the N-terminal 120 residues leads to splicing defects and severely impaired growth. This N-terminal truncation does not significantly affect Brr2's helicase activity. Brr2-D120 can be successfully assembled into the tri-snRNP (albeit at a lower level than the WT Brr2) and the spliceosomal B complex. However, the truncation significantly impairs spliceosomal activation, leading to a dramatic reduction of U5, U6 snRNAs and accumulation of U1 snRNA in the B^{act} complex. The N-terminal domain of Brr2 does not seem to be directly involved in regulating U1/5'ss unwinding. Instead, this N-terminal domain seems to be critical for retaining U5 and U6 snRNPs during/after spliceosomal activation through its interaction with snRNAs and other spliceosomal proteins, revealing a new role of Brr2 in spliceosomal activation in addition to U4/U6 unwinding.

505 hnRNP L inhibits CD44 V10 exon splicing through the cis elements in its upstream intron.

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CD44 is a complex cell adhesion molecule that mediates communication and adhesion between adjacent cells as well as between cells and the extracellular matrix. CD44 pre-mRNA produces various mRNA isoforms through alternative splicing of 20 exons, among which exons 1-5 (C1-C5) and 16-20 (C6-C10) are constant exons, whereas exons 6-15 (V1-V10) are variant exons. CD44 V10 exon has important roles in breast tumor progression and Hodgkin lymphoma. Here we show that increased expression of hnRNP L inhibits V10 exon splicing of CD44 pre-mRNA, whereas reduced expression of hnRNP L promotes V10 exon splicing. In addition, hnRNP L also promotes V10 splicing of endogenous CD44 pre-mRNA. Through mutation analysis, we demonstrate that the effects of hnRNP L on V10 splicing are abolished when the CA-rich sequence on the upstream intron of V10 exon is disrupted. However, hnRNP L effects are stronger if more CA-repeats are provided. Furthermore, we show that hnRNP L directly contacts the CA-rich sequence. Importantly, we provide evidences that hnRNP L inhibits U2AF65 binding on the upstream Py tract of V10 exon. Our results reveal that hnRNP L is a new regulator for CD44 V10 exon splicing.

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506 CD44V6 isoforms are regulated by hnRNP A1 in metastatic breast cancer cells which followed by increased cell death and motility.

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CD44 is a transmembrane receptor for hyaluronic acid. CD44 pre-mRNA contains 19 exons, 9 of which are alternatively spliced. Among the CD44 spliced variants, V4-7 variant, one of V6 exon containing isoforms that contains variable exon 4, 5, 6 and 7, confers metastatic potential to non-metastatic cells. Splicing of CD44 and the function of CD44 isoforms are different in breast cancer cells. hnRNP A1 is a ubiquitously expressed protein with an inhibitory function in pre-mRNA splicing. We show that CD44V6 proteins has a highest expression level in non-metatatic breast cancer cells (MCF7) than metastatic breast cancer cells (MDA-MB-231) and normal breast cells (MCF 10A). Furthermore we show that hnRNP A1 regulates splicing of CD44 differently in breast cancer cells. We show here the CD44 isoform expression is completely different in MDA-MB-231 from that in MCF7 and MCF 10A, whereas MCF7 and MCF 10A cells has a similar expression pattern of CD44 isoforms. RT-PCR analysis of CD44V6 shows that MCF7 MCF 10A cells express C5V6V7V8V9V10C6 and C5V6V8V9V10C6 isoforms. However MDA-MB-231 cells also express, in addition to these two isoforms, C5V6C6. We also found that knockdown of hnRNP A1 significantly reduced the expression of C5V6V7V8V9V10C6, C5V6V8V9V10C6, promoted the expression of C5V6C6. hnRNP A1 knockdown significantly induced cell death. In addition, hnRNP A1 induced the decrease of cell invasion of MDA-MB-231 cells. Our results are different from the effects of hnRNP A1 knowdown on hepatocellular carcinoma and CD44V6 expression. Our results indicate that the function of hnRNP A1 has a specific function on the splicing of CD44 in the breast cancer cells.

507 A genome landscape of SRSF3-regulated splicing events and gene expression in human osteosarcoma U2OS cells

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Alternative RNA splicing is an essential process to yield proteomic diversity in eukaryotic cells, and aberrant splicing is often associated with numerous human diseases and cancers. We recently described serine/arginine-rich splicing factor 3 (SRSF3) being a proto-oncogene. However, the SRSF3-regulated splicing events responsible for its oncogenic activities remain largely unknown. By global profiling of the SRSF3-regulated splicing events in human osteosarcoma U2OS cells, we found that SRSF3 regulates 182 splicing events in at least 164 genes, including EP300, PUS3, CLINT1, PKP4, CHK1, SMC2, CKLF, MAP4K4, Sp1, and SRSF1, and the expression of additional 60 genes including ERRFI1, ANXA1, and TGFB2, which are primarily associated with cell proliferation or cell cycle. SRSF3 also significantly regulates the expression of at least 20 hsa-miRNAs, including a subset of oncogenic or tumor suppressive miRNAs. We found that the expression of two oncogenic splicing factors, SRSF1 and SRSF3, are mutually dependent and coexpressed in normal tissues and increasingly in tumor tissues/cells. These data indicate that SRSF3 affects a global change of gene expression to maintain cell homeostasis.

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508 Unmasking Alternative Splicing inside Protein-Coding Exons Defines Exitrons and Their Role in Proteome Plasticity

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Alternative splicing (AS) diversifies transcriptomes and proteomes and is widely recognized as a key mechanism for regulating gene expression. Previously, in the analysis of intron retention events in *Arabidopsis*, we found unusual AS events inside annotated protein-coding exons (Marquez et al. 2012). Here, we also identify such AS events in human. We use these two sets to analyse their features, regulation, functional impact and evolutionary origin. As these events involve introns with features of both introns and protein-coding exons, we name them exitrons (**ex**onic **i**n**trons**). Though exitrons were detected as a subset of retained introns, they are clearly distinguishable, and their splicing results in transcripts with different fates. About half of the 1002 *Arabidopsis* and 923 human exitrons have sizes of multiples of three nucleotides. Splicing of these exitrons results in internally deleted proteins and affects protein domains, disordered regions and various post-translational modification sites thus broadly impacting protein function. Exitron splicing is regulated across tissues, in response to stress and in carcinogenesis. Intriguingly, intronless genes can be also alternatively spliced via exitron usage. We demonstrate that at least some exitrons originate from ancestral coding exons and that their evolution involved intron loss. Based on our findings, we propose a "splicing memory" hypothesis whereby intron loss and imprints of former exon borders defined by vestigial splicing regulatory elements could drive the evolution of exitron splicing. Altogether, our studies show that exitron splicing is a conserved strategy for increasing proteome plasticity in plants and animals complementing the repertoire of AS events.

509 A Functional Analysis of Alternative Splicing in Response to Transcription Stress in HeLa cells

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Alternative splicing is a common way of diversifying the proteome and regulating protein functions without increasing genome size. Chemotherapeutic agents, such as cisplatin and camptothecin cause changes in the splicing of proliferation-associated exons, which have the potential of changing the proliferative phenotype of cancer cells. The mechanisms by which transcriptional stress alters the functions of splicing machinery are unknown. Thus, we tested the effects of the transcription elongation inhibitor DRB (5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole) on the splicing of several inducible exons. These exons were identified through genomewide searches for a splicing code that is sensitive to the splicing factors hnRNP A1 and hnRNP H. We found that the *HNRNPH1* exon 4 and its paralog *HNRNPH3* exon 3 skipping increased upon DRB treatment in HeLa cells, as did the *GRIN1* CI, a brain-region-specific rat exon previously studied in our lab. We chose single and combined mutations to monitor effects of DRB on splicing, since shared RNA motifs include two exonic UAGG motifs and a GGGG close to 5′ splice site. Two GGGG mutants had a bigger increase in skipping, compared to the wild-type, upon DRB-induced transcription inhibition. These data point to a role for this splicing code in response to transcription stress.

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510 To what extent do Sat III RNA induction and nuclear stress bodies formation contribute to alternative splicing modulation by heat shock?

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When human cells are exposed to various stresses such as heat shock, exposure to UV or heavy metal, osmotic and oxidative stresses, transcription of Satellite III (Sat III) RNAs by RNA polymerase II is activated. These RNAs are transcribed from tandem arrays of Sat III DNA which are located in the pericentromeric heterochromatin of chromosomes 9, 12 and 15. Sat III RNAs remain at their sites of transcription leading to nuclear stress bodies (nSBs) formation. The function of nSBs still remains to be fully characterized. They are thought to participate in rapid, transient and global reprogramming of gene expression through different mechanisms including chromatin remodeling and trapping of transcription and splicing factors. Indeed, a subset of splicing factors including SR proteins (SRSF1, SRSF9 and SRSF7) and hnRNP (HAP, M) are efficiently recruited to nSBs by direct or indirect interaction with Sat III RNAs.

For a better characterization of the impact of Sat III RNA expression on global changes of gene expression in response to stress, we performed a genome-wide transcriptome analysis on heat shocked HeLa cells using Human Junction ArraYs (HJAY) from Affymetrix. Our results highlight alternative splicing variations of about 450 pre-mRNAs, several of them encode proteins involved in the control of mRNA fate. To test the contribution of Sat III RNAs to the detected variations, we settled an efficient protocol for Sat III depletion using LNA gapmers. In these conditions, neither hnRNP HAP, nor SRSF1 were present in the nuclear HSF1 foci observed after heat shock. Surprisingly, heat shock modulation of alternative splicing was still observed after Sat III RNA depletion. Sat III depletion did also not affect the return to normal splicing pattern after a long recovery time (24 h at 37°C). These results indicate that Sat III RNAs are not required for alternative splicing changes following heat shock, even for splicing events regulated by SRSF1. Moreover, we observed opposite effects of heat shock and SRSF1 depletion on splicing of SRSF1 mRNA targets. Altogether, our results lead us to reconsider the hypothesis of alternative splicing regulation by SRSF1 trapping within nuclear stress granules after heat shock.

511 ESRP2 Regulates A Conserved And Cell-Type-Specific Splicing Program to Support Postnatal Liver Maturation.

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While the major genetic networks controlling early liver specification and morphogenesis are known, the mechanisms responsible for postnatal hepatic maturation are poorly understood. Here we employed high-resolution RNA-seq analyses of mouse liver transcriptome to identify a highly conserved and temporally coordinated cell type-specific splicing program, which is activated in part by epithelial-specific splicing regulatory protein 2 (ESRP2) during postnatal development. Combining detailed expression analyses with gain- and loss-of-function studies we demonstrate ESRP2 controls postnatal switch of approximately 20% of splice isoforms in mouse and human hepatocytes. Strikingly, the normal shift in splicing coincides tightly with dramatic postnatal induction of ESRP2 in hepatocytes and the knockout of Esrp2 in mice results in a failure of neonatal-to-adult splicing transitions in a spectrum of genes involved in liver maturation. Consistent with failure of neonatal-to-adult splicing transitions, Esrp2 null mice exhibit persistent expression of fetal markers and loss of mature hepatocyte characteristics. Phenotypic and biochemical characterization of Esrp2 null mice identified defects in cell cycle exit, abnormalities in liver zonation, and reduction in albumin production. Importantly, forced expression of ESRP2 in immature mouse or human hepatocytes results in a complete and reciprocal switch of the developmentally regulated splicing program. Thus, our results unveil an essential role for ESRP2 in generation of conserved repertoires of adult splice isoforms that support terminal differentiation, functional competence and postnatal maturation of hepatocytes.

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512 Network of splice factor regulation by alternative splicing coupled with nonsense mediated mRNA decay

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Nonsense-mediated mRNA decay (NMD) is an RNA surveillance pathway that degrades aberrant transcripts harboring premature termination codons. However, this pathway also has physiological targets: many genes produce alternative isoforms containing premature termination codons. In this mode of regulation, a splicing factor can induce splicing of an alternative isoform with an early stop codon. These isoforms will be degraded by NMD, resulting in lower protein expression. Regulation of alternative splicing involves complex interactions between many splice factors, and so splice factor levels must be carefully regulated. Splicing coupled to NMD allows for an additional level of post-transcriptional regulation for these genes. For example, splicing factors such as SRSF1, SRSR2, SRSF3, and SRSF7 are known to regulate their own expression and expression of other splice factors by coupling alternative splicing and NMD. hnRNP L, hnRNP LL, PTBP1, and PTBP2 are regulated in the same manner.

After an extensive literature search, we generated a splicing factor regulatory network that encompasses current knowledge of splice factor regulatory interactions. The currently available data shows that the majority of the SR proteins and a few hnRNP splicing factors are known to be regulated by another splicing factor via alternative splicing coupled with NMD. Since all the SR proteins and many hnRNP splicing factors produce isoforms degraded by NMD, we predict that this mode of regulation is pervasive in this dense splicing factor regulatory network. In addition, CLIP-seq data reveals extensive splicing factor-mRNA interactions, providing an additional hint that many more splicing factors might be regulated by other splicing factors via alternative splicing coupled with NMD. Further work will establish the true extent of regulation by alternative splicing coupled to NMD of splicing factors by building a comprehensive regulatory network model.

513 SRSF1 Negatively Regulates Alternative Splicing of *MDM2* Under Damage

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Murine Double Minute 2 (MDM2) is an E3 ubiquitin ligase and negative regulator of the tumor suppressor protein p53. Under normal conditions, *MDM2* is constitutively spliced to generate a full-length protein, which promotes the proteasome-mediated degradation of p53. However, under stress *MDM2* undergoes alternative splicing, generating splice variants that are unable to bind and regulate p53. Subsequently, p53 becomes upregulated and activates downstream targets involved in apoptosis and cell cycle arrest. *MDM2-ALT1*, which consists of only the two terminal coding exons 3 and 12, is the most frequently observed of these splice isoforms. Despite studies characterizing *MDM2-ALT1* as a dominant negative regulator of full-length *MDM2* and its pervasiveness in cancers, there is very little known about the regulation of *MDM2* alternative splicing in cancer and under stress. There is therefore a critical need to understand the regulation of *MDM2* alternative splicing in to modulate its splicing. *We hypothesize that there are negative splicing regulatory elements that are responsible for stress-induced alternative splicing of* MDM2.

In order to study the alternative splicing of *MDM2* we have developed a stress-inducible minigene system. The *MDM2* 3-11-12s minigene recapitulates the splicing of the endogenous gene by excluding exon 11 under genotoxic stress. Using a SELEX-based bioinformatics program, we identified predicted binding sites for SRSF1 in this regulated exon. We report that the binding of SRSF1 to this site is increased under damage and its mutation is sufficient to ablate damage-induced exon 11 exclusion in a three-exon minigene system both *in vitro* and in cell-based assays. Furthermore, SRSF1 overexpression promoted exclusion of exon 11 while its siRNA-mediated knockdown prevented the stress-induced alternative splicing of endogenous *MDM2*. Additionally, we observed elevated SRSF1 levels under stress and in tumors correlating with the expression of *MDM2-ALT1*. Notably, we demonstrate that *MDM2-ALT1* splicing can be blocked by targeting SRSF1 sites on exon 11 using antisense oligonucleotides.

These results present conclusive evidence supporting a negative role for SRSF1 in *MDM2* alternative splicing. Importantly, we define for the first time, a clear-cut mechanism for the regulation of damage-induced *MDM2* splicing and present potential strategies for manipulating *MDM2* expression via splicing modulation.

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514 Proofreading the Branch Site: Implications for First Step Splicing Fidelity and Alternative Branch Site Selection

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Maintaining fidelity in cellular processes is essential to ensure cell viability. Fidelity of pre-mRNA splicing, an essential step in gene expression ubiquitous to eukaryotes, is maintained by the spliceosome through DExD/H-box ATPases, a class of ATP-dependent RNA helicases that are conserved from yeast to humans. One such DExD/H-box ATPase, Prp16, acts to maintain fidelity of the first of two transesterification steps in splicing. In addition to ensuring first step splicing fidelity, Prp16 is required for the second step of splicing and has been implicated in mediating rearrangements required for transitioning to the second step.

We provide evidence that Prp16 facilitates both branch site fidelity and substrate rearrangement through a general mechanism of undocking of the branch site from the branch point binding sequence of U2 snRNA. Additionally, as mutations that destabilize the U2-branch site interaction can be suppressed by prp16 mutations defective in ATP hydrolysis and unwinding, Prp16 may ensure first step splicing fidelity through a kinetic proofreading model in which a variable rate of U2-branch site disruption competes with the first chemical step of splicing, the rate of which can also vary. We further show Prp16 crosslinks downstream of the U2-branch site interaction at the first chemical step and provide evidence that Prp16 translocates toward but not through this duplex to undock the branch site. This suggests Prp16 acts as a molecular winch to interrogate the stability of the U2-branch site interaction during proofreading and to mediate substrate rearrangement to prime the spliceosome for the second step of splicing.

Similarly, the DExD/H-box ATPase Prp22 both promotes mRNA release and maintains fidelity of the second step of splicing through a general mechanism of undocking of the 3' splice site. Surprisingly, the proofreading activities of Prp16 and Prp22 promote alternative branch site and 3' splice site selection, respectively, indicating a role for proofreading in not only rejecting splice sites but also selecting splice sites and implicating proofreading as an attractive target for alternative splicing regulation. We discuss experiments to test the kinetic proofreading model directly and to elucidate the mechanism of Prp16-dependent alternative branch site selection.

515 The impact of snRNA mutations and modifications on U2 stem II toggling

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The splicing of precursor messenger-RNA (pre-mRNA) is carried out by an assembly of five small nuclear ribonucleoproteins (snRNPs): U1, U2, and the U4/U6.U5 tri-snRNP. Each snRNP contains a specific RNA (snRNA) and associated proteins. The binding of the intronic branchpoint sequence is carried out by the U2 snRNP. For binding to occur, the U2 snRNA must be in the stem loop IIa conformation. This stem loop has been observed in equilibrium between two stem loop conformations: IIa, necessary for binding to pre-mRNA, and IIc, needed for catalysis. This equilibrium is shifted by the RNA-binding protein Cus2 which binds stem loop IIa[1]. Cus2 is thought to select for the stem loop IIa conformation to promote the binding of the branchpoint to promote prespliceosome assembly. The stem loop II then needs to toggle to the IIc conformation for the first catalytic step in splicing. It is thought that stem loop II must then toggle back to the IIa conformation during the rearrangement preceding the second catalytic step. The toggling of stem loop II has been proposed to be important for progression through the splicing pathway.

The mechanisms governing the equilibrium between stem loops IIa and IIc are of particular interest, specifically mutations and modifications to the Cus2 and the U2 snRNA. We have expressed and purified two Cus2 mutants (D282N and L284F) originally identified as suppressors of mutations destabilizing stem IIa (G53A)[2]. We are testing how wild-type and mutant Cus2 proteins bind wild-type U2 snRNA, G53A U2 snRNA, and pseudouridylated U2 snRNA (Ψ56 and Ψ93) by EMSA and single molecule assays. Two fluorophores have been ligated to the U2 snRNAs that enable us to detect the relative occupancy of the two stem II conformations via single-molecule FRET. We plan to investigate what, if any, effects the mutations to Cus2, U2 snRNA, and pseudouridylated U2 snRNA have on the stem IIa-IIc equilibrium.

1. Tretbar, U.S., Rodgers, M.R., and Hoskins, A.A. 2015. To be published.

2. Yan, D. et al. 1998. CUS2, a yeast homolog of human Tat-SF1, rescues function of misfolded U2 through an unusual RNA recognition motif. Mol. Cell. Biol. 18: 5000-5009.

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516 Identifying factors that control alternative splicing in fission yeast

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Alternative splicing (AS) is a critical regulator of gene expression and allows organisms to generate multiple protein isoforms from the same gene, yet the mechanisms by which this process is regulated have not been widely elucidated. The fission yeast *Schizosaccharomyces pombe* represents a powerful, genetically tractable system in which to study splicing regulation. In many ways, splicing in *S. pombe* closely resembles that seen in higher eukaryotes, including the prevalence of multi-intronic genes, degenerate splice site sequences, and the presence of bona fide SR proteins. Importantly, our lab has recently identified a handful of fission yeast genes which are subject to mammalian-like AS in the form of environmentally regulated cassette exons.[1] Here we describe a genetic screen we have undertaken in *S. pombe* to identify regulators of one of these events, AS of the *srrm1* gene. We chose to examine AS of the *srrm1* gene for a number of reasons including: its deep evolutionary conservation; its demonstrated AS in mammals; its environmental regulation in *S. pombe*; and because the *srrm1* gene itself encodes an SR protein. To screen for regulators of *srrm1* splicing, we designed robotic-assisted quantitative RT-PCR protocols to specifically quantify the cellular abundances of *srrm1* splice isoforms in a collection of 4000 randomly mutagenized strains selected for temperature sensitivity. We identified mutant strains in which the skipping of the alternative exon in the *srrm1* gene is increased by as much as 4-fold. Interestingly, by simultaneously assessing the splicing efficiency of several canonical introns in each of these strains, it is clear that we have identified 'general' splicing factor mutations which affect both canonical and AS events, as well as mutations which appear to specifically alter *srrm1* AS. We are using a sequencing-based approach to map and clone the causative mutations in these strains in order to understand how the genes in which these mutations reside regulate AS events.

[1]Awan et al., *PNAS* 2013

517 Ubiquitously expressed C/D box snoRNAs regulate alternative splicing

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C/D box snoRNAs (SNORDs) are small non-coding RNAs whose best-understood function is to target the methyltransferase fibrillarin to rRNA, resulting in 2'-O-methylation. Several non-canonical functions of SNORDs were previously reported, including generation of miRNAs, regulation of cytosolic lipid metabolism and the regulation of alternative splicing of the serotonin receptor 2C pre-mRNA.

To examine the biochemical nature of non-canonical SNORD action, we fractionated HeLa nuclear extract made under native conditions and found co-purification of numerous SNORDs with pre-mRNA splicing complexes. These fractions are devoid of fibrillarin and NOP58, two essential components of the SNORD-RNP involved in rRNA methylation. Detailed analysis of SNORD2, SNORD27, SNORD60 and SNORD78 that have been previously implicated in rRNA methylation showed that a considerable fraction of these SNORDs do not associate with fibrillarin and form distinct ribonuclear protein complexes.

Computational predictions showed multiple SNORD binding sites located near known alternative exons in pre-mRNAs. SNORDs were predicted to interact with pre-mRNAs through parts of their entire sequence, including C, D and antisense boxes. This further suggests that SNORDs act in non-canonical protein complexes to change pre-mRNA splicing. An influence of the SNORDs on alternative splice site selection was validated by genome-wide RNAseq and RT-PCR. The complementarity between pre-mRNAs and SNORDs was not evolutionary conserved, suggesting a species-specific regulation.

Interestingly, previously unknown, "silent" exons where activated by SNORD knockdown, suggesting that SNORD expression is needed to repress "cryptic" exons.

Detailed analysis using compensatory mutations showed that SNORD27 regulates alternative splicing of the transcription factor *E2F7* pre-mRNA through direct RNA:RNA interaction without 2'-O-methylation likely by competing with U1 snRNP. The SNORD27 sequences targeting *E2F7* and rRNA are distinct.

This suggests that SNORD expressing units generate not only canonical snoRNAs regulating rRNA methylation, but also novel RNAs targeting hnRNPs to pre-mRNA, which regulates exon recognition. Thus SNORDs are an example of RNAs regulating pre-mRNA splicing, which largely increase the number of splicing regulators.

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518 Towards elucidating the role of the splicing factor DDX39B in the development of autoimmunity

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Multiple Sclerosis (MS) is a progressive and potentially debilitating autoimmune disorder of the central nervous system, with genetics playing a large role in disease susceptibility. We previously established that a genetic variant (rs6897932) within exon 6 of the interleukin-7 receptor alpha gene (IL7Rα) is a risk factor for MS. We further showed that the risk allele of this variant increases skipping of exon 6, resulting in enhanced expression of soluble IL7Rα, the latter of which has been shown to exacerbate clinical progression in a mouse model of the disease. These findings implicated splicing of IL7Rα pre-mRNAs in the pathogenesis of MS and prompted us to postulate that *trans*-acting factors which regulate exon 6 splicing are candidate genes for MS susceptibility. By combining RNA affinity chromatography, mass spectrometry and RNAi, we uncovered the RNA helicase DDX39B as an important *trans*-activator of IL7Rα exon 6 splicing. In conformity with our hypothesis, we found a large number of SNPs within the DDX39B gene are strongly associated with increased MS risk, one of which appears to decrease DDX39B expression by influencing the use of alternative transcription start sites. Moreover, we found strong correlation of this SNP with IL7Rα rs6897932, in which carriers of the risk allele at both loci are at greater risk to develop the disease. Interestingly, other genetic studies have associated DDX39B with numerous autoimmune diseases. Considering this widespread association of DDX39B with autoimmunity, our results suggest that DDX39B could be the master regulator of a splicing program critical in the development of autoimmunity.

519 ESRP1 and hnRNPM compete to regulate alternative splicing of APLP2 during EMT

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The epithelial-mesenchymal transition (EMT) is a fundamental biological process where epithelial cells transit into fibroblastic mesenchymal cells capable of migrating and invading to other tissues. Although EMT is a normal developmental process, aberrant activation of the pathway has been increasingly implicated in pathological fibrosis as well as cancer metastasis. Alternative mRNA splicing is emerging as an essential mechanism that governs EMT in human disease.

ESRP1 and hnRNPM are splicing factors that mediate global changes in alternative splicing by driving epithelial or mesenchymal phenotypes, respectively. By examining previously published as well as our own RNA-seq datasets, we discovered that the amyloid precursor-like protein 2 (APLP2) is one of the most differentially spliced genes during EMT, and this splicing occurs in an ESRP1 and hnRNPM dependent manner. Exon 7 of APLP2 encodes a Kunitz Protease Inhibitor (KPI) domain that undergoes exon-skipping during EMT, yielding a long isoform (APLP2-L) and short isoform (APLP2-S). In cell culture models of EMT, APLP2 exhibits a gradual isoform switch from APLP2-L to APLP2-S. Knockdown experiments illustrate that ESRP1 is necessary to maintain APLP2-L expression in epithelial cells while hnRNPM is essential for sustained APLP2-S expression in mesenchymal cells. Using a splicing minigene construct containing APLP2 exon 7 and surrounding intronic sequences, a dose-dependent increase in exon-skipping occurs with hnRNPM transfection. While transfecting ESRP1 alone does not significantly affect APLP2 exon 7 splicing, co-transfection of hnRNPM with ESRP1 partially attenuates hnRNPM-mediated skipping. RNA pull-down assays identify two GU-rich sequences immediately downstream of APLP2 exon 7 that recruit ESRP1 and hnRNPM directly to the APLP2 pre-mRNA. Mutation of these sequences results in significant reduction of hnRNPM-mediated exon skipping and loss of protein binding.

Our results suggest that ESRP1 antagonizes the activity of hnRNPM in the alternative splicing of APLP2, perhaps through direct competition for cis-acting splicing regulatory sequences. APLP2 is expressed in many tissues throughout the body and has been implicated in cell migration, adhesion, proliferation, and wound healing. This profile of activity coupled with the tightly regulated APLP2 isoform switching that occurs during EMT suggests a functional role for APLP2 isoform switching in EMT-associated processes.

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520 The Role of LUC7L2 in Bone Marrow Failure and Splicing

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Myelodysplastic syndromes (MDS) are a group of disorders characterized by bone marrow failure and frequent progression to acute myeloid leukemia (AML). MDS is the most common hematological malignancy in patients over 65, with only 35% surviving three years after diagnosis. Recent next generation sequencing studies have shown that over 65% of MDS patients harbor recurrent point mutations in several proteins involved in pre-mRNA splicing. The most commonly mutated proteins include SF3B1, U2AF1 and SRSF2. Here we report that another poorly characterized splicing-related protein, LUC7L2, appears to play a role in MDS. Up to14% of MDS patients are deficient in LUC7L2 expression due to truncating point mutations, chromosomal deletions or other mechanisms. Low expression of LUC7L2 is correlated with a decrease in overall patient survival, however it is unknown how LUC7L2-deficiency contributes to pathogenesis. LUC7L2 is an ortholog of yeast protein LUC7, which is involved in 5' splice site recognition and required for formation of the U1 snRNP complex. RNA-Seq studies of LUC7L2-deficient patient bone marrow detected differential splicing of at least 44 transcripts compared to non-deficient MDS patients. Therefore we hypothesize that LUC7L2 is involved in recognition of the 5' splice sites of a subset of transcripts, and deficiency of LUC7L2 results in aberrant splicing of oncogenes and tumor suppressor genes that drive the pathogenesis of MDS. To test this hypothesis, we performed cross-linked immunoprecipitation followed by sequencing (CLIP-Seq). CLIP-Seq is an *in vivo*, high throughput technique to identify interactions of LUC7L2 with RNA on a genome-wide basis. Preliminary data shows that LUC7L2 crosslinks to pre-mRNA. High throughput sequencing is underway and the results will be presented.

521 A network-based analysis of colon cancer splicing changes reveals a regulatory pathway emanating from ELK1

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Splicing aberrations are prominent drivers of cancer, yet the regulatory pathways controlling them are mostly unknown. Here we develop a method that integrates physical interaction, gene expression and alternative splicing data to construct the largest map of transcriptomic and proteomic interactions leading to cancerous splicing aberrations defined to date. Applying our method to colon adenocarcinoma uncovered a novel regulatory pathway involving the transcriptional induction of the transcription factor *MYC* by the transcription factor ELK1, and the subsequent induction of the alternative splicing factor *PTB* by both transcription factors. We reveal that PTB promotes specific *RAC1*, *NUMB*, and *PKM* isoforms that are major triggers of colon tumorigenesis. We found *ELK1*, *MYC*, and *PTB* to be overexpressed in conjunction with oncogenic *KRAS* mutations in tumor samples, and that these mutations increased *ELK1* levels through the RAS-MAPK pathway. Our method can be applied to other cancers to identify regulatory pathways promoting splicing aberrations.

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522 NTR1 is required for transcription elongation checkpoints at alternative exons in *Arabidopsis thaliana*

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The interconnection between transcription and splicing is a subject of intense study. We report that *Arabidopsis thaliana* homologue of spliceosome disassembly factor NTR1 is required for correct expression and splicing of DOG1, a regulator of seed dormancy. Global splicing analysis in *atntr1* mutants revealed a bias for downstream 5' and 3' splice site selection and an enhanced rate of exon skipping. A local reduction in PolII occupancy at misspliced exons and introns in *atntr1* mutants suggests that directionality in splice site selection is a manifestation of fast PolII elongation kinetics. In agreement with this model, we found AtNTR1 to bind target genes and co-localise with PolII. A minigene analysis further confirmed that strong alternative splice sites constitute an AtNTR1- dependent transcriptional roadblock. Plants deficient in PolII endonucleolytic cleavage showed opposite effects for splice site choice and PolII occupancy compared to *atntr1* mutants, and inhibition of PolII elongation or endonucleolytic cleavage in the *atntr1* genetic background resulted in partial reversal of splicing defects. We propose that AtNTR1 is part of a transcription elongation checkpoint at alternative exons in *Arabidopsis thaliana*.

523 Role of the Ubiquitin-like Protein Hub1 in Splicing

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Hub1 is a highly conserved small ubiquitin-related protein found in all eukaryotes. Distinct from other canonical ubiquitin-like proteins, Hub1 is not conjugated to proteins but rather binds non-covalently. Previously, we have shown that Hub1 interacts through a specific surface with the spliceosomal tri-snRNP protein Snu66 via HIND (Hub1-interaction domain) elements found in the N-terminal domain of Snu66. In *S. cerevisiae,* Hub1 is required for splicing of introns harboring certain suboptimal 5' splice sites, particularly modulating alternative splicing of *SRC1* pre-mRNA with no impact on general splicing (Mishra *et al.,* 2011). Similarly, Hub1 promotes alternative splicing in humans as well (Ammon *et al.,* 2014). However, the precise molecular mechanism of Hub1 function in pre-mRNA splicing remains unsolved.

By using yeast *in vitro* splicing assays, we recently found that Hub1 is also indispensable for splicing of pre-mRNA substrates with suboptimal 5' splice sites. Extracts lacking Hub1 are severely impaired in spliceosome assembly already before the first step of splicing on a suboptimal splicing substrate. Accordingly, Hub1 interacts with the pre-spliceosomal E and A complexes prior to Snu66 and tri-snRNP recruitment and is important for the proper formation of pre-catalytic B complex. Intriguingly, *in vitro* splicing assays using extracts from cells overexpressing Hub1 results in both new mis-cleavage products at positions -1, -2, -3, and -4 and activation of a nearby cryptic splice site in the upstream of the normal 5' splice site even when optimal splicing substrates are used.

Our results suggest a model in which Hub1 alters spliceosomes before the first step of splicing to relax proofreading, thereby allowing the usage of suboptimal splicing substrates. Hub1 might compete and antagonize spliceosomal DExD/H ATPases/helicases, which are known to promote splicing fidelity by rejecting suboptimal substrates.

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Ammon, T., Mishra, S.K., Kowalska, K., Popowicz, G.M., Holak, T.A., and Jentsch, S. (2014). The conserved ubiquitin-like protein Hub1 plays a critical role in splicing in human cells. *J. Mol. Cell Biol*. **6**, 312-323.

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524 Mutant SF3B1 results in altered expression of key genes related to Myelodysplastic Syndromes (MDS)

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Myelodysplastic Syndromes (MDS) are clonal disorders of bone marrow characterized by dysregulated maturation of hematopoietic precursors leading to reduced blood counts. About half of MDS are now shown to be associated with single-allele non-synonymous mutations in various splicing proteins (SF3B1, SRSF2, U2AF1 or ZRSR2). SF3B1, the most commonly mutated protein is a component of both U2 and U12 spliceosomes. SF3B1 mutations are clustered in its HEAT domain repeats. Interestingly, the same mutations have been described in other clonal diseases such as chronic lymphocytic leukemia (CLL) and uveal melanoma. The obvious explanation for the role of these mutations would be the aberrant splicing of key transcripts during hematopoietic maturation, but evidence of splicing anomalies caused by mutant SF3B1 is lacking. To address this, we conducted transcriptome-wide analysis (paired-end RNA-Seq) in blood cells with enforced expression of either wild-type or mutant SF3B1 (K700E, the most common mutation in MDS). Our analysis revealed differential expression of several key genes associated with MDS in SF3B1 mutant cells compared to wild-type. These include EGR1, SLC25A37 and DLK1. EGR1 is a transcription factor critical to maintenance of hematopoietic stem and progenitor cells, loss of which results in stem cell expansion. Protein levels of EGR1 were unexpectedly reduced in SF3B1 mutant cells (despite unchanged splicing pattern and high transcript abundance) suggesting that EGR1 may be targeted for degradation in SF3B1 mutant cells, and the increased transcript levels are likely a compensatory response. SLC25A37 (mitoferrin1) is a mitochondrial transporter of iron, and its loss results in severe anemia. When transcriptomic data was analyzed for changes in splicing, we observed a modest number of events (732 in total) changing between wild-type and mutant SF3B1 expressing cells of which cassette exon was the predominant event type. We also analyzed RNA-Seq from 8 SF3B1-mutated MDS patients and 5 healthy controls, which revealed similar patterns for altered splicing events. In summary, dysregulated expression of several key genes was noted in blood cells expressing mutant SF3B1. Changes in splicing per-se was relatively modest; this suggests that molecular mechanisms other than pre-mRNA splicing may be involved in the pathogenesis of SF3B1-mutated MDS.

525 Ptbp2 controls an alternative splicing program necessary for male germ cell survival.

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The polypyrimidine tract binding protein 2 (Ptbp2) is a tissue-restricted RNA-binding protein abundant in brain and testis. Mouse models have previously shown that Ptbp2 is essential for postnatal survival and is necessary for proper control of alternative mRNA splicing in the nervous system (1,2). Here, we demonstrate that Ptbp2 regulates alternative splicing of germ cell mRNAs and is essential for male germ cell development.

Using a Cre-lox conditional knockout (cKO) approach and RNA-Seq, we identified alternative splicing defects associated with the loss of Ptbp2 expression. Interestingly, genes whose mRNAs are most affected by Ptbp2 loss clustered into a functionally-related network important for the transport and localization of membrane proteins.

In parallel, we developed an improved procedure to separate, count, and collect germ cells in different stages of development. This approach utilizes transgenic mice bearing a dual fluorescence reporter, Cre-lox technology, and flow cytometry. We show that loss of Ptbp2 results in spermatogenic arrest due to defects at two different stages. The first defect occurs during meiosis I, with a subset of cKO germ cells undergoing apoptosis. Those cKO cells that do complete meiosis yield haploid cells that prematurely detach from somatic support cells and become giant multinucleated cells. This second block is complete and occurs in the earliest steps of haploid cell differentiation.

Collectively, the data suggest that Ptbp2 is an essential factor that controls an alternative splicing program critical for germ-somatic cell adhesion and transduction of trophic signals necessary for mammalian germ cell survival.

1. Licatalosi et al., 2012. *Genes and Development.*
2. Li et al., 2014. *eLife.*

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526 JNK-induced Alternative Splicing of MKK7 Generates a Positive Feedback Loop to Amplify JNK Signaling in Activated T cells

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Alternative splicing is widespread during T cell activation and is enriched for genes encoding signaling molecules; however, the functional consequence of differential isoform expression remains largely unknown. We find that in response to T cell activation, the JNK-kinase MKK7 is alternatively spliced to favor an isoform that lacks exon 2. This isoform restores a MAPK docking site within the protein that is disrupted in the larger isoform. Consistently, we show that skipping of exon 2 enhances JNK pathway activity, as indicated by c-Jun phosphorylation and upregulation of the c-Jun target gene TNF-alpha. Moreover, we find that activation-induced repression of MKK7 exon 2 itself occurs specifically in response to signaling through JNK. Notably, signaling through JNK itself is necessary and sufficient to promote activation-induced repression of exon 2 of the MKK7 gene. Thus, MKK7 alternative splicing represents a positive feedback loop through which JNK promotes its own signaling. Importantly, ~25% of T cell receptor mediated alternative splicing events are dependent on JNK signaling, demonstrating a widespread role for JNK in splicing regulation. Furthermore, through minigene based analysis we have identified sequences within both the upstream and downstream introns of MKK7 exon 2 that are necessary and sufficient to promote activation-induced skipping. Either intron in isolation is not sufficient for this activity, suggesting that these act cooperatively to repress exon 2. Analysis of RNA-protein interactions has identified several RNA binding proteins, including CELF2, hnRNPC, HuR and SRp20 that bind to the MKK7 introns and coordinately regulate MKK7-E2 inclusion levels. We find that increased binding of CELF2 accompanied by a loss of hnRNP C in activated cells is a major driver of MKK7-E2 activation-induce skipping.

527 The Drosophila Rbfox protein Ataxin 2 Binding Protein-1 (A2bp1) mediates immune responses

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In diverse animal species, innate immunity acts as the first line of defense against pathogenic microbes. Innate immune cells express germline-encoded receptors that recognize molecular patterns common to microorganisms, allowing immediate and robust responses to a multitude of pathogens. The fruit fly Drosophila melanogaster relies on innate immune responses to defend against infection. To identify novel genes and signaling pathways involved in phagocytosis of bacteria, we screened a subset of the Drosophila Genetic Reference Panel (DGRP) for defects in the ability of hemocytes to phagocytose the Gram-positive bacteria Staphylococcus aureus. Natural variants in Rbfox family member Ataxin 2 Binding Protein-1 (A2bp1) affect phagocytosis in our screen. In humans, Rbfox1, 2, and 3 are neuronal splicing factors that are also expressed in polymorphic mononuclear cells. A2bp1 is the only member of the Rbfox family in Drosophila. It has been shown to regulate the nervous system, wing and germline development.

In this study, we elucidate an immune-specific role for A2bp1. To mount an effective immune response to S. aureus, A2bp1 transcript levels are tightly regulated in hemocytes. Loss of A2bp1, in mutant flies, or flies in which blood cell A2bp1 levels are knocked down using RNA-interference (RNAi), causes reduced phagocytosis and increased susceptibility to S. aureus infection. Interestingly, overexpression of specific isoforms of A2bp1 in hemocytes also leads to cellular immune dysfunction. Concomitantly over-expressing A2bp1 specifically in hemocytes can rescue the phagocytosis phenotypes of A2bp1 mutants.

The homology shared by Drosophila A2bp1 and its human homologs implies that characterization of the role of A2bp1 in fly immunity may provide valuable insights into Rbfox function. A2bp1 may regulate the expression of receptors or plasma membrane-associated signaling proteins. To determine the molecular mechanisms by which A2bp1 mediates the cellular immune response, we have sequenced RNA from hemocytes isolated from wild-type and A2bp1 RNAi flies (both infected and uninfected), obtaining over 10 million mapped reads for each of nine conditions in triplicate. Analysis is underway. We hope to identify A2bp1 target genes that underlie the efficient phagocytosis of S. aureus.

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528 Dissecting a Role for Protein Arginine Methylation in Pre-mRNA Splicing

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Splicing of pre-mRNAs is a critical step in eukaryotic gene expression. Previously, we have established a role for protein arginine methylation in promoting proper cotranscriptional recruitment of pre-mRNA splicing factors to their genomic targets in *S. cerevisiae*. Works by other labs have identified Npl3, a SR-/hnRNP-like protein, as a robust substrate for Hmt1, which is the predominant protein arginine methyltransferase enzyme in *S. cerevisiae*. In addition, our previous work has identified a subunit of U1 snRNP, Snp1, as an *in vitro* substrate for Hmt1. However, the molecular basis by which Hmt1 modulates the recruitment of splicing factors remains to be determined.

Using a directed chromatin immunoprecipitation approach, we determined the effects of Hmt1 on the *in vivo* occupancy of early splicing factors across three different intron-containing genes (ICGs) – *ECM33*, *SUS1*, and *SCS22*. In cells lacking Hmt1, the recruitment of Npl3 is increased across these ICGs whereas the U1 snRNP component Snp1is decreased. Assaying the splicing efficiency for all three ICGs in Hmt1-null cells revealed decreased splicing efficiency for only *SUS1* and *SCS22*, but not *ECM33*. Using a methylarginine-specific mutant of Npl3 (termed $Npl3^{RK}$), we showed that recruitment of Snp1 is restored to the wild-type levels in cells expressing only $Npl3^{RK}$ regardless of Hmt1 status. Furthermore, splicing of *SUS1* pre-mRNA, but not *SCS22*, is restored in cells that express $Npl3^{RK}$. Overall, our data implicate a role of Hmt1 in promoting splicing at a transcript-specific level and underscores a level of regulation in pre-mRNA splicing by post-translational modification.

Keywords: splicing, methylation, Npl3

529 Withdrawn

530 Investigating the Fidelity of Splice Site Selection via Lariat Sequencing in *S. pombe*

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The spliceosome has to maintain a fine balance between tolerance and rejection of suboptimal splice sites with the aid of proofreading components, especially in organisms with degenerate splice sites. An accurate global estimate of the level of spliceosome's tolerance and the impact of loss of proofreading factors on this balance is difficult to assess, largely because the spliced mRNA products from many of these events are known to be rapidly degraded in the cell. Our lab has previously shown that global splicing events can be monitored with high sensitivity by isolating and sequencing the excised lariats that are generated during each splicing reaction[1]. When sequencing lariat introns, a subset of reads can be identified which traverse the branch point adenosine, thereby revealing the sequences of both the 5' splice site and branch point which were used to generate that spliced lariat. Here we have used these high information reads to assess the frequency with which the spliceosome activates 'incorrect' splice signals. We are examining the fidelity of the spliceosome in the background of mutants predicted to impact splice site selection. One particular mutant of interest is SF3b-14, the U2-snRNP associated component recently demonstrated to bind to the bulged adenosine at the branch point and poise it for the first transesterification reaction in splicing[2]. Because of the unique ability of lariat sequencing to identify the nucleotide used at the branch point, we are now determining the global defects associated with branch point selection in the absence of SF3b-14.

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2. Schellenberg, M.J., Edwards, R.A., Ritchie, D.B., Kent, O.A., Golas, M.M., Stark, H., Lührmann, R., Glover, J.N.M., and MacMillan, A.M. (2006). Crystal structure of a core spliceosomal protein interface. Proc Natl Acad Sci U S A 103, 1266–1271.

531 Coordinated Tissue-Specific Regulation of Adjacent Alternative 3' Splice Sites in *C. elegans*

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Adjacent alternative 3' splice sites, those separated by ≤18nt, provide a unique problem in the study of alternative splicing regulation; there is overlap of the *cis*-elements that define the adjacent sites. Identification of the intron's 3' end depends upon sequence elements that define the branchpoint, polypyrimidine tract and terminal AG dinucleotide. Starting with RNA-seq data from germline-enriched and somatic cell-enriched *C. elegans* samples, we identify hundreds of introns with adjacent alternative 3' splice sites. We identify 203 events that undergo tissue-specific alternative splicing. For these, the regulation is mono-directional, with somatic cells preferring to splice at the distal 3' splice site (furthest from the 5' end of the intron) and germline cells showing a distinct shift towards usage of the adjacent proximal 3' splice site (closer to the 5' end of the intron). Splicing patterns in somatic cells follow consensus rules of 3' splice site definition, using sites with a short stretch of pyrimidines preceding an AG dinucleotide. Splicing in germline cells occurs at proximal 3' splice sites that frequently lack a polypyrimidine tract or, occasionally, the AG dinucleotide. We provide evidence that use of germline-specific proximal 3' splice sites is conserved across Caenorhabditis species. We propose that there are differences between germline and somatic cells in the way that the basal splicing machinery functions to determine the intron terminus.

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532 SF3b1 modulates branch site selectivity during spliceosome assembly

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Two lines of evidence link SF3b1, an essential component of U2 snRNP, to human disease and altered splicing. First, deep sequencing data from Myelodysplastic Syndrome (MDS) patients have identified mutations in the C-terminal domain of SF3b1, of which K700E is predominant. Secondly, our laboratory performed a screen in S. cerevisiae to identify factors that improve the splicing of a sub-optimal branch-flanking substrate (reduced base-pairing with U2 snRNA), identifying SF3b1 as one of several U2 snRNP components that can contribute to altered splicing. Aberrant patterns of alternative splicing have been observed in patients, but the mechanistic consequences of SF3b1 mutations on U2 snRNP interactions and spliceosome fidelity are not known.

U1 and U2 snRNPs are principal participants in intron and exon definition, their interaction being critical for juxtaposition of 5'SS and branch site. Our laboratory previously characterized a U1-U2 snRNP interaction network, placing Prp5, an ATPase, as a central player for pre-spliceosomal assembly and splicing fidelity. Specifically, it was shown that the U2 snRNP SF3b complex, of which SF3b1 is the largest component, interacts with Prp5. In addition, splicing of introns containing sub-optimal branch sites are strongly improved by prp5 mutants, either by slowing ATPase activity or by impairing SF3b interactions.

To test the consequence of MDS-related SF3b1 mutations, specifically positions K666 and T663, we inserted disease mutations into the Sc. SF3b1 homologue. SF3b1 MDS-related mutations alter the splicing of sub-optimal branch-flanking regions but do not alter the splicing of BS or 3'SS. Moreover, the altered splicing of sub-optimal branch region is position specific. Mutations in the K666 residue inhibit splicing of the mutant branch-flanking splicing reporter (U257C); in contrast, changes in T663 enhance splicing of the same reporter. Immunoprecipitation (IP) assays, testing the efficiency and stability of Prp5-U2 snRNP interactions, showed an altered interaction between SF3b1 mutants and Prp5. Specifically, K666 mutations improved Prp5-U2 snRNP co-IP in contrast to T663 mutants that decreased it. These results lead us to hypothesize that changes in Prp5-U2 snRNP interaction due to SF3b1 mutations result in either delayed or advanced Prp5 ATPase activity, both contributing to altered splicing fidelity.

533 Expression of alternative splicing factors decrease with loss of circulating 17β-estradiol in the hypothalamus of aged female rats

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The loss of the major circulating estrogen, 17β-estradiol (E2), contributes to the decline of cognitive function including mood disorders and memory impairment observed in postmenopausal women, and longer periods of E2 deprivation correlate with poor outcomes from E2 replacement therapy. Recent studies have observed increased alternative splicing (AS) of Estrogen Receptor β (ERβ), an important nuclear steroid receptor that mediates the actions of E2, as a result of long periods of E2 deprivation were linked to decreased neurogenesis and depressive-like behaviors. This may be due to an alternative exon included within the ligand binding domain of ERβ that decreases its affinity for ligand. Aging increases AS in the brain of both healthy and neurodegenerative individuals. Loss of circulating E2 associated with menopause further compounds the effects of aging in women, yet increases in age-related AS have not been studied in females. Age-related changes in the expression of the CNS-specific RNA-binding splicing factor NOVA1 have been implicated in increased AS events in male-only studies. There are many important splicing-related factors, like NOVA1, present in the brain that may be affected not only by aging, but also by loss of circulating E2. We hypothesized that splicing factor expression decreases with aging and longer periods of E2 deprivation in the hypothalamus. In our model of surgically-induced menopause, 18 month old animals were ovariectomized (OVX), and then, after varying deprivation periods (1 wk, 4 wks, 8 wks, 12 wks), were treated with either vehicle or 2.5 ug/kg E2 for 3 consecutive days. All animals were sacrificed 24 hours after the last treatment for tissue collection and data analysis. Expression of the splicing-related factors HNRNPH1, DDX17, CELF4, and RBFOX1 all decreased with longer periods of E2 deprivation in the aging hypothalamus. Interestingly, all four of these factors significantly increased following E2 treatment early in the deprivation (1-4wks). Contrary to our hypothesis, NOVA1 expression significantly increased up to 8 weeks post-OVX, however expression was nearly undetectable at the 12 week time point. These data suggest that both age and E2 contribute to changes in splicing-related factors that may contribute to increased AS in the hypothalamus.

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534 Sam68 regulates S6K1 alternative splicing during adipogenesis

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Src-associated substrate during mitosis of 68kDa (Sam68) is an RNA binding protein (RBP) of the KH domain family. Sam68 is a sequence-specific RBP that binds repeats of U(U/A)AA sequences. The binding of Sam68 near alternative splice junctions in pre-mRNAs has been shown to regulate splice site selection and regulate the usage of alternative exons. Sam68 null mice leads to increased energy expenditure, decreased number of early adipocyte progenitors, and defective adipogenic differentiation, resulting in mice having a lean phenotype protected against dietary induced obesity. The lack of Sam68 results in mTOR (mammalian target of rapamycin) intron 5 retention and the production of a short transcript (named $mTOR_{i5}$) leading to reduced mTOR protein levels resulting in defects in insulin-stimulated S6 and Akt phosphorylation.

The Sam68-deficient pre-adipocyte defect is partially rescued by the ectopic expression of the full-length mTOR expression, suggesting that there may be other splicing events regulated by Sam68 in the mTOR signaling pathway. To identify these alternative splicing events that contribute to the differentiation defects of Sam68-deficient pre-adipocytes, we monitored the presence of spliced isoforms in the mTOR signaling pathway. Herein, we report that Sam68 regulates isoform expression of *ribosomal S6 kinase* (*Rps6kb1*). Sam68-deficient adipocytes express *Rps6kb1-002* and its encoded p31S6K1 protein, in contrast to wild type adipocytes that do not express this isoform. Sam68 binds an RNA sequence encoded by *Rps6kb1* intron 6 and prevents serine/arginine-rich splicing factor 1 (SRSF1)-mediated alternative splicing of *Rps6kb1-002*, as assessed by crosslinking and immunoprecipitation (CLIP) and minigene assays. Depletion of p31S6K1 with siRNAs partially restored adipogenesis of Sam68-deficient pre-adipocytes. The ectopic expression of p31S6K1 in wild type 3T3-L1 cells resulted in adipogenesis differentiation defects, showing that p31S6K1 is an inhibitor of adipogenesis. Our findings indicate that Sam68 is required to prevent the expression of p31S6K1 in adipocytes for adipogenesis to occur. This work is funded by a grant from the Canadian Institute of Health Research.

535 Characterization of the regulation of CD46 alternative splicing

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In this project, we characterize the regulation of CD46 alternative splicing. CD46 is a membrane-bound complement control protein with two mutually exclusive cytoplasmic tails derived from the alternative splicing of exon 13, a cassette exon. The two cytoplasmic tails have different functions in regulating the response of T_{H1} cells, which are the adaptive immune effectors against intracellular pathogens. As altered exon 13 splicing has been implicated in autoimmune diseases, understanding the regulation of exon 13 splicing could have therapeutic applications in addition to improving current knowledge in splicing. The CD46 splice isoforms analyzed by semi-quantitative PCR showed variations in their relative abundances in 20 different human tissues, indicating that CD46 splicing is tissue specific. Linker scan experiment identified strong ESEs and ESSs distributed along exon 13, and one strong ISEs located at immediate downstream of exon 13 5'ss in addition to several weak silencers and enhancers in the intronic sequences. Several *trans*-acting factors have been identified through RNA-pulldown and functional assays, which either promote or repress the recognition of exon 13 through the *cis*-acting elements found in the linker scan experiment. Finally, we show that splicing of exon 13 can be modulated by anti-sense oligonucleotides (ASOs) in a dose-dependent manner, and these ASOs should be useful to study the functions of each cytoplasmic tail in T-cell response.

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536 Oncoprotein EWS-FLI1 drives alternative splicing in Ewing sarcoma through a unique protein network that can be disrupted by YK-4-279

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Alternative splicing has been implicated as an oncogenic process and provides both a categorization of cancer as well as an opportunity for more effective targeted treatments. Spliceosomal network interactions, including proteins that recognize splice enhancer and silencer regions, are critical for the regulation of alternative splicing leading to oncogenic protein isoforms. Interrogation of complete protein networks remains challenging because it is difficult to modify single interactions while preserving overall network architecture. We hypothesized that EWS-FLI1, Ewing sarcoma (ES) oncoprotein, modulates post-transcriptional gene regulation through novel protein interactions. EWS-FLI1 has multiple connections to the spliceosome and reduction of EWS-FLI1 alters significant numbers of exon skipping and intron inclusion events identified from RNA-seq. We validated the effect of EWS-FLI1 on alternative splicing (AS) using putative oncogenic genes including *CLK1, PPFIBP1, CASP3* and *TERT*. In the presence of EWS-FLI1, the γ-isoform of hTERT is expressed and RNA immunoprecipitation reveals EWS-FLI1 binding *TERT* transcripts. We have validated a small molecule probe, YK-4-279, as an enantio-specific inhibitor of EWS-FLI1 that directly disrupts both RHA and p68 (*DDX5*). In addition, YK-4-279 reverts alternative splicing changes seen in the presence of EWS-FLI1, which was not an effect of altering RNA pol II activity (as shown by BruDRB-seq). We also determined that splicing patterns from 75 ES patients match splicing patterns for 10 genes in cell line models supporting the clinical relevance of our findings. Further, while overall gene expression levels did not stratify for overall survival, principal component isoform specific analyses did segregate survivors from those who died. These experiments establish oncogenic aspects of splicing which are specific to cancer cells and thereby illuminate potentially oncogenic splicing shifts as well as provide a potential stratification mechanism for ES patients. Assessment of aberrant splicing driven by EWS-FLI1 may inform oncogenesis and reciprocally, EWS-FLI1 activities may inform splicing mechanisms.

537 U12-U6atac catalytic core formation is impaired in patient cells with defects in the minor spliceosome component.

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Recently we described biallelic disease-causing mutations in the RNPC3 gene encoding for U11/U12-65K protein (Argente et al. (2014), a specific protein component of the minor spliceosome and part of the U11/U12 di-snRNP that functions in the U12-type intron recognition. RNPC3 mutations are associated with Isolated Growth Hormone Deficiency (IGHD), which leads to pituitary hypoplasia and growth retardation. Analysis of patient lymphoblasts revealed defects in splicing of U12-type introns and destabilization of U11/U12 di-snRNP. Unexpectedly, Northern analysis revealed that U4atac snRNA levels were 2-fold elevated in patients cells compared to the controls. To investigate whether the U4atac/U6atac di-snRNA levels were also affected, whole cell lysates were mildly deproteinized, run on native gels and analysed by Northern blotting. The results confirmed 2-fold upregulation of U4atac/U6atac in patient cells. Rather surprisingly, another snRNA complex was also detected, and subsequently identified as a U12/U6atac snRNA duplex. Because U12 and U6atac snRNAs form an analogous catalytic core structure as the U2 and U6 snRNAs in the major spliceosome, our results suggested that we may have identified the U12-type catalytic core structures, which are stable in native gel conditions. Probing of the same filter with U2 and U6 did not reveal a similar signal, suggested that such interactions for major spliceosomes are not stable in the same conditions. To our knowledge, catalytic core structures isolated from the cellular material have been not detected earlier in mammalian system. We have confirmed the U12/U6atac complex identity using oligonucleotide targeting and by 2D-native gel analysis. Interestingly, patient cells which showed a 2-fold increase in U4atac/U6atac also showed reduced 2-fold reduction of the U12-U6atac catalytic core structures. This suggests that there may be a general defect in the catalytic structure formation in the IGHD patient cells.

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538 Controlling Bcl-X alternative splicing by G-quadruplex ligands - new opportunities for cancer therapies?

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G-tracts are well-characterized cis-acting splicing elements that are recognized by hnRNP F/H. G-tracts can also form secondary structures called G-quadruplexes (G4s). We have shown previously that G4 formation or hnRNP F/H binding are mutually exclusive and suggested that G-quadruplexes could modulate alternative splicing of the Bcl-x pre-mRNA by antagonizing hnRNP F/H (1,2). G-tracts near the 5' end of an intron can stimulate splicing. This has been attributed to U1 recruitment (3) or, in PAX 9 intron 1, quadruplex formation (4).

A major problem in establishing a role for quadruplexes in RNA is that they have been shown to form in short RNA fragments but not in intact pre-mRNA, where secondary structures might interfere. To address this, we have used a novel strategy based on comparing the RNA footprints of normal and deaza-7-GTP-substituted pre-mRNA to identify quadruplexes within the 2D structures of Bcl-X, which has two alternative 5'ss (X_S and X_L). This revealed the presence of two G4s, one 3' of the X_S site and the other 5' of the X_L site. Mutagenesis strategies designed to preserve the 2D structures suggested that these affect splice site selection.

To confirm the functionality of these G4s, we screened the effects of more than 30 G4-binding ligands on Bcl-X splicing. Eight shifted splicing towards the pro-apoptotic Bcl-X_S isoform, both *in vitro* and *in vivo*. The most potent has independent and opposite effects on the two splice sites, decreasing X_L and increasing X_S mRNA. Moreover, it binds to the 5'ss-containing fragments of the RNA and affects the RNA footprints around the splice sites. We conclude that G4s play a significant role in the alternative splicing of *Bcl-X* and that specific G4 ligands augment this effect.

These results are highly significant because the active G4s-binding ligands are promoting a shift from an anti-apoptotic to the pro-apoptotic isoform of *Bcl-X* and could be developed as anti-cancer treatments that target pre-mRNA splicing.

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539 A positive feedback loop couples aberrant TGF-beta signaling and CD44 alternative splicing

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The TGFb signaling pathway plays a critical role in cell invasion, inflammation, and extracellular matrix deposition. Aberrantly activated TGFb signaling is responsible for numerous pathological conditions, including chronic fibrosis, cardiovascular disease and cancer. Therefore, developing therapeutic strategies to impair constitutively activated TGFb signaling is highly relevant to human health and disease. Studies in recent years have connected growth factor and cytokine mediated signaling pathways to changes in RNA alternative splicing. These alterations in alternative splicing are increasingly recognized as causative drivers of human pathologies. Investigation of the intersection between cell signaling and alternative splicing will reveal attractive targets for the development of new therapieutics.

Previous work in our lab demonstrated that treatment of TGFb in epithelial cells causes CD44 splice isoform switching from CD44 variable exon containing variants (CD44v) to the non-variable exon containing CD44 standard (CD44s). We showed that TGFb-induced CD44 isoform switching is critical for epithelial-mesenchymal transition (EMT). We also identified that regulation of isoform switching is mediated by competition between two splicing regulators ESRP1 and hnRNPM. In epithelial cells, ESRP1 binds to CD44 pre-mRNA, precluding hnRNPM binding and resulting in the production of CD44v. Following TGFb treatment, TGFb-induced Snail expression represses ESRP1 transcription, allowing the competing splicing factor hnRNPM to assess CD44 pre-mRNA and promote CD44 variable exon skipping. Excitingly, we recently discovered that the CD44s splice isoform functionally activates the TGFb pathway. Manipulating hnRNPM, ESRP1, and CD44s directly affects TGFb activation, suggesting a positive feedback loop between TGFb signaling and CD44 alternative splicing. Hence, our results suggest an alternative splicing-mediated positive feedback loop that sustains TGFb signaling for an EMT phenotype, a hallmark of numerous pathological conditions. Disrupting this feed-forward loop provides an opportunity to target aberrantly activated TGFb signaling.

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540 Re-Introduction of Tumor-Suppressor miR-34a Shows Therapeutic Efficacy in Triple Negative Breast Cancer

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Triple-negative breast cancer (TNBC) accounts for a disproportionate share of the total breast cancer morbidity because of its aggressive behavior and lack of effective targeted therapies to treat the disease. MicroRNAs, global regulators of survival and proliferation pathways important in tumor development and maintenance, are highly dysregulated in cancer. We identified miR-34a to be aberrantly lost in TNBC lines when compared to both a luminal cancer subtype as well as normal breast cells. Re-introduction of miR-34a in TNBC lines results in inhibition of cell proliferation and invasion, reactivation of senescence, and enhanced sensitivity to apoptosis-inducing agents. Furthermore, intratumoral delivery of miR-34a into subcutaneous tumors in nude mice, as well as systemic delivery of poly(amine-co-ester) PACE-loaded miR-34a in an orthotopic setting, delayed tumor growth. In conclusion, re-introduction of miR-34a in TNBC promotes potent anti-tumorigenic phenotypes in vitro and in vivo, and could be a promising targeted therapeutic agent to treat the disease.

541 Broad Spectrum RNA Inhibitors of Reverse Transcriptase

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Reverse transcriptase (RT) inhibitors are a core component of highly active antiretroviral therapy. While effective at curtailing the spread of HIV infection, the high mutation rate of the virus, coupled with the selection pressure introduced by the RT inhibitor, allows for the rapid emergence of viral drug resistance and necessitates a new class of inhibitors with a high genetic barrier to viral resistance. RNA aptamers selected to bind RT can also inhibit it by outcompeting viral RNA for access to the enzyme's active site. These aptamers have shown strong biochemical and biological inhibition against the subtype and strain originally selected for, but not against phylogenetically diverse RT. We hypothesize that RNA aptamers that bind to features conserved across phylogenetically diverse viral strains, rather than features that differentiate one strain from another, will offer a high genetic barrier to viral resistance. To identify these broad-spectrum RNA inhibitors we designed a novel selection strategy termed "Poly-Target SELEX," in which a pre-existing aptamer library, enriched for a specific RT (HIV-1 M:B), was subjected to three additional rounds of independent selections against a phylogenetically diverse panel of RT from HIV-1, HIV-2, and SIV. Following the selections, populations were subjected to high-throughput sequencing. Data was analyzed using the recently developed FASTAptamer toolkit for combinatorial selections and identified several candidate aptamers that co-enriched across multiple selection trajectories. Biochemical assays then validated cross-clade RT inhibition for these candidate aptamers and "winners" were truncated to study their minimal sequence requirements and further investigated in cell culture assays to confirm their ability to suppress viral replication. Our results reveal that a previously identified "6/5" motif, and several new motifs, may be responsible for broad-spectrum inhibition. Several aptamers, including "88.1," demonstrated robust inhibition of all strains used in the selection and even against an RT that was outside of the training set. Taken together, we demonstrate that Poly-Target SELEX allows for the identification of broad-spectrum RNA inhibitors of RT and that pre-enriched aptamer libraries may contain sufficient untapped diversity to allow for selection of additional functions.

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542 Making the Optimal Messenger RNA for Gene Therapy Applications: Evaluation of Novel Nucleotide Modifications for Improved Activity

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Recently, there has been significant interest in the use of messenger RNA (mRNA) based expression systems for gene therapy applications. Several groups have shown that mRNA is an attractive vehicle for therapeutic gene expression in mammals (Nat. Biotechnol (2011) 29, 154; Molecular Therapy (2012) 20, 948). Messenger RNAs are translated in the cytoplasm of cells which may improve protein expression in difficult to transfect, non-dividing cells. For example, highly efficient induced pluripotent stem cell (iPSC) generation by transfection of mRNAs encoding reprogramming factors was demonstrated (Cell Stem Cell (2010) 7, 618). The authors suggested that iPSCs generated in this manner should be safer than iPSCs derived by plasmid transfection or viral transduction because mRNA poses no risk of insertional mutagenesis and subsequent oncogenesis. In addition, transient expression from mRNA is desirable for applications such as genome editing using zinc-finger nucleases, TALENs and Cas9/CRISPR. Lastly, there is considerable interest in using mRNAs for vaccines (Expert Rev Vaccines (2015) 14, 151).

A key insight was the recognition that mRNA induces innate immune responses in transfected cells. Kariko et al. showed that substitution of uridine and cytidine residues with pseudouridine and 5-methylcytidine dramatically reduced innate immune recognition of mRNA (Molecular Therapy (2008) 16, 1833). Pseudouridine modified mRNA also translated more efficiently.

These studies highlight the importance of the development of stable, non-immunogenic mRNA. Activity and immunogenicity of mRNAs likely depends on the chemical modification pattern, route of delivery and cell type or tissue transfected. To date, however, there have been few studies to assess novel chemical modifications of mRNAs. We synthesized numerous novel nucleotide triphosphates (NTPs). Twenty-seven combinations of modifications were used to synthesize eGFP and luciferase mRNAs and incorporation by T7 RNA polymerase was assessed. The translation potential of the mRNAs was evaluated in rabbit reticulocyte lysates. Activity and toxicity were measured in six primary and immortalized cell lines. While trends were seen, cell type specific differences in expression were also observed. These studies greatly expand our knowledge of the optimal chemical modification of mRNA required to achieve maximal expression in different cell types.

543 Selective activation of gene expression by targeting long non-coding RNA

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Long non-coding RNA (lncRNA) can regulate transcription by recruiting epigenetic modifying complexes to target genes. Polycomb repressive complex 2 (PRC2), an epigenetic transcriptional repressor, is recruited to its target genes via lncRNA. PRC2 represses transcription through the action of the histone methyltransferase EZH2, which methylates histone H3 at lysine 27 (applying the H3K27me3 repressive mark). Using RIP-Seq, we identified the genome-wide pool of RNA sequences that interact with PRC2. We design short single-strand oligonucleotides that hybridize to an individual lncRNA, and sterically block its binding to PRC2. These oligonucleotides selectively block PRC2 binding to a specific lncRNA, thereby de-repressing the expression of an associated target mRNA, resulting in increased amounts of the therapeutic protein. This approach was used to increase SMN2 transcription as a potential treatment for Spinal Muscular Atrophy (SMA). Oligonucleotides that target an antisense lncRNA increase SMN2 mRNA and protein levels in SMA patient cells *in vitro* and in a mouse SMA model that carries the human SMN2 locus. Data support a mechanism of action whereby the oligonucleotides block recruitment of PRC2 to SMN2 chromatin, decrease the H3K27me3 repressive chromatin mark, and increase RNA polymerase II association along the SMN2 gene. This therapeutic approach potentially can be used to elevate the expression of many genes whose gene products are limiting in various human diseases.

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544 Silencing gene expression by recruiting RISC.

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To trigger effective RNAi in vivo, a double-stranded siRNA must (1) retain function but resist nucleolytic degradation; (2) load its antisense strand into Argonaute2 protein, the only one of the four mammalian Argonautes that can mediate RNAi; and (3) silence the target gene only in the intended cell type or target tissue. Redirecting endogenous microRNAs (miRNA) to silence genes that they do not normally target solves these challenges that limit the successful use of siRNAs in vivo. We are exploring a new approach to gene silencing that takes advantage of the well understood stability and delivery properties of antisense oligonucleotides and the cell-type specific expression of endogenous miRNAs. Our strategy employs a stabilized, synthetic oligonucleotide tether that recruits an endogenous miRNA to a specific target mRNA. The oligonucleotide tether contains one region complementary to the target mRNA and another to an abundant endogenous miRNA. The tether binds endogenous miRNA-loaded Argonaute complexes and links them to the mRNA. In vivo in mouse liver and in cultured human hepatocyte cell lines, a tether can readily silence the target luciferase reporter expressed from rAAV without altering expression of the control luciferase. Tethers designed to recruit miR-122 to the 3′ UTR of an endogenous mRNA reduced mRNA abundance in cultured human hepatocytes by ~50%. Our results suggest that it is possible to (1) redirect an endogenous miRNA to silence an mRNA with no binding sites for that miRNA and (2) design the tether to silence only in specific tissues by choosing a miRNA with an appropriate expression pattern. We will present results of our ongoing in vitro and in vivo efforts to optimize tether design and chemistry.

545 Promoter Compatibility Enables Viral Vectors to Deliver Antiviral RNA Aptamers

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HIV-1 reverse transcriptase (RT) copies the RNA genome into double-stranded DNA during viral replication. Nucleic acid aptamers that bind RT inhibit its enzymatic activity in biochemical and biological assays, and they efficiently suppress viral replication in cell culture. Delivery of aptamer-expressing genes into appropriate cells is a necessary central question for moving aptamer-based strategies into pre-clinical contexts. Ex vivo transductions with HIV-derived lentiviruses allow efficient gene delivery and stable expression. However, delivery of anti-HIV aptamers might induce self-targeting, in which expression of the antiviral cargo gene during vector production interferes with efficient gene delivery. We propose that regulated promoters are more suitable than constitutive promoters for delivering anti-RT aptamer genes by viral vectors. Specifically, promoters that are strong in T cells are expected to suppress replicating virus most effectively, while promoters that are weak in producer cells are expected to yield the highest transduction efficiencies by minimizing self-targeting during particle production.

To establish relative promoter strength in producer HEK293FT cells, we cloned constitutive and regulated promoters (CMV, CMV-TO, EF1α, UbC, CD4 and U6) to drive the expression of either a fluorescent aptamer (stabilized tetramer-Broccoli) or EGFP. Baseline promoter strength (MFI) varied widely in HEK293FT cells, with strong expression under CMV, intermediate expression under EF1α and UbC, and almost no expression under CD4. Regulated expression was achievable under CMV-TO by co-transfecting a plasmid expressing TetR Repressor. This suggests that promoters with lower levels of expression in producer cells, such as CD4 and CMV-TO, might overcome self-targeting and become optimal for production of lentiviral vectors.

We next evaluated each promoter/aptamer combination on viral infectivity by measuring EGFP expression. Aptamer-expressing constructs were transiently transfected into producer cells (HEK293FT) along with proviral and helper plasmids. Virus harvested from aptamer-expressing cells after 48h was less able to infect epithelial (HEK293FT) and T cell lines (CEM-T4) than virus harvested from cells expressing an arbitrary RNA, in each case correlating inversely with promoter strength in the producer cells. These infectivity data correlates with promoter strength, suggesting that EGFP and stabilized tetramer-Broccoli are informative surrogates for viral inhibition.

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546 Combinatorial Aptamer Transcripts (CATs) for HIV RT Suppression

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The Human Immunodeficiency Virus (HIV) Reverse Transcriptase (RT) is a DNA polymerase encoded by the viral genome and it is preferentially targeted by current therapeutics due to its critical role in the viral life cycle. High affinity RNA aptamers that bind RT out-compete viral genome for access to the active site and thereby inhibit replication. Aptamer binding to RT during viral formation is believed to drive aptamer encapsidation into the budding virus, which leads to significantly reduced infectivity. Co-transcribing multiple aptamer modules as combinatorial transcripts is expected to increase avidity and packaging, resulting in greater net viral suppression. To test this hypothesis, we built a series of Combinatorial Aptamer Transcripts (CATs) carrying multiple, co-transcribed aptamer modules (homodimeric/trimeric UCAA and 6/5 structural motifs) and compared their inhibitory capabilities as a function of valency.

In general, transcripts with more modules exhibited moderately increased RT inhibition in-vitro and increased net binding affinity in Electrophoretic Mobility Shift Assays (EMSAs).The EMSAs do not show evidence of binding multiple RT per transcript, and preliminary cell-based assays do not indicate that this approach to multivalency improves net viral suppression. In contrast, an alternative design appears to be more promising, in which individual aptamer are modularly incorporated into a stable 3-Way Junction (3WJ). The 3WJ structural core is expected both to isolate the individual aptamer structures from each other (thereby reducing misfolding) and to allow sufficient separation to prevent steric hindrance in the binding of multiple RT. Additional design features simplify the operational requirements of swapping individual modules or integrating libraries of aptamers. Initial data based on this design are encouraging and incorporation of fluorescent aptamers (dBroccoli) will allow for monitoring of packaging efficiency by relative signal intensity.

Combinatorial transcripts are expected to block evolutionary escape by forcing the virus to acquire multiple simultaneous mutations, especially for hetero-multimer designs. It is well established that combinations of small molecule drugs or of siRNA improves their targeting, delivery, and potency, while reducing their susceptibility to escape mutations. Evaluating multimerization designs will inform downstream utilization of CATs as a therapeutic strategy in the treatment of HIV.

547 Antisense oligonucleotide therapies for the treatment of Huntington's disease

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Huntington's disease (HD) is a dominantly inherited neurodegenerative disease caused by a CAG expansion in the huntingtin gene. Carriers of the mutant huntingtin gene experience loss of motor, cognitive and psychiatric functions and ultimately die ~15-20 years after onset. All therapeutic strategies for HD available or in trials today target the symptoms of the disease and not the underlying disease mechanism, and although important, they do not alter the course of disease or the ultimate outcome. Antisense oligonucleotides (ASOs) offer an alternative method to modulate traditionally 'undruggable' targets, and in the case of HD can target huntingtin, the source of this intractable disease. ASOs are primarily used to modulate RNA either through the recruitment of RNAseH which degrades the target RNA, or by obstructing interactors of the target RNA to inhibit translation or modulate splicing. Exploiting different ASO mechanisms, we have explored multiple ways to modulate huntingtin RNA with ASOs. This includes, RNAseH mediated suppression of total huntingtin RNA. In animal models of HD, this can both prevent disease progression and reverse existing disease. Moreover, RNAseH huntingtin ASOs delivered directly into the CSF of large animals distribute broadly in CNS tissues and suppress huntingtin RNA in the spinal cord, cerebral cortex and additional brain regions. ASOs have also been utilized to selectively suppress the mutant huntingtin allele, either by targeting SNPs in linkage disequilibrium with the CAG expansion or by targeting the CAG repeat directly. Here, I will focus on the state of ASO therapeutics for the treatment of Huntington's disease and the multiple approaches explored.

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548 RNA Nanoparticles for Immune Modulation

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Modulation of immune response through cytokine induction is a vital process in cancer immunotherapy. It is desirable to develop potent and non-toxic immunomodulators to promote successful cancer immunotherapy. Here we report the development of novel immunomodulators by engineering rationally designed RNA nanoparticles based on the pRNA of the bacteriophage phi29 DNA packaging motor. RNA nanoparticles with triangular, square and pentagonal shape were successfully constructed based on the three-way junction of the pRNA. Changing of the length of one RNA strand induced the stretching of the angle of the pRNA three-way junction from 60° to 90° or 108°, resulting in self-assembly of elegant RNA triangular, square, and pentagonal polygons as revealed by AFM imaging. When immunoactive CpG DNAs were incorporated into the RNA nanoparticles, their immunomodulation effect for cytokine TNF-α and IL-6 induction was greatly enhanced both *in vitro* and *in vivo*, while RNA nanoparticle controls induced unnoticeable cytokine induction. Moreover, the RNA nanoparticles were delivered to macrophages specifically and the degree of immunostimulation greatly depended on the size, shape, and the number of payload per RNA nanoparticles. Stronger immune response was observed when the number of adjuvants per nanoparticle was increased, demonstrating the advantage of shape transition of the RNA nanoparticles from triangle into pentagon which can carry five adjuvants. This finding demonstrates that RNA nanotechnology such as developing RNA nanoparticles based on pRNA has great potential to develop novel immunomodulators for cancer immunotherapy.

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549 Ribonuclease P-associated external guide RNAs effectively inhibit hepatitis B virus gene expression and replication in vitro and in vivo

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External guide sequences (EGSs) are RNA molecules that consist of a sequence complementary to a target mRNA and recruit intracellular ribonuclease P (RNase P), a tRNA processing enzyme, for specific degradation of the target mRNA. We have previously used an *in vitro* selection procedure to generate EGS variants that efficiently induce human RNase P to cleave a target mRNA *in vitro*. In this study, we constructed EGSs from a variant to target pregenomic RNA (pgRNA) of hepatitis B virus (HBV). Furthermore, an attenuated *Salmonella* strain was constructed and used for delivery of anti-HBV EGS in cells and in mice. Substantial reduction in the levels of HBV gene expression and viral DNA was detected in cells treated with the *Salmonella* vector carrying the functional EGS construct. Furthermore, oral inoculation of *Salmonella* carrying the EGS construct led to an inhibition of about 97% in the levels of HBV gene expression and a reduction of about 300,000 fold in viral DNA level in the livers and sera of the treated mice transfected with a HBV plasmid. Our results provide direct evidence that EGSs are effective in inhibiting HBV replication in vitro and in vivo and can be used for gene-targeting applications such as anti-HBV therapy.

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550 GalNAc-conjugated siRNAs as a new paradigm in RNAi therapeutics

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Synthetic small interfering RNAs (siRNAs) act as therapeutic agents through the RNA interference (RNAi) pathway and are specific and potent inhibitors of gene expression. These agents may be designed to target disease pathways previously considered "undruggable". Recently, delivery of therapeutic siRNAs to liver hepatocytes upon subcutaneous administration has been achieved by conjugating chemically modified siRNAs with multivalent N-acetylgalactosamine (GalNAc) residues that are recognized by the asialoglycoprotein receptor (ASGPR). siRNA-GalNAc conjugates efficiently target and silence disease-causing genes produced in liver hepatocytes in animal models and humans. Using this conjugation platform, Alnylam is advancing several RNAi agents specific for liver targets through pre-clinical and clinical development to address diseases with unmet medical need. Our progress with the chemistry of siRNA-GalNAc conjugates and applications in several therapeutic areas will be presented.

551 Guanabenz (Wytensin™) selectively enhances uptake and efficacy of hydrophobically modified siRNA

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One of the major obstacles to the pharmaceutical success of oligonucleotide therapeutics (ONTs) is efficient delivery from the point of injection to the intracellular setting where functional gene silencing occurs. In particular, a significant fraction of internalized ONTs are nonproductively sequestered in endo-lysosomal compartments. Here, we describe a two-step, robust assay for high-throughput de novo detection of small bioactive molecules that enhance cellular uptake, endosomal escape, and efficacy of ONTs. Using this assay, we screened the LOPAC (Sigma-Aldrich) Library of Pharmacologically Active Compounds and discovered that Guanabenz acetate (Wytensin™), an FDA-approved drug formerly used as an antihypertensive agent, is capable of markedly increasing the cellular internalization and target mRNA silencing of hydrophobically modified siRNAs (hsiRNAs), yielding a ~100-fold decrease in hsiRNA IC_{50} (from 132 nM to 2.4 nM). This is one of the first description of a high-throughput small-molecule screen to identify novel chemistries that specifically enhance siRNA intracellular efficacy, and can be applied towards expansion of the chemical diversity of ONTs.

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552 Exploring molecular features of viral RNA *trans*-splicing for therapeutic strategies

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Viruses are the smallest replicating specimens and for most viruses, economy in genome size is a common theme. Alternative splicing, a mechanism that doesn't enlarge the genome size, represents a key mechanism that is recruited by most DNA viruses, and nuclear replicating RNA viruses to generate the full repertoire of protein functions. Spliceosome-mediated RNA *trans*-splicing describes a special form of alternative splicing in which sequences of distinct pre-mRNA transcripts are joined *in trans* triggering the formation of chimeric mRNA and gene products. *Trans*-splicing is increasingly being explored for genetic therapy as a molecular tool that allows mRNA repair or labeling. However, these attempts suffer from dissatisfying *trans*-splice activity and specificty. Recently, we reported that viruses, i.e. the simian virus 40 (SV40), employ the mechanism of RNA *trans*-splicing to generate new sequences and protein functions [1-2]. In the case of homologous SV40 RNA *trans*-splicing, two identical early viral transcripts are joined triggering the formation of a 100kD super tumour antigen. The SV40 *trans*-splice reaction was described to be supported by various SV40-intrinsic molecular helper functions which render this reaction highly efficient with about 50 to 70% of the viral pre-mRNA transcripts being involved in *trans*-splicing. We combined computational RNA secondary structure design with molecular features associated with viral RNA *trans*-splicing to design *trans*-splicing RNA suitable to label cancer- or virus-specific transcripts with a death signal for suicide gene therapy. *Trans*-splicing towards these abberant transcripts enabled expression of the HSVtk which then converted the drug ganciclovir into a toxic compound triggering cell death. Our molecular targets were the a-fetoprotein pre-mRNA which is associated with hepatocellular carcinoma or HPV-16 transcripts. Advanced RNA design significantly improved both activity and specificity of RNA *trans*-splicing and efficiently triggered target cell death. This is the first example demonstrating that RNA *trans*-splicing towards a natural endognous target can trigger a therapeutically relevant phenotype. This technology is being further optimised and may in the future lead towards therapy of cancer or infections with integrating viruses.

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553 Discovery of Novel PIWI/AGO Families in the Mediator Complex and a Mobile, Three-Gene Operon: Roles in Eukaryotic Transcription Initiation and Bacterial Genome Conflict

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The PIWI/AGO superfamily of proteins bind guide strands which target complementary nucleic strands, thereby functioning as critical components of several cellular pathways including germline maintenance, chromatin organization, regulation of splicing, RNA interference, and virus suppression. We recently discovered two divergent, novel families of the PIWI/AGO superfamily, the first such to be described since the initial characterization of the superfamily over a decade ago. Both families conserve the amino acid residues necessary for the binding of oligonucleotide guide strands. The first family, found only in eukaryotes and named the MedPIWI family, is the core conserved module of the Med13 protein, a subunit of the CDK8 subcomplex of the transcription regulatory Mediator complex. Med13 functions as part of a regulatory switch through which the CDK8 subcomplex modulates transcription at Mediator-bound promoters of highly transcribed genes and we propose this switch is activated via RNA recognition by MedPIWI. The second family, the bacterial PIWI_RE family, is one part of a highly-mobile, three-gene operon which also includes a restriction endonuclease (REase) fold enzyme and a helicase of the DinG family. The presence of the DinG family helicase, which specifically acts on DNA-RNA hybrid-forming R-loops, implicates PIWI_RE as a sensor in a novel RNA-dependent restriction system potentially targeting invasive DNA from phages, plasmids, or conjugative transposons.

Placement of these novel families in the broader context of PIWI/AGO evolutionary history throws extensive light on functional trajectory of the PIWI/AGO superfamily: ancestral members functioned in bacterial conflict systems targeting DNA substrates while the shift to RNA-targeting occurred in the course of superfamily diversification. We present evidence that a subset of prokaryotic PIWI/AGO proteins are likely to act on RNA substrates, implying that RNA-targeting began prior to PIWI/AGO recruitment to RNAi systems during eukaryogenesis. Identification of the MedPIWI family reveals that the PIWI/AGO superfamily was recruited on at least two occasions to distinct functional contexts in the early phases of eukaryotic evolution.

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554 The genome-wide effects of hypomorphic mutations in six Sen1-dependent transcription termination factors are surprisingly diverse

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In the yeast *Saccharomyces cerevisiae*, the Sen1-dependent termination pathway terminates RNA polymerase (Pol) II-mediated transcription of many short non-coding RNA genes. It also regulates expression of some protein-coding genes, and restricts promiscuous transcription arising from intergenic regions. Although this termination pathway has been the subject of several recent studies, our understanding of its mechanism and biological functions remain incomplete. To assess the functional relationships of different termination factors in this pathway, we performed transcriptome analyses of strains bearing hypomorphic mutations in six different factors involved in Sen1-mediated transcription termination. Four of the mutant alleles, *sen1-E1597K*, *nrd1-V368G*, *ssu72-G33A*, and *rpb11-E108G*, were obtained in genome-wide selections for read-through of specific Sen1-dependent terminators. The other two mutations, *nab3-F371L/P374T* and *hrp1-L205S*, have been shown to elicit read-through at certain Sen1-dependent terminators.

Overall, the *sen1-E1597K* substitution induces the strongest and broadest terminator read-through among the six factors. Surprisingly, this substitution derepresses meiotic genes in vegetative cells, suggesting that Sen1 is important for silencing meiotic genes. Pairwise correlation analyses show that transcriptome changes caused by mutations in Sen1, Nrd1, and Nab3, which compose the core termination complex, and Ssu72, a Pol II C-terminal domain (CTD) phosphatase, are most highly correlated, consistent with the model that the CTD recruits the core complex for termination. Our results confirm that termination of sn/snoRNA genes are widely Sen1-dependent, but only a subset of these genes are affected by substitutions in Nrd1 or Nab3. We are currently investigating if the insensitivity of some snoRNA genes to Nrd1 and Nab3 mutations is due to redundant function of these factors, or if other RNA-binding proteins recognize some snoRNA terminators. Sen1 appears to have a separate function that is also affected by the substitution in Rpb11, the second smallest subunit of Pol II. The *rpb11-E108G* substitution strongly derepresses the *FLO1* gene and decreases expression of the arginine biosynthetic genes. Hrp1 exhibits little overlap with the Sen1 pathway, but appears to be important for the processivity of transcription on protein-coding genes. Our results suggest that transcription termination by Pol II is under complex, combinatorial control, similar to transcription initiation at Pol II promoters.

555 Interpretation of the RNA-mediated transcription attenuation mechanism through all-atom and coarse-grained MD simulations

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In RNA-mediated transcriptional attenuation, the elongation of a nascent mRNA is halted prematurely by the binding of a non-coding, antisense RNA to a corresponding hairpin in its 5'UTR. This process has been utilized for the engineering of RNA-only gene expression networks. The creation of increasingly complex networks has been facilitated by the development of new, chimeric transcriptional attenuators. However, the regulatory response of these synthetic attenuator systems can vary greatly with subtle changes in sequence. In-cell SHAPE-seq reactivity measurements, performed by our collaborators in the Lucks lab at Cornell, indicate that bulges in sense strand hairpin are critical for desired levels of activity. Here we employ all-atom and coarse-grained molecular dynamics (MD) simulations to provide a mechanistic interpretation of the changes observed by SHAPE-seq reactivities between active and inactive chimeric attenuator systems. All-atom, replica exchange MD simulations show significantly increased intra-strand base pair occupancy in the would-be fusion region of the sense strand of an inactive chimera. These base pairs increase the energetic barrier to binding of the antisense regulator and are in good agreement with reduced SHAPE-seq reactivates. Additionally, coarse-grained simulations of sense-antisense association show extended inter-strand base pairing and the formation of stable intermediates for an active chimera, while a sequentially similar inactive chimera fails to form base pairs beyond an initial contact complex.

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556 Analysis of Maelstrom function in piRISC-mediated transcriptional silencing of transposons

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Transposons are well-known selfish genetic elements, existing universally in the genomes of eukaryotes, which move around the genome and often cause genomic instability. PIWI-interacting RNAs (piRNAs), endogenous small RNAs specifically expressed in the reproductive cells of animals, form a complex with the protein PIWI to repress transposons. The mechanism of action is highly conserved among multiple species, and is indispensable for stabilizing the genomes of reproductive cells.

Drosophila follicle cells express Piwi protein localized in the nucleus. After Piwi interacts with piRNAs in the cytoplasm to form a piRNA-induced silencing complex (piRISC), it is translocated to the nucleus, where it represses transposons. It has become clear from the recent analyses of Brennecke et al. that the protein Maelstrom (Mael) is indispensable for piRISC-dependent transposon silencing in the nucleus, but the functional mechanism remains unknown. Mael consists of two domains: the HMG-box and the MAEL domain. The HMG-box has been shown to have RNA-binding activity but it is dispensable for transposon silencing in the soma. Therefore, the Mael domain is sufficient to repress transposons. Interestingly, the Mael domain has RNase activity in vitro, but it is not indispensable for transposon silencing. Therefore, it is presumed that Mael functions as a scaffold. Here, we detected a Mael–Piwi interaction in nuclear extracts of ovarian somatic cells (OSCs), and identified the other nuclear factors present in this complex. At this meeting, we will discuss the results of this research. We will also discuss the relationship between Maelstrom and chromatin at transposon loci.

557 Structure and Mechanism of the T-box Riboswitch, Bacterial Sensors of Amino Acid Starvation

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Bacterial T-box riboswitches sense intracellular amino acid availability by directly binding tRNAs and monitoring their aminoacylation status. It remains unknown how the T-box recognizes its cognate tRNA, detects its aminoacylation state, and directs genetic switching. We define the T-box Stem I as the minimal domain necessary and sufficient for specific, high-affinity tRNA binding, and report its co-crystal structure complexed with cognate tRNA at 3.2 Å resolution. The C-shaped Stem I cradles the L-shaped tRNA forming an extended interface, simultaneously recognizing the anticodon and elbow of tRNA. There are noteworthy structural parallels with the ribosome and RNase P. Using chemical analogs, we find that the T-box detects the molecular volume of tRNA 3'-substituents. Combined calorimetric and fluorescence lifetime analyses demonstrate that this discrimination results from intimate, coaxial stacking between the tRNA acceptor end and a helix in the T-box 3' domain. This intermolecular stacking, selective for uncharged tRNA, stabilizes the antiterminator and allows transcription readthrough into downstream coding regions.

Taken together, the T-box paradigm demonstrates that non-coding RNAs can recognize each other not just through sequence complementarity, but through structural specificity using mutually induced fit between precisely positioned RNA motifs. It also provides a proof of principle that compact RNA domains can sense minute chemical changes on another RNA (such as tRNA aminoacylation), and alter gene expression based on that readout. These findings hint at the possible existence of networks of interconnected non-coding RNAs and a potential for RNA devices to directly read and act on epigenetic marks.

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558 The Regulation of RNAPII Transcription Termination by FUS and TARDBP

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The C-terminal domain (CTD) of RNA polymerase II (RNAPII) is a platform for modifications specifying the recruitment of factors that regulate transcription, mRNA processing, and chromatin remodeling. We have found that a CTD Arginine residue (R1810) is symmetrically dimethylated (me2s), allowing it to recruit the Tudor domain of the SMN protein, which is mutated in spinal muscular atrophy. SMN can oligomerize and interact with Senataxin (Setx), which is mutated in Amyotrophic Lateral Sclerosis 4 (ALS4). We found that R1810me2s and SMN, like Setx, are important for resolving R-loops created by RNAPII in transcription termination regions. FUS and TDP-43(TARDBP) are DNA/RNA binding proteins that are involved in numerous aspects of gene regulation. They are known to interact with each other and with SMN, and both have been implicated in ALS and (Fonto-temporal Dementia). We have found that FUS and TDP-43 act downstream of the CTD R1810me2s-SMN pathway. Through RNAPII ChIP and R-loop (DNA:RNA hybrid) DIP, we found that TDP-43 and FUS are also involved in resolving R-loops that are enriched in RNAPII termination regions. Defects in FUS and TDP-43 recruitment to RNAPII may lead to R-loop accumulation and DNA damage, which may contribute to neurodegenerative disorders like SMA and ALS/FTD.

559 Dissecting the role of eIF3 during mRNA recruitment and scanning

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Translation initiation in eukaryotes is distinguished by the formation of a 43S pre-initiation complex (PIC) that is recruited to the 5' end of the mRNA and is then thought to scan in the 3' direction in search of the AUG start codon. Upon recognizing the AUG codon, the PIC arrests the scanning process and is joined by the 60S subunit for form the 80S ribosome, thus committing the ribosome to the elongation cycle. Eukaryotic initiation factor 3 (eIF3) is the largest of the initiation factors and has been shown to bind the solvent face of the 40S subunit and project arms near both the mRNA entry and exit channels located near the A and E sites, respectively. eIF3 modulates events throughout the initiation pathway, and in particular plays a crucial role in the recruitment of the PIC to the 5' cap structure, as well as subsequent transit to the start codon. In collaboration with the Hinnebusch and Valášek labs, we have assembled a library of *S. cerevisiae* eIF3 mutants spanning its five core subunits. Using our *in vitro* reconstituted system, we have begun to dissect the contribution of these subunits to the role played by eIF3 during translation initiation. Together with previous *in vivo* studies, our experiments shed light on a diverse interaction network — coordinated by eIF3 — that helps drive mRNA recruitment and includes the PIC, mRNA, and the eIF4 factors.

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560 Translation Initiation Factor eIF3 Promotes Programmed Stop Codon Readthrough

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Programmed stop codon readthrough is a post-transcriptional regulatory mechanism that specifically increases proteome diversity by creating a pool of C-terminally extended proteins. During this process, the stop codon is decoded as a sense codon by a near-cognate tRNA, which programs the ribosome to continue elongation. The efficiency of competition for the stop codon between release factors (eRFs) and near-cognate tRNAs is largely dependent on its nucleotide context, however, the molecular mechanism underlying this process is unknown. Unexpectedly, translation initiation factor eIF3 critically promotes programmed stop codon readthrough on all three stop codons in the unfavorable termination context. In order to do so, it must associate with pre-termination complexes (pre-TCs) where it interferes with decoding of the 3rd position of the stop codon, thus allowing incorporation of near-cognate tRNAs with the mismatch at the same position. Importantly, this role is conserved between yeast and humans.

561 Withdrawn

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562 The conserved GTPase LepA contributes mainly to translation initiation in *Escherichia coli*

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LepA is a paralog of EF-G found in all bacteria. Deletion of *lepA* confers no obvious growth defect in *E. coli*, and the physiological role of LepA remains unknown. Here, we identify nine strains (Δ*dksA,* Δ*molR1,* Δ*rsgA,* Δ*tatB*, Δ*tonB*, Δ*tolR,* Δ*ubiF*, Δ*ubiG*, or Δ*ubiH*) in which Δ*lepA* confers a synthetic growth phenotype. These strains are compromised for gene regulation, ribosome assembly, transport and/or respiration, indicating that LepA contributes to these functions in some way. We also use ribosome profiling to deduce the effects of LepA on translation. We find that loss of LepA alters the average ribosome density (ARD) for hundreds of mRNA coding regions in the cell, substantially reducing ARD in many cases. By contrast, only subtle and codon-specific changes in ribosome distribution along mRNA are seen. These data suggest that LepA contributes mainly to the initiation phase of translation. Consistent with this interpretation, the effect of LepA on ARD is related to the sequence of the Shine-Dalgarno region. Global perturbation of gene expression in the Δ*lepA* mutant likely explains most of its phenotypes.

563 Intrinsic RNA-binding preferences of eukaryotic translation initiation factor eIF4G contribute to competitive discrimination of different mRNAs

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Translational control of gene expression plays essential roles in cellular stress responses and organismal development by enabling rapid, selective, and localized control of protein production. Translational regulation depends on context-dependent differences in the translational efficiencies of mRNAs, but the key mRNA features that distinguish efficiently translated mRNAs are largely unknown. Here we comprehensively determined the RNA-binding preferences of the central initiation factor eIF4G to assess whether core translation initiation factors have intrinsic sequence preferences that contribute to preferential translation of specific mRNAs. We identified a simple sequence motif – oligo-uridine – that mediates high-affinity binding to eIF4G. Oligo(U) motifs occur naturally in the transcript leaders of hundreds of yeast genes and are conserved between yeast species. Notably, mRNAs containing oligo(U) motifs resist translational repression upon depletion of eIF4G, demonstrating the impact of these motifs in vivo. Together, our data suggest a mechanism for selective translational control mediated by core initiation factors.

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564 Charting the Thermodynamic Landscape of Nucleotide Binding of the Universally Conserved Molecular Switch, Elongation Factor Tu

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During protein synthesis Elongation Factor (EF) Tu plays a critical role in maintaining translation fidelity. EF-Tu functions as a molecular switch gating the entrance of a new aminoacyl (aa)-tRNA into the translating ribosome This process is dependent on the nucleotide bound state of EF-Tu. During the EF-Tu facilitated aa-tRNA binding to the ribosomal A site, correct codon-anticodon interactions stimulates EF-Tu to hydrolyze GTP to GDP and Pi and to undergo a conformational change releasing the bound aa-tRNA. To prevent nucleotide exchange on the ribosome and to therefore prevent ribosome stalling EF-Tu has evolved to have a 40 fold higher affinity for GDP than GTP. To this end we investigate what thermodynamic and structural dynamics features of EF-Tu give rise to the differences in nucleotide affinity to understand how this property of EF-Tu contributes to translational fidelity.

By studying the temperature dependence of the rate constants describing the nucleotide association and dissociation we have determined thermodynamic parameters governing this process. We find that EF-Tus affinity to GTP and GDP binding differ mainly due differences in the transition state energy barriers of dissociation and not association. Surprisingly we also observe that the EF-Tu•GDP complex is enthalpically favored while the EF-Tu•GTP complex is entropically favored. This is in agreement with previous work in which we showed that the GTP dissociation is entropically driven1. To provide a structure interpretation of the different thermodynamic contributions to nucleotide binding we utilized Molecular Dynamic simulations of EF-Tu in its various nucleotide bound forms. These simulations highlight the role of differences in the structural dynamics of EF-Tu to fine-tune the thermodynamic parameters governing nucleotide binding. We identify a hydrogen-bonding network within EF-Tu as the likely contributor to stabilizing the GDP conformation, whereas differences in water coordination seem to favor the GTP conformation. Our findings show that EF-Tu has evolutionarily modulated both the entropic and enthalpic contributions to the transition state barrier of nucleotide dissociation for the fine-tuning of nucleotide-binding affinities.

1. Evan Mercier, Dylan Girodat, Hans-Joachim Wieden. A conserved P-loop anchor limits the structural dynamics that mediate nucleotide dissociation in EF-Tu. Scientific Reports 5, 1-9 (2015).

565 The reaction landscape of transition from the initiation to elongation phases of translation

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The rearrangement of the 30S initiation complex (30S IC) into the translating 70S ribosome (70S IC) provides an important checkpoint for mRNA selection. Here, we study the timing and control of the reactions which occur during 70S IC formation by rapid kinetic techniques using a toolbox of fluorescence-labeled translation initiation components. We find that IF3 dynamics on the 70S IC are independent of the IF2-mediated reactions, whereas IF1 and IF3 allosterically affect each other. IF1 dissociation is inhibited in the absence of GTP hydrolysis. IF2-dependent reactions such as Pi release, tRNA release from IF2 into the P site, GDP release from IF2 and the first peptide bond are unaffected by IF1 or IF3, but rely heavily on GTP hydrolysis. In the presence of non-hydrolysable GTP analogs, the IF2-dependent pathway is reversible with the equilibrium shifted towards a stable 70S-mRNA-IF2-fMet-tRNAfMet complex. We present a kinetic model for the events which drive the transition of 30S IC into an elongation-competent 70S IC.

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566 Resolving a paradox on the importance of the Shine-Dalgarno sequence to translation efficiency

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Numerous experimental studies have demonstrated that a short sequence motif-the Shine-Dalgarno (SD) sequence-located directly upstream of the start codon is critical for translation initiation in bacteria. Paradoxically, two recent systems biology approaches have reported no significant association between the SD motif and translation efficiencies of endogenous mRNAs measured via ribosome profiling. Unresolved, this discrepancy raises concerns about the reliability and accuracy of data-driven approaches to biological discovery, and calls into question the degree to which targeted experimental findings can be generalized in a quantitative manner at the genome-scale. To address these concerns, we re-analyze ribosome profiling data from two prokaryotes and investigate the relationship between translation initiation sequence features and translation efficiency. Contrary to the findings of the two recent systems-level studies, we find that ribosome profiling data strongly supports the hypothesis that the SD motif enhances translation. The scale of this analysis allows us to determine that the average enhancement is on the order of 15-25% depending on the precise definition of the SD sequence. Further, we show that a multiple-regression model is able to predict translation efficiencies by accounting for several sequence features related to translation initiation and elongation. Remarkably, we find that a model trained on one organism is able to predict translation efficiencies in another bacterial organism, illustrating the conservation of translation initiation mechanisms among bacteria and the importance of the SD sequence.

567 Structural insights into open-reading-frame maintenance on the 70S ribosome

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Ribosomes decode (translate) messenger RNA to synthesize polypeptides. During translation initiation, the open reading frame of mRNA is established via decoding of the AUG start codon in the P site, however non-AUG codons are employed in a subset of mRNAs. In subsequent elongation steps, sense codons are decoded in the ribosomal A site, however miscoding events occur despite the stringent aminoacyl-tRNA selection mechanism. To gain insights into non-AUG-dependent initiation and mRNA miscoding during elongation, we have determined a crystal structure of the bacterial 70S ribosome complex, formed in the presence of mismatched tRNA-mRNA interactions. This work provides the structural basis for understanding open-reading-frame establishment and maintenance in the course of translation.

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568 Studies of protein synthesis in live cells - one molecule at a time

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Protein synthesis has been studied extensively over the years, and the combination of traditional biochemistry, structural approaches, and more recently single-molecule fluorescence based *in vitro* techniques, have led to a detailed picture of the molecular mechanisms of ribosome catalyzed protein synthesis. However, we have very sparse information about the dynamics of protein synthesis, in particular inside living cells, one major problem being the vast number of ribosomes in the cell pursuing different tasks at any given moment. The sheer complexity of the translational system (do we know all the players yet?), and its interplay with other processes, make it very hard to connect the molecular details of protein synthesis with cell physiology and population biology, i.e. with the level at which selection pressure applies. Our research aims at connecting all these dots, in space and time, to get a coherent picture of one of the most fundamental processes of life. To do this, we are developing new fluorescence based methods to study mRNA-specific protein synthesis dynamics on the single-molecule level with high temporal and spatial resolution in living bacterial cells.

569 Structural insights into translational control on the ribosome during cellular stress

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Protein synthesis (translation) is accomplished by ribosomes in all living cells. Ribosomes decode genetic information encoded in messenger RNA and catalyze peptide-bond formation. We study the mechanisms of translation translation regulation, using structural and biochemical methods. Here, we will present new structural insights into regulation of translation on the ribosome under cellular stress and/or disease.

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570 Initiation factor 2 stabilizes the ribosome in a semi-rotated conformation

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Conformational rearrangements of the ribosome during the initiation phase of translation in bacteria are not well understood. Here we use single-molecule Förster resonance energy transfer (smFRET) to monitor intersubunit rotation and the inward/outward movement of the L1 stalk of the large ribosomal subunit during the subunit-joining step of translation initiation. We show that upon subunit association, the ribosome adopts a distinct conformation in which the ribosomal subunits are in a semi-rotated orientation and the L1 stalk is observed in a half-closed state. The formation of the semi-rotated intermediate requires the presence of an aminoacylated initiator fMet-tRNA^{fMet} and IF2 the in GTP-bound state. Our results suggest that positioning subunits in a semi-rotated orientation facilitates subunit association and support a model in which L1 stalk movement is allosterically coupled to intersubunit rotation and IF2 binding.

571 Roles of Helix H69 of 23S rRNA in 70S initiation complex formation

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Assembly of 70S initiation complex (70SIC) involves complex interplay between the ribosome, mRNA, initiator tRNA, and initiation factors. The molecular mechanisms that govern the process of 70SIC formation are not fully understood. Here, we show that deletion of helix H69 (ΔH69) of 23S rRNA suppresses functions of IF3 in regulating 50S docking to the 30SIC, due to delay of IF3 dissociation from ΔH69 ribosomes. Mutation ΔH69 also inhibits a subsequent step of 70SIC formation, consequences of disrupting at least two different mechanisms. Our data provide direct evidence that steric clash between IF3 and H69 is critical for IF3 regulation of 70SIC formation, and support a model in which IF3 dissociation is triggered by H69 after initial 50S docking. We also find that H69 is necessary for subsequent positioning of initiator tRNA as indicated by severely reduced rate of overall initiation in ΔH69 ribosomes. These large effects of ΔH69 on initiation provide an explanation for the dominant lethal phenotype of the mutation.

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572 Human eIF4AIII mediates translation of nuclear cap-binding complex-bound mRNAs by facilitating derangement of RNA secondary structures in 5'UTR

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Generally, intron-containing or spliced mRNAs have been thought to be translationally more active than intronless mRNAs, identical to mRNAs not generated by splicing. The splicing-dependent enhancement of translation is mediated, in part, by the exon junction complex (EJC). Nonetheless, the molecular details of how each EJC component contributes to the translational enhancement remain elusive. Here, we show that human eukaryotic translation initiation factor 4AIII (eIF4AIII), one of the core components of EJC, has the novel role in mRNA translation bound by the nuclear cap-binding complex (CBC), a heterodimer of cap-binding protein 80 (CBP80) and CBP20. Human eIF4AIII, an ATP-dependent DEAD-box RNA helicase, is recruited onto the 5′-end of mRNAs bound by the CBC via direct interaction with the CBC-dependent translation initiation factor (CTIF) regardless of introns (deposited EJCs after splicing). Polysome fractionation, tethering assay, and in vitro reconstitution experiments using recombinant proteins show that eIF4AIII participates in efficient disruption of RNA secondary structures in 5′UTR, and consequently facilitates CBC-dependent translation in vivo and in vitro. Therefore, our results suggest that human eIF4AIII is a specific translation initiation factor for CBC-dependent translation.

573 *Triticum Mosaic Virus* contains a translational enhancer in its 5' UTR and requires eIF4F for its translation

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Instead of a 5'cap, many RNA viruses use translational enhancers that recruit the translation machinery to the mRNA, and sometimes in a 5'- and/or cap-independent manner. Here, we show that *Triticum Mosaic Virus* (TriMV, *Potyviridae*) contains cap-independent and 5' end-independent translational enhancer in its 739-nucleotide 5' untranslated region (UTR). We tested the translational ability of the TriMV 5'UTR in vitro under limited cap-binding conditions by adding exogenous m7GTP cap-analog to the translation reaction. Under these conditions, TriMV 5'UTR-mediated translation was largely unaffected compared to a capped mRNA control. In a competition assay against a capped mRNA in translation, free TriMV 5'UTR RNA was able to inhibit translation of the capped RNA. Addition of eIF4F or its large subunit eIF4G was sufficient to recover this loss to nearly 100%, while the addition of eIF(iso)4F recovered translation to about 50%. To reveal factor dependency of TriMV 5' UTR-mediated translation, we reconstituted translation in wheat germ extract depleted of the cap-binding complexes. Our data suggests that eIF4G alone is able to restore TriMV 5' UTR-directed translation. Together, our data reveal that the TriMV 5'UTR contains a translational enhancer that requires eIF4F for its translation. Our long-term goal is to identify all the required canonical translation initiation factors required for TriMV translation and identify trans-acting factors that enhance viral translation.

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574 The unique anticodon stem of initiator $tRNA^{fMet}$ imparts the selectivity of start codon recognition seen in bacteria

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In bacteria, genes begin with AUG, GUG, or UUG. Initiation from GUG or UUG start codons is generally less efficient than from AUG but considerably more efficient than from near-cognate codons such as AUU, AUC and CUG. Start codon recognition occurs in the P site, which may help explain the first position degeneracy of natural start codons. However, the molecular basis of start codon selectivity remains poorly understood. Here, we analyze the effects of start codon substitutions on the stabilities of 30S•mRNA•$tRNA^{fMet}$ and 30S•mRNA•$tRNA^{Met}$ ternary complexes. We employ the toeprinting technique, which allows detection of the specific complex of interest, and measure the overall equilibrium binding constant and the dissociation constant (k_{off}) for mRNA. We find that, with initiator $tRNA^{fMet}$, the sequence of the start codon influences complex stability over a ~100-fold range, following the trend AUG > GUG, UUG > CUG. The effects of these start codon substitutions on the intrinsic stability of the 30S•mRNA•$tRNA^{fMet}$ ternary complex correlate remarkably well with their effects on initiation rate *in vivo*. Interestingly, with elongator $tRNA^{Met}$, the codon-dependence of binding differs, with complex stabilities following the trend AUG > UUG > GUG, CUG. In other words, $tRNA^{Met}$ fails to discriminate between GUG and CUG, despite having the same anticodon as $tRNA^{fMet}$. A unique feature of initiator $tRNA^{fMet}$ is a series of three G-C base pairs in the anticodon stem, which are known to be important for efficient initiation *in vivo*. A mutation targeting the central of these G-C base pairs causes the mRNA binding specificity pattern to change in a way reminiscent of elongator $tRNA^{Met}$. However, close inspection of the toeprints shows that the mutant tRNA allows non-conventional pairing to nucleotides downstream of the start codon. Furthermore, the mutant $tRNA^{fMet}$ shows clear defects in initiation, particularly at an early stage of the process. These data help explain the contribution of the unique stem of $tRNA^{fMet}$ to both the speed and fidelity of initiation.

575 40S recruitment in the absence of eIF4G/4A by EMCV IRES refines the model for translation initiation on the archetype of Type II IRESs

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Initiation of translation on Type II IRESs, such as those of EMCV and FMDV viruses, has been well documented in the recent years. For EMCV, the current model argues for a mechanism in which the key interaction necessary for the pre-initiation complex recruitment is eIF4G binding to the central J-K domains of EMCV-IRES. Here we demonstrate that, in contrast with the current model, the molecular mechanism of EMCV-IRES involves direct recruitment of the 40S subunit. Importantly, we identified a specific structural element that prevents the correct positioning of the initiation codon in close vicinity of the ribosomal P site. This work clarifies how this interaction could not be anticipated by earlier studies and allows us to propose a new model for initiation complex assembly on EMCV-IRES. The role attributed to eIF4G/4A can thus be refined as stabilizing/promoting the conformational changes that are necessary for IRES function; thus resembling the role conventionally assigned to ITAFs. This raises the interesting possibility that IRESs are primarily ribosome binders, some of which having partly lost the ability to fold into the active structure without the help of proteins.

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576 Synonymous codons direct co-translational folding towards different protein conformations.

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The genetic code is degenerate with several codons specifying incorporation of the same amino acid into the protein. Synonymous codons are translated with different rates. Non-uniform translation elongation rates are thought to influence co-translational folding of nascent polypeptides. In this study, we investigated the influence of synonymous codons on the kinetic of translation elongation and co-translational folding of the model protein gamma-B crystallin (γBC). We compared the unmodified bovine nucleotide sequence (U) coding for γBC to a sequence harmonized for *E. coli* codon usage (H). We detected higher yield of recombinant protein in *E. coli* and less degradation products occurring post synthesis upon expression of the H variant compared to the U variant. Using a completely reconstituted *E. coli* translation system, we showed that both sequence variants are indeed translated with different rates. Probing the folding state during *in-vitro* translation by using a pulse-proteolysis approach revealed different conformational sampling of the U and H ribosome-bound chains over time and different yields of folded full length γBC after release from the ribosome. We were able to experimentally link differences in translation elongation rates to conformational sampling on the ribosome and finally to yield of correctly folded protein released from the ribosome.

577 Ribosome Induces Conformational Change in Release Factor 1

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The ribosome is one of the largest cellular machines known to date and its task is to translate the genetic information from messenger RNAs (mRNAs) into proteins with the help of transfer RNAs (tRNAs). This process can be dissected into four main steps: initiation, elongation, termination and recycling. The termination phase of protein synthesis is initiated when a stop codon appears on the mRNA. The stop codon is recognized by class I release factors (RF1 and RF2 in *E. coli*) that bind to the ribosome and catalyze the release of the newly synthesized protein.

The goal of this study is to determine the mechanism used by release factors to achieve high fidelity stop codon recognition. The proposed hypothesis is that conformational changes in RF1/RF2 play a critical role in discriminating between stop and sense codons.

By utilizing new fluorescence resonance energy transfer (FRET) experiments, the changes in RF1 structure following binding to the ribosome were monitored.

Our results indicate that RF1 undergoes a large conformational change from a closed to an open state upon binding to the ribosome. The results suggest that high termination fidelity is achieved by linking conformational changes in RF1 to stable binding to the ribosome and catalysis of peptide release. These studies have medical significance because ribosomes are a well-known target of antibiotics and the results present novel insights in translation.

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578 Dissecting eIF4 factor interactions with the yeast translation preinitiation complex

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Recruitment of mRNAs to the ribosomal translation preinitiation complex (PIC) in eukaryotic cells serves as a major point of regulation of gene expression, and requires the activity of multiple eukaryotic translation initiation factors (eIFs). The current model for mRNA recruitment hinges on a strong role for the eIF4 group of factors, which are proposed to bind to the 5'end of an mRNA and activate it for translation. Our recent data support a model in which yeast eIF4B binds directly to the small ribosomal subunit in a manner that promotes both eIF4F function and a receptive state of the 40S mRNA binding channel, rather than by acting as a ssRNA-binding protein. To further dissect the molecular functions of eIF4F and eIF4B, we have generated fluorescently labeled versions of several proteins in the complex, and are using these reagents to probe interactions among eIF4F, eIF4B, and components of the PIC. Coupled with genetic and structural studies of the interactions of the PIC with components of the mRNP, these studies report on the architecture and dynamics of the 48S PIC, and provide insights into the molecular mechanisms underlying eukaryotic translational control.

579 Oxidized bases in the ribosome's peptidyl transferase center and their effects on translation

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The ribosome is central to protein biosynthesis and the focus of extensive research. Recent biochemical and structural studies, especially detailed crystal structures and high resolution Cryo-EM in different functional states have broadened our understanding of the ribosome and its mode of action. However, the exact mechanism of peptide bond formation and how the ribosome catalyzes this reaction is not yet understood. Also, consequences of direct oxidative stress to the ribosome and its effects on translation have not been studied.

So far, no conventional replacement or even removal of the peptidyl transferase center's bases has been able to affect *in vitro* translation. Significant contribution to the catalytic activity seems to stem from the ribose-phosphate backbone, specifically 2'OH of A2451. Using the technique of atomic mutagenesis, novel unnatural bases can be introduced to any desired position in the 23S rRNA, surpassing conventional mutagenesis and effectively enabling to alter single atoms in the ribosome. Reconstituting ribosomes *in vitro* using this approach, we replaced universally conserved PTC bases with synthetic counterparts carrying the most common oxidations 8-oxorA, 5-HOrU and 5-HOrC. To investigate the consequent effects on translation, the chemically engineered ribosomes were studied the in various functional assays.

Incorporation of different oxidized bases into the 70S ribosome affected the ribsosomes in differnet ways. Depending on the nucleobase modified, the reconstituted ribosomes exhibited radical deceleration of peptide bond formation, decrease of synthesis efficiency or even an increase of translation rate. These results may further our understanding of the residues involved in the peptide bond formation mechanism, as well as the disease-relevant effects of oxydative stress on the translation machienery.

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580 Analysis of allostery within the 30S ribosomal subunit

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During decoding, the ribosome selects the correct (cognate) aminoacyl-tRNA (aa-tRNA) from a large pool of incorrect aa-tRNAs. In the initial selection stage, interaction between codon and anticodon in the 30S A site leads to activation of GTPase domain of elongation factor EF-Tu and GTP hydrolysis, which allows release of aa-tRNA from EF-Tu. The mechanism by which codon recognition stimulates GTPase activation of EF-Tu remains unclear. In previous work, a number of ribosomal ambiguity (*ram*) mutation in 16S rRNA were isolated, largest subset mapping to helices h8 and h14. These helices interact with each other and with the 50S subunit to form bridge B8. Biochemical and structural studies have shown that disruption of bridge B8 is an important aspect of GTPase activation, and mutation G299A in h12 allosterically destabilizes B8 from 80 Å away.

Here, we investigate the functional dependence of various *ram* mutations on B8, using genetic epistasis. We combined mutation h8Δ2bp (truncation of h8, disrupting B8) with each *ram* mutation and measured miscoding rates of the double-mutant ribosomes *in vivo*. The miscoding rates of most double-mutant ribosomes are substantially higher than the corresponding single-mutant ribosomes. These data suggest that *ram* mutations located in h12, h16, h21, h27, h34 and h44 act, at least in part, independently of B8.

To further explore allostery within the 30S subunit, we tested the impact of two B8 destabilizing mutations, G347U (at B8) and G299A (in h12), on A-tRNA binding. The results show that mutations G347U and G299A stabilize tRNA in the 30S A site. These findings suggest conformational coupling not only between B8 and h12, but also between B8 and the 30S A site and between h12 and the 30S A site. Taken together, these data shed light on the role of the 16S rRNA in the decoding process.

581 Probing the mechanism of ATP utilization in mRNA recruitment to the eukaryotic ribosome

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Accurate protein synthesis is a fundamental process necessary for life. Perturbation is connected to developmental defects, neurodegenerative diseases, cancer, and viral infections. Most of translational control is achieved during the initiation phase. In eukaryotes, a dozen Initiation Factors (**eIF**) form a pre-initiation complex (**PIC**) with the small subunit of the ribosome (**40S**) and an mRNA. Eukaryotic translation Initiation Factor 4A (**eIF4A**) is an RNA-dependent ATPase and RNA helicase, thought to remove mRNA structure and promote mRNA recruitment to the PIC. The enzyme is well characterized in isolation, but the mechanism of how it promotes mRNA recruitment in the context of the PIC is not clear. Using an *in vitro* reconstituted *S. cerevisiae* translation initiation system we are trying to understand how eIF4A utilizes ATP and contributes to recruitment of mRNAs to the ribosome. Rates of eIF4A ATPase were compared in the absence and presence of various components of initiation machinery as well as the entire PIC. In addition, we are able to follow a labeled mRNA during recruitment to the ribosome via a gel shift assay. Our preliminary data suggest that the activity and mechanism of eIF4A during mRNA recruitment to the PIC may be distinct from what is observed when eIF4A acts on RNA substrates and ATP in isolation.

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582 How the ribosome moves the tRNAs during EF-G-catalyzed translocation

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Translocation of messenger RNA and transfer RNAs through the ribosome, catalyzed by elongation factor EF-G, is one of the most critical steps in protein synthesis. We recently solved the 3.8 Å crystal structure of the 70S ribosome trapped in mid-translocation bound with two tRNAs. The 30S subunit head undergoes 21° counterclockwise rotation while the 30S body undergoes 2.7° rotation relative to the 50S subunit. The P-site tRNA has moved into a pe/E chimeric state and the A-site tRNA has into an ap/ap chimeric state. The structure shows how ribosomal RNA undergoes conformational changes to move the tRNA from the A site and P sites to the P site and E sites.

583 Regulation of hepatitis C virus by microRNA-122 and host protein factors

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An estimated 2-3% of global population is living with Hepatitis C virus (HCV) infection and 60-90% of these infections become chronic, which ultimately leads to cirrhosis and liver cancer. While new antiviral therapies are emerging, a better understanding of how HCV interacts with host cells is important. HCV is a hepatotropic positive sense RNA virus. The HCV 5' untranslated region (UTR) contains two binding sites for a highly abundant liver-specific microRNA, miR-122. In contrast to the canonical function for microRNAs in binding to 3'UTR sites, leading to mRNA degradation and translational repression, HCV uses miR-122 as an essential positive regulator of viral replication. The mechanism of this regulation remains uncertain, with viral translation, RNA stability and replication all implicated in different studies.

We have investigated the mechanism of HCV regulation by miR-122 and the host protein factors involved in this regulation. Eukaryotic initiation factor (eIF)4AII has been described as a binding partner of HCV RNA polymerase enzyme (NS5B) and is involved in miRNA regulation of translation at 3'UTR sites. We find that depletion of eIF4AII leads to a reduction in HCV RNA level and in miR-122-mediated regulation of HCV translation, suggesting that these host factors cooperate to regulate HCV. By co-immunoprecipitation, we find that eIF4AII interacts with HCV RNA and miR-122 in a miR-122-dependent manner**.** Finally, we have investigated how miR-122 interacts with and regulates HCV RNA during the viral replication cycle.

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584 Lysine-encoding A-rich sequences control gene expression through stalling and frameshifting

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Gene expression is strictly regulated at each step in the biosynthesis of mRNA and protein products. Translational regulation, in particular, plays a central role in determining the stability of mRNA and abundance of proteins. We have identified a novel translational regulation mechanism that works through lysine encoding A-rich mRNA sequence motifs[1,2]. These sequences stall the translation apparatus and induce ribosomal frameshifting, which consequentially reduces mRNA transcript stability as well as protein output. Altering the number of consecutive adenosine bases in mRNA results in variations of the protein abundance. These effects were observed in both reporter constructs and recombinant genes with naturally occurring A-rich motifs [1,2].

Approximately two percent of genes in the human genome contain A-rich motifs which potentially regulate their expression by this novel mechanism. Conservation of these regulatory sequences between humans and other eukaryotes suggests a selective advantage to maintain the composition of adenosine bases in the motifs. The majority of these genes are involved in nucleic acid binding, cell differentiation, and proliferation. We hypothesized that the presence an A-rich motif in these genes is used for fine-tuning their expression in a translation dependent manner.

Our preliminary results show that synonymous changes of lysine AAA and AAG codons in these genes show substantial effects on protein expression. Additionally, we have noticed the presence of alternative -1 frame-shifted protein products from several A-rich motif containing genes. We are currently focusing on the mechanism of this gene dosage effect by targeting NMD and NGD factors. Furthermore, by analyzing effects of synonymous mutations in A-rich motifs of genes involved in mRNA splicing we are examining in the physiological role of A-rich motif genes in cellular homeostasis.

1. Arthur, Laura L., et al. "Translational control by lysine coding A-rich sequences" *in submission*

2. Koutmou, Kristin S., et al. "Ribosomes slide on lysine-encoding homopolymeric A stretches." eLife (2015): e05534.

585 Pumilio proteins repress mRNAs through multiple mechanisms

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Protein expression can be regulated by sequence-specific RNA binding proteins, such as the eukaryotic PUF (Pumilio and Fem-3 binding factor) family. To understand mRNA regulation, we study Drosophila Pumilio (Pum), which recognizes specific sequences in the 3' Untranslated Region (UTR) of target mRNAs and represses protein expression. Regulation by Pum is likely extensive, as it binds hundreds of different mRNAs and controls diverse biological processes including neurological function, stem cell maintenance, and fertility. We aim to dissect the mechanisms through which Pum limits protein expression. Using cell-based and biochemical assays, we identified multiple domains of Pum which cause repression. The highly conserved RNA binding domain (RBD) is responsible for poly(A)-dependent repression. The RBD antagonizes the translation activity of poly(A) binding protein (pAbp) and recruits the Ccr4-Not complex to accelerate deadenylation. However, the major repressive activity of Pum is conferred by three repression domains (RDs) located in the protein's N-terminus. These RDs act independently to inhibit protein synthesis. The RDs are small, modular, and potently reduce protein and mRNA levels when tethered to a reporter mRNA. Our results demonstrate that the RDs do not target the poly(A) tail and pAbp. In addition, each individual RD is sufficient for repression when fused to the RBD. Our ongoing work seeks to determine the mechanism by which the Pum N-terminal domains repress and identify protein partners which function as corepressors.

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586 Inhibitory Codon Pairs Affect Translation by Distinct Mechanisms.

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The choice of synonymous codons used to encode a polypeptide influences the rate and efficiency of translation as well as folding of the nascent polypeptide. However, the mechanisms by which codon choice impairs translation are not understood, in part because neither the identity nor properties of inhibitory codons or codon combinations are known. We previously implicated CGA-CGA codon pairs as strong inhibitors of translation in yeast due to wobble decoding [1], finding that adjacent CGA codons are substantially more inhibitory than separated CGA codons.

To identify other inhibitory codon pairs, we generated a library of superfolder GFP variants carrying an insertion of three randomized codons in the RNA-ID reporter [2] and subjected yeast with these integrated variants to fluorescence-activated cell sorting into GFP expression bins, followed by deep sequencing, similar to methods used to analyze protein and tRNA function [3,4]. For each three-codon variant, a syn-GFP^{SEQ} score was calculated, based on the distribution of that variant in the bins and normalized to the highest expressing synonymous variant, yielding 1395 variants with syn-GFP^{SEQ} scores 3 or more standard deviations from the mean. Thirteen inhibitory codon pairs, including the known CGA-CGA codon pair, were identified based on the expectation that inhibitory pairs cause reduced GFP in most variants containing that pair, and were substantiated by additional computational and experimental evidence.

Distinct molecular mechanisms of codon-mediated inhibition are suggested by two types of analyses. First, genes implicated in CGA-mediated inhibition modulate the inhibitory effects of only a fraction of these codon pairs. Second, mRNA levels are reduced by some, but not all, inhibitory codon pairs. Thus, all of the inhibitory pairs are sufficient for inhibition, but work in different ways to cause reduced translation.

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587 The role of structural elements of the 5'-terminal region of p53 mRNA in translation under stress conditions assayed by the antisense oligonucleotide approach.

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The p53 is one of major factors responsible for cell cycle regulation and stress response. Due to its functions p53 protein is strictly controlled at many expression levels. In the 5'-terminal region of p53 mRNA an IRES element(s) has been found which takes part in expression regulation of translational isoforms of p53. Previously, we have proposed the secondary structure model of the 5'-terminal region of p53 mRNA (Blaszczyk and Ciesiolka, 2011, Biochemistry 50, 7080). Two characteristic hairpin motifs are present in this region, one in which the first AUG codon is embedded, and the second one, which has been earlier shown to interact with Hdm2. We have also used antisense oligomers complementary to this region of mRNA to modulate FLp53 and ΔNp53 expression at RNA and protein level (Gorska *et al.,* 2013, PLoS One 8, e78863). In the current study oligomers hybridizing to the 5'-terminal region of p53 mRNA were applied to assess the role of structural motifs present in this region in translation initiation process upon stress conditions. The oligomers had 2′-*O*Me modifications in each ribose position and were unable to activate RNase H. Thus they could only act by inducing RNA misfolding or imposing a steric hindrance to the translating ribosome. Structural changes in the 5'-terminal part of p53 mRNA upon oligomers' binding were monitored by the Pb^{2+}-induced cleavage method. The impact of antisense oligomers on the synthesis of FLp53 and ΔNp53 was analysed in the HT-29, MCF-7 and HepG2 cells, in normal and under stress conditions, as well as *in vitro* in rabbit reticulocyte lysate. The results revealed that the hairpin U180-A218 and single-stranded region A219-A228 region were predominantly responsible for high efficacy of IRES-mediated translation in the presence of stress factors. These motifs play a role of *cis*-acting elements which are enable to modulate IRES activity, likely, *via* interactions with various proteins.

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588 Identification of TOP mRNA translational regulators by site-selective UV crosslinking

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The synthesis of many proteins of the translational apparatus is coordinately and selectively regulated in a growth-dependent manner at the translational level. This was first observed over 3 decades ago for messenger (m)RNAs that encode ribosomal proteins. These mRNAs contain a 5' **t**erminal **o**ligo**p**yrimidine tract (5' TOP) that mediates this regulation, thus they are termed TOP mRNAs. TOP-mediated translational control has been well-studied and important advances on signalling pathways and functionally relevant features of the TOP element have been made. However, it is still debated what trans-acting factors regulate TOP mRNAs. This has been in large part due to technical challenges of isolating specific RNAs with their bound protein partners. We developed a novel strategy based on site-selective UV-crosslinking, generating a TOP element selectively labelled with 4-thio uridine (4SU) that was fused by splint ligation to the body of an mRNA and then incubated in an in vitro translation system from HeLa cells. The extracts with the 4SU TOP and control mRNAs were subjected to Photoactivatable-Ribonucleoside-Enhanced Crosslinking (PAR-CL), RNA capture and quantitative proteomics. We (re-) identified a known TOP mRNA regulator, Larp1, and several novel factors. SiRNA-mediated depletion of Larp1 and one of the novel factors results in specific reduction of TOP mRNA encoded proteins in vivo without changing the levels of the respective TOP mRNAs. We have identified TOP RNA Binding Proteins (RBPs) that are functionally involved in the regulation of TOP mRNA translation, addressing a long-standing question in the field of translational control.

589 Five eIF4E isoforms from Arabidopsis thaliana are characterized by distinct features of cap analogs binding

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The assembly of the ribosome on majority of eukaryotic mRNAs is initiated by the recruitment of eIF4E protein to the mRNA 5' end cap structure. Flowering plants use two eIF4E isoforms, named eIF4E and eIF(iso)4E, as canonical translation initiation factors and possess a homolog of mammalian 4EHP (or eIF4E-2) termed nCBP. Plants from Brassicaceae family additionally conserve a close paralog of eIF4E which in Arabidopsis thaliana has two copies named eIF4E1b and eIF4E1c. In order to assess the efficiency of plant non-canonical (eIF4E1b/1c and nCBP) and canonical (eIF4E and eIF(iso)4E) eIF4E proteins to bind mRNAs we utilized fluorescence titrations to determine accurate binding affinities of five Arabidopsis thaliana eIF4E isoforms for a series of cap analogs. We found that eIF4E binds cap analogs from 4-fold to 10-fold stronger than eIF(iso)4E, while binding affinities of nCBP and eIF(iso)4E are comparable. Furthermore, eIF4E1c interacts similarly strongly with the cap as eIF4E, but eIF4E1b binds cap analogs ca. 2-fold weaker than eIF4E1c, regardless of the 95% sequence identity between these two proteins. The use of differentially chemically modified cap analogs in binding studies and a detailed analysis of the obtained homology models gave us insight into the molecular characteristic of varying cap-binding abilities of Arabidopsis eIF4E isoforms.

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590 Intron Inhibits Ribosomal Scanning on the 5′-Untranslated Region of *HAC1* mRNA

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An intervening sequence (intron) base-pairs with the 5′-untranslated region (5′-UTR) and keeps yeast *HAC1* mRNA translationally inert in the cytoplasm. Under conditions of endoplasmic reticulum (ER) stress, an endonuclease Ire1 cleaves the *HAC1* pre-mRNA at two specific sites, resulting in removal of the intron and generation of a new open reading frame (ORF). The new ORF then encodes a transcription factor that activates expression of a set of genes involved in the ER stress response. From a genetic screen, we identified that a single mutation in the 5′-UTR (*i.e.*, C_{-32} to A, the adenine of AUG start codon assigned as 1 and the positive sign for upstream nucleotides whereas the negative sign for downstream nucleotides) or in the intron (*i.e.*, $G_{771}A$) eliminated translational block in the *HAC1* pre-mRNA. Subsequent site-directed mutagenesis in the 5′-UTR-intron interaction site supported the genetic observation. These results led us to hypothesize that the single mutation disrupted the 5′-UTR-intron interaction and allowed ribosomes to reach the normal ORF, thus translating the Hac1 protein from the *HAC1* pre-mRNA. In other words, the 5′-UTR-inter interaction inhibits the initiation of translation. Supporting this hypothesis, we showed that *HAC1* mRNA in an Ire1 protein null strain was separated in a lighter ribosomal fraction in the sucrose density gradient. To further confirm our results, we inserted an AUG start codon at the 5′-UTR before the 5′-UTR-intron interaction site (*i.e.*, AUG_{-60}) and mutated the normal AUG (*i.e.*, A_1G) in a single *HAC1* allele. Interestingly, we observed that the *HAC1-AUG*$_{-60}$,A_1G mRNA was able to produce Hac1 protein without removal of intron, suggesting that (1) the pre-initiation complex was formed on the AUG_{-60} codon, (2) an elongating ribosomes was able to disrupt the 5′-UTR-intron interaction and (3) the major block of translation occurred at the initiation stage. Taken together, our results suggest that the 5′-UTR-inter interaction impedes ribosomal scanning on the 5′-UTR of *HAC1* mRNA.

591 RNA recognition by the Bicaudal-C translational repressor

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Bicaudal-C (Bic-C) is an important RNA binding protein required for embryonic development and the development and function of specific adult organs in species ranging from flies to humans. Bic-C is an mRNA-specific translational repressor and its' RNA binding functions selectively recognize specific mRNAs for repression. Previous work from our lab established that Xenopus Bic-C binds to a 32 nucleotide region within the 3'UTR of the maternal mRNA encoding the Cripto-1 protein (also called xCR1). The Cripto-1 protein is a ligand of the nodal signaling pathway, and Bic-C binds to the Cripto-1 mRNA and represses its translation. This cell-type specific repression ensures the spatially restricted expression of Cripto-1 that is necessary for normal embryonic patterning and formation of the body axes. We defined the 32 nucleotide Bic-C binding site in the Cripto-1 3'UTR, the first binding site defined for any Bic-C ortholog, and have shown that this site is necessary and sufficient to direct Bic-C-dependent translational repression. Our data suggest that this binding site is complex requiring both RNA secondary structure and sequence elements. We are defining the important sequence features of the Bic-C binding site at nucleotide resolution using a mutational approach in conjunction with electrophoretic mobility shift and fluorescence anisotropy assays to measure Bic-C binding. The data from these analyses will significantly increase our understanding of the Bic-C protein/RNA interface, facilitate the use of this binding site to identify other Bic-C target mRNAs and advance our understanding of how Bic-C modulates cell-fate decisions during vertebrate development.

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592 Comparative Genomic Analysis of Alternative Splicing-Coupled Translational Control (AS-TC)

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Alternative pre-mRNA splicing (AS) is a powerful cellular mechanism for regulation of gene expression and protein diversity. Our laboratory recently developed a cellular fractionation and high throughput sequencing approach (Frac-Seq) for quantification of isoform-specific mRNA recruitment to polyribosomes[1]. Initial experiments revealed 597 alternative splicing events that correlate with preferential polyribosome association of the minor spliced isoform. Our results demonstrated that alternative splicing remodels cis-regulatory elements important for determining the cytoplasmic fate of particular mRNA isoforms. Here, we test the alternative splicing-coupled translational control (AS-TC) hypothesis using a comparative genomics analysis of isoform-specific polyribosome association across human, rhesus and murine embryonic stem cells. Ultimately our goal is to identify instances of dynamically regulated AS-TC that are fundamental (conserved) to neural differentiation as well as events that are primate- or human-specific.

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593 Dynamic interaction of the signal recognition particle receptor and the translocon

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Cotranslational targeting of membrane proteins is mediated by the signal recognition particle (SRP) pathway. This is an evolutionary conserved pathway in which SRP is recruited to ribosomes synthesizing membrane proteins and targets them to the translocon (SecYEG in bacteria) in the membrane guided by an interaction with the SRP receptor (FtsY in bacteria), which is associated with SecYEG. FtsY comprises two domains: the N-terminal A domain, which interacts with both membrane lipids and SecYEG, and the C-terminal NG domain which interacts with the homologous NG domain of SRP. The details of the targeting process at the membrane are poorly understood and the detailed role of FtsY is unclear.

We have studied the interaction of FtsY and SecYEG in the context of the lipid environment by utilizing nanodiscs, performing fluorescence resonance energy transfer (FRET) measurements and site-specific crosslinking. We observe that unbound FtsY or FtsY bound to membrane lipids is present in a closed conformation in which A and NG domains are engaged in an intramolecular interaction. The interaction is rather strong, as verified for the complex of the isolated domains. On binding to SecYEG the domains come apart and FtsY adopts an open conformation, in which both the A and the NG domains interact with the lipid-embedded translocon. Forming the open conformation presumably facilitates SRP binding to FtsY and, thereby, the docking of the translating ribosome on the SecYEG in a quaternary targeting complex.

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594 Functional Conservation of Ligand-Responsive RNA Switches Regulating IRES-Driven Translation in Viruses

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RNA viruses including the hepatitis C virus (HCV) and Seneca Valley virus (SVV) depend on an internal ribosome entry site (IRES) to recruit host cell ribosomes for protein synthesis. We have discovered ligand-responsive conformational switches which are functionally conserved in many viral IRES elements. Modular RNA motifs of greatly distinct sequence and local secondary structure serve as switches that are involved in viral IRES-driven translation and may be captured by identical cognate ligands. The modular RNA motifs in viral IRES elements constitute a new paradigm for ligand-captured switches that differ from metabolite-sensing riboswitches with regard to their small size as well as intrinsic stability and structural definition of the constitutive conformational states. These viral RNA modules represent the simplest form of ligand-responsive mechanical switches in nucleic acids.

595 Ded1 and eIF4A Cooperate to Promote Translation.

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Our previous work suggests that the DEAD-box ATPase, Ded1, promotes translation via two sequential functions. First, the N and C termini of Ded1 facilitate the formation of an mRNP that includes eIF4F complex, but not the ribosome; in vivo, this complex can accumulate in stress granules. Second, the ATPase domain promotes translation of this complex. We postulate that Ded1 primes mRNAs for translation by facilitating binding of eIF4F and then promotes translation via its ATPase activity. Cold sensitive *ded1* mutants are defective in translation in vivo. To identify other factors that promote Ded1 function, we screened for over-expression suppressors of these cold-sensitive mutants and identified Dbp1, a paralog of Ded1, as expected from Jamieson and Beggs (1991). Additionally, overexpression of eIF4A suppressed the cold sensitive growth defect, suggesting that eIF4A cooperates with Ded1 in vivo. While over-expression of Dbp1 can suppress lethal ATPase mutants, over-expression of eIF4A shows very low levels of suppression, suggesting that, while eIF4A and Ded1 cooperate, they do not perform the same function in vivo.

We previously showed that Ded1 and eIF4G directly interact and now find that Ded1 facilitates the binding of eIF4E/eIF4G to small RNAs and to the cap. Additionally, we observe a direct physical interaction between Ded1 and eIF4A. In vitro, Ded1 helicase activity is stimulated by eIF4A, and stimulated more in the presence of eIF4A, eIF4E, and eIF4G. Taken together, these results suggest that eIF4F can modulate Ded1 function.

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596 Translational repression in *Toxoplasma gondii* operates under different stringencies in tachyzoites and bradyzoites

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Toxoplasma gondii is the world's most widely spread and successful parasite. In the host, this obligate intracellular protozoan pathogen must adapt to a variety of stressful stimuli. For example, after undergoing multiple replicative cycles, the rapidly growing tachyzoite stage of the parasite causes a physical tension in the host leading to its lysis and the release of tachyzoites into the extracellular milieu. The activation of the host's immune system confers a sustained stress upon the parasite and induces the conversion of tachyzoites into quasi-dormant, immunologically protected bradyzoites that persist for the life of the host. Interestingly, both of these stressful stimuli cause the induction of a generalized translational repression (TR) mediated through the phosphorylation of eIF2α. This research is directed towards identifying the differences between the short-term (extracellular) and long-term (bradyzoite) stress responses. Our previous research has established that the lactate dehydrogenase 1 (*LDH1*) transcript specifically undergoes TR in bradyzoites through the presence of a small regulatory RNA hairpin in its 5'UTR. This system was therefore used to investigate the differences between the TR in extracellular tachyzoites and intracellular bradyzoites. Using a dual luciferase reporter assay, we show that, while the presence of the *LDH1* 5'UTR is necessary for TR in bradyzoites, it is unnecessary for repression in extracellular tachyzoites indicating that TR in the latter condition is likely more stringent. Furthermore, using a fluorescently-tagged poly(A) binding protein we show that stress granules form in in response to short-term stressful stimuli but are not visible in bradyzoites. This occurs despite *LDH1* maintaining an elongated poly(A) while it is directed for sustained TR in bradyzoites. These results suggest that although eIF2α phosphorylation maintains a generalized TR under both conditions, the parasite alters the stringency of TR in response to a sustained stressful stimulus. Possible mechanisms are discussed with an emphasis on their involvement in developmentally controlled gene expression and parasite differentiation.

597 Ribosome-mediated ligand-dependent RNA conformational rearrangement for translational reading-frame switch regulation

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RNAs fold into specific structures to perform their biological functions. However, RNA not only folds into its structure according to RNA free energy landscape, but also undergoes conformational changes induced by the binding of proteins, metabolites or metals. For example, RNA riboswitch can adopt alternative structures in response to the binding of specific metabolites to regulate gene expression in prokaryotic systems. In addition, structure within mRNA is unwound by ribosome for decoding during translation elongation and the ribosome-mediated conformation remodeling has been demonstrated in the regulatory circuit of tryptophan operon attenuation. We have recently shown that a refolding hairpin upstream of the translational frameshifting site can attenuate mammalian -1 programmed ribosomal frameshifting (PRF) efficiency. We also designed ligand-dependent conformational switches to regulate mammalian -1 PRF attenuation via controlling hairpin refolding. However, the functional roles of the ligand and ribosome in RNA hairpin unwinding and co-translational refolding remain to be further defined.

Using enzymatic mapping, we found that the addition of ligand was not sufficient to rearrange RNA conformation in the designed ligand-responsive -1 PRF regulatory element in transcript transcribed *in vitro* although it could restore upstream hairpin attenuation of -1PRF in mammalian cells. This means the required RNA remodeling is mediated by other cellular factors and particularly the elongating ribosome. Consistently, the probed RNA structures are different with or without the incubation of ribosome, implicating the switch of a ligand binding conformation requires translational machinery. To further address this issue, we designed an *in vitro* translation system allowing single-round translation to monitor RNA conformation remodeling during translation elongation. In combined with DMS/DEPC modification and coupled with reverse transcription, the RNA conformation switch mediated by ribosome was tracked by stalling the ribosome at different positions along the mRNA of interest in the absence or presence of ligand. This approach thus provides a mean to study co-translational mRNA structure remodeling.

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598 GLD-1, FOG-2, and the Emergence of Self-fertility in *C. elegans*

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Germ cell sex determination in *Caenorhabditis* nematodes is regulated by a network of translational repressors. In species where the XX female has evolved self-fertility, this network is reconfigured to allow limited spermatogenesis. This is achieved by changes to both repressor proteins and in the *cis*-regulatory sites of their client mRNAs. Here, we focus on a highly conserved mRNA-binding protein, GLD-1, and its *C. elegans*-specific cofactor, FOG-2. Previous work has implicated both proteins in the translational regulation of *C. elegans tra*-2, a key promoter of female fate. The STAR family protein GLD-1 targets hundreds of mRNAs in addition to *tra-2*. Consistent with this, GLD-1 is not only required for XX spermatogenesis, but for a several aspects of oocyte differentiation and development. These oocyte-related roles are conserved across the *Caenorhabditis* genus. In contrast, while FOG-2 is also essential for XX spermatogenesis and forms a heterodimer with GLD-1, its function is restricted to specification of XX spermatogenesis. FOG-2 is a recent gene duplicate found only in *C. elegans*, indicating it is a new gene that was essential to the emergence of a new phenotype. We seek to understand the molecular mechanism of how FOG-2 affects sex determination, and in particular how it modifies the activity of GLD-1. We will present results from both genetic and biochemical experiments that address the mechanism and specificity of FOG-2 action. Surprisingly, our preliminary data suggest that FOG-2 may have *tra-2*-independent effects on sex determination.

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599 O6-Methyguanosine leads to position-dependent effects on ribosome speed and fidelity

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Nucleic acids are under constant assault from endogenous and environmental agents that alter their physical and chemical properties. While nucleobases can be modified at a number of sites, methylation of the O6 position of guanosine (m^6G) is notable for its high mutagenicity during DNA replication in which thymidine is readily incorporated opposite m^6G instead of cytosine. Yet, while it has been established that m^6G accumulates in both DNA and RNA, the consequences of m^6G lesions in RNA remain poorly understood. Here, we investigate the effects of m^6G on the decoding process using a high-resolution reconstituted translation system. Similar to DNA polymerases, m^6G in the first codon position decreases the accuracy of tRNA selection and incorporates m^6G:U near-cognate aminoacyl-tRNAs (aa-tRNAs). In contrast, m^6G at the second position slows cognate peptide-bond formation by >1000-fold but does not promote near-cognate aa-tRNAs incorporation. Exploring this second position defect further, we found that m^6G does not affect maximal rates of peptide release and that the related modified base N6-methyladenosine (m^6A) has only minimal effect on both tRNA selection and peptide release. This suggests that the decoding center's second position is exquisitely sensitive to the unique geometries of the m^6G:C and m^6G:U base pairs and not merely the presence of an additional methyl group. This is particularly interesting given how little the m6G:T base pair deviates from canonical Watson-Crick geometry. Together, our data support a model in which alkylated mRNAs alter translation by modifying the decoding process, highlight the deleterious effects that these adducts pose to cellular fitness, and suggest that cells may have evolved mechanisms to resolve these damaged nucleobases.

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600 A novel role for PolyA tail binding protein C1 in cardiac development and hypertrophy

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The poly(A) binding protein C1 (PABPC1) is a highly conserved and ubiquitously expressed cytoplasmic regulatory factor that facilitates mRNA translation by stabilization of a “closed loop structure” between the 5’-cap and the 3’-poly(A) tail. Here we report that PABPC1 protein expression in human and mouse heart is developmentally regulated and is reduced by approximately 800-fold within the first four weeks after birth. Remarkably, the postnatal silencing of PABPC1 protein expression is post-transcriptional, cardiomyocyte specific, and evolutionarily conserved. We demonstrate that silencing of PABPC1 in cardiac myocytes is driven by a decrease in its own poly(A) tail length, which results in reduced polysome association and translation of *Pabpc1* transcripts in adult heart.

Strikingly, PABPC1 protein levels are up regulated under cardiac hypertrophic conditions and we show that forced expression of PABPC1 in adult hearts of transgenic mice is sufficient to stimulate hypertrophic growth. Furthermore, we demonstrate that PABPC1 depleted mouse neonatal cardiomyocytes are viable; but incapable of undergoing physiologic or pathologic hypertrophy due to their inability to enhance basal protein synthesis. Taken together, our results illustrate a cell-type and developmental stage-specific role for PABPC1 and highlight its importance in controlling cardiac growth during normal development and in hypertrophy.

601 Role of the Insulin 5'UTR binding factors in Insulin gene regulation

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Insulin regulates the glucose homeostasis in mammals and its biosynthesis is regulated by glucose. In the initial phase of glucose stimulation, insulin biosynthesis is regulated mainly at the translation level. 5'UTR of the insulin mRNA plays important role in insulin translation regulation where some of the trans-acting factors from β-Cells of the pancreas bind to it and regulate its translation. Previously, we have identified Protein Disulfide Isomerase (PDI), Poly A binding protein (PABP) and pancreatic amylase as insulin 5'UTR-binding proteins by biotin RNA pull down assay followed by mass spectrometry. We have shown that upon glucose stimulation, PDI gets phosphorylated and the activated PDI rearranges the disulfide bonds of the PABP and forms a complex with insulin mRNA (5'UTR) to increase insulin biosynthesis.

Here we want to explore the role of pancreatic amylase in insulin translation regulation. So, to check the role of Pancreatic amylase in insulin translation we have characterized the insulin 5'UTR binding activity of amylase by yeast 3 hybrid, gel shift assay with pancreatic tissue extract and recombinant amylase. Specificity of 5'UTR binding was confirmed by competitive RNA-EMSA and Immunodepletion of amylase followed by gel shift assay. Luciferase reporter assay suggest that amylase down-regulates the insulin translation. Although pancreatic amylase is not expressed in mouse insulinoma cell lines in the normal conditions but the high level of Th1 cytokines (TNF-α and IFN-γ) induces the amylase expression in these cell lines which eventually reduces the insulin production. There are reports which suggest that patients with chronic or acute pancreatitis disease have high levels of Th1 cytokines in pancreatic β cells. So we hypothesize that this leads to amylase induction followed by loss of insulin production and eventually to apoptosis and diabetes in these patients.

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602 Regulation of the fidelity of start codon recognition by environmental cues

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Changes in the environmental conditions can affect gene expression at many levels. The execution of critical genetic programs that determine a cell's fate and function - including many developmental processes - frequently begins at the translation level. The recognition of the start codon in the mRNA transcript is a vital step in gene expression and is responsible for determination of the N-terminus of the protein as well as the reading frame of decoding. We set out to determine whether external conditions, signals or internal cues can modulate the fidelity of start codon recognition to alter gene expression at the level of translation. We found that elevation of the ambient temperature from 30 to 37°C led to loss of the fidelity of start codon recognition by 1.5-2 fold. Similarly, lowering the temperature from 30 to 20°C led to a 3-fold increase in the fidelity of start codon recognition. In order to probe the mechanism behind this phenomenon, we screened ~125 kinase deletion strains of yeast for effects on the changes in stringency of start codon selection with alterations in temperature. The initial screening identified several kinases whose deletion led to the loss of the effect of temperature on start site selection. The screening also identified some novel kinases whose deletion led to severe impairment in the accuracy of start codon selection. Apart from changes in the ambient temperature, several other stress conditions also led to loss in the accuracy of start codon selection viz. changes in the pH of the medium and reducing-stress by DTT. Conversely, glucose starvation led to increase in the stringency in start site selection. Initial experiments indicated that SNF1 protein might be a key regulator in this process and mediates its effect through phosphorylation of eIF1, a key molecule in start codon recognition. Thus, these key experiments indicate that apart from the genetic and biophysical interactions among the initiation factors, external cues might also regulate the crucial event of start codon recognition and thus can potentially have a significant impact on the cell physiology.

603 Regulation of gene expression by ligand-inducible –1 ribosomal frameshifting

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Most RNA has a potential to form complex structures by pairing with itself. RNA pseudoknots play crucial roles for several stages of translation of bacterial and viral RNA, auto regulation and ribosomal frameshifting. A small molecule that binds specifically to target RNA and induces pseudoknot structures might be a promising tool for gene regulation and gene therapy.

We have developed a series of synthetic small molecules, Naphtyridine carbamate tetramer (NCTn), selectively binds to (CGG)n sequence. We previously revealed that NCTn could induce a pseudoknot structure in mRNA by the simultaneous binding between the loop region and the single-stranded tail. Here, we investigated whether NCTn-inducible formation of RNA pseudoknot can regulate translation of target gene by -1 ribosomal frameshifting (–1RF) in the cell.

We used VPK pseudoknot, which causes –1RF in mouse mammary tumor viruses, as the structural template for engineering to NCTn-inducible pseudoknots. –1RF is the translational recoding mechanism used in many viruses, which is promoted by a slippery sequence and an adjacent mRNA secondary structure, most often an mRNA pseudoknot. The ribosome is stalled at a pseudoknot and forced to shift one nucleotide backwards into an overlapping reading frame and to translate an entirely new sequence of amino acids. We designed Dual-luciferase plasmid, containing a slippery sequence and NCTn-inducible pseudoknot sequence between the *renilla* luciferase and firefly luciferase. After transfection of dual-luciferase plasmid to HeLa cell, transfected cells were treated with NCT8. –1RF efficiency increased by NCTn-induced pseudoknot folding can be detected by increase in luciferase activity. As a result, addition of NCT8 increased –1RF efficiency. These results suggested that NCTn could induce pseudoknot formation in mRNA and modulate –1RF efficiency. Moreover, we demonstrated localization of specific proteins was regulated by NCTn-inducible –1RF in the cell. The knowledge obtained here indicate that ligand-inducible –1RF would become an important component of the synthetic biology toolbox for precisely controlling gene expression and constructing synthetic gene networks.

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604 Developmentally regulated RBP10 acts as a translation repressor and associates with an eIF4E-binding protein

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Sequence specific translation regulation is essential during cell development. RNA binding proteins (RBPs) are crucial to this process. Using *Trypanosoma brucei* as our model organism, we studied RNA binding protein 10 (RBP10). The single RRM containing protein, RBP10, is essential and only expressed in the mammalian-infective bloodstream forms; it is undetectable in the vector-infective procyclic forms. Tethering of RBP10 to a reporter mRNA reduces its abundance and represses its translation. The reporter mRNA shows a rapid reduction in abundance and is shifted from heavy polysomes to the top fractions of sucrose gradient. In addition, we have evidence by yeast two hybrid and co-immunoprecipitation that RBP10 interacts with eIF4E interacting protein (4E-IP). Interestingly, in Leishmania, 4E-IP binds to one of the four eIF4Es, eIF4E-1; and eIF4E-1 does not interact with any eIF4G homologues. We have confirmed that trypanosome eIF4E-IP interacts with trypanosome eIF4E-1. Both of these proteins act as repressors of translation when tethered to mRNA. Intriguingly, deletion of the consensus eIF4E binding peptide (YXXXXLΦ) resulted in loss of this interaction between 4E-IP and eIF4E-1, but the truncated 4E-IP was still a strong suppressor in the tethering assay. Normally, eIF4E1 may serve to recruit 4E-IP to mRNAs. The minimum region of 4E-IP that has the repressor effect is a C terminal region containing PQ repeats; and for RBP10 also, a C terminal part with a low complexity region is sufficient to suppress reporter expression. Low complexity domains are known to facilitate protein aggregation, and indeed RBP10 can self interact in a yeast 2-hybrid assay. Furthermore, trypanosome eIF4E-1 is found in P-bodies. Taken together, we propose a repressive "closed loop" model of RBP10 target mRNAs; where RBP10 gets recruited in a sequence-specific fashion to elements in the 3' UTR, it then binds to 4E-IP, resulting in translation repression and degradation of the mRNA.

605 Programmed Ribosome Frameshifting in the *H. neapolitanus* CsoS2 Encoding Transcript Gives Rise to Two Functionally Distinct Carboxysome-associated

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Carboxysomes are bacterial microcompartments found in a wide variety of bacteria such as *Halothiobacillus neapolitanus.* Carboxysomes host the carbon-fixation pathway in these organisms by concentrating carbonic anhydrase and RuBisCO to their respective substrates bicarbonate and ribulose-1, 5-bisphosphate thereby increasing metabolic flux of the system. Given the ability of microcompartments to optimize biochemical pathways, synthetic biologists would like to repurpose these structures into synthetic organelles that can create desired biochemical products. In order to repurpose the carboxysome, however, more needs to be known about how enzymes are targeted to the microcompartment. The carboxysosome-associated protein CsoS2 is potential candidate for binding to RuBisCO and the carboxysome shell thereby localizing RuBisCO to the carboxysome interior. Here we demonstrate that *H. neapolitanus* produces two distinct isoforms of the CsoS2 protein. A consensus ribosome slip sequence 'CCCAAAG' is responsible for a ribosome frameshifting event that gives rise to the two isoforms of CsoS2. We predict that the two isoforms are functionally distinct and the frameshifting event controls the stoichiometry of RuBisCO localized to the carboxysome interior.

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606 In vitro translation of mRNAs that are associated to their native ribonucleoprotein complex (mRNPs)

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After synthesis in the nucleus and export to the cytoplasm, the mature mRNA is covered with hundreds of RNA binding proteins to constitute the messenger ribonucleoprotein complex (mRNP). The composition of this mRNP is influenced by many nuclear post-transcriptional events such as splicing, EJC deposition…that have a global impact on RNA translation. However, the lack of relevant in vitro systems has himpered to directly measure these effects. By using a recently described *in vitro* hybrid translation assay (Panthu et al, 2015), we have now been able to evaluate precisely the effects of mRNP composition on translational efficiency; for instance, the use of intron containing mRNA constructs showed that splicing strongly enhanced translation. Our data reveal a strong influence of the mRNP composition on translational efficiency and this is due both to the nature and the nuclear history of the transcript.

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607 Defining the RNA-binding specificity of the Bicaudal-C translational repressor

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The selective and regulated translation of maternal mRNAs drives key cell-fate decisions during the earliest stages of animal development. Moreover, accumulating evidence reveals the relevance of translational regulation to the formation and maintenance of internal organ systems during later development and in adults. The importance of such regulation is clear, yet many of the most basic issues, including how specific mRNAs are targeted for repression, remain poorly understood. We are focused on the Bicaudal-C protein (Bic-C), a conserved RNA binding protein and developmental regulator in metazoans. Bic-C functions to selectively repress the translation of specific mRNAs. This repression is essential for normal embryogenesis in the model vertebrate Xenopus laevis. Despite Bic-C's relevance to biology, there is a paucity of experimental structure-function data about this regulatory protein, especially with regards to how it binds specific mRNAs. The N-terminal half of Bic-C contains three KH-domains and two KH-like domains and this region is necessary and sufficient for binding to known Bic-C target mRNAs in vivo as assayed with RIP RT-QPCR. This result and assay system provides a unique opportunity to dissect the Bic-C protein and define the minimal region sufficient for specific in vivo RNA binding. Analysis of different mutant Bic-C proteins indicates that deleting any of the KH or KHL domains significantly reduces the efficiency of RNA binding. Canonical KH domains contain GXXG motifs that for other RNA binding proteins are important determinants of RNA recognition; changing these to GDDG eliminates RNA binding. Analysis of Bic-C mutant proteins where individual GXXG motifs have been changed to GDDG revealed minimal defects in RNA binding indicating that the different KH domains may function redundantly to recognize specific RNA substrates. Bic-C proteins containing double combinations of GDDG mutations are more deficient, but not inactive for RNA binding. Only changing all three GXXG motifs eliminated RNA binding. These results indicate that RNA recognition by Bic-C is complex and the multiple KH domains may function redundantly.

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608 Switching off protein synthesis in dormant bacterial cells via oligoglutamylation of ribosomal protein S6

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Survival of bacteria during famine periods depends on repression of protein biosynthesis. We demonstrated that oligoglutamylation of *Escherichia coli*'s ribosomal protein S6 is accomplished by the RimK enzyme in the stationary phase of bacterial culture. Oligoglutamylation of S6 cause repression of protein biosynthesis *in vivo*. Failure to suppress stationary phase translation activity in D*rimK* cells results in the elimination of the mutant strain from the stationary phase cultures mixed with the isogenic parental strain. Transition of the stationary phase cultures to a fresh media resulted in a simultaneous onset of protein biosynthesis in D*rimK* cells, while the isogenic parental strain cells split into the quiescent and actively growing populations. Inability to form a dormant state lead to increased antibiotic sensitivity of a strain devoid of S6 oligoglutamylation. Inhibition of S6 modification in clinically relevant pathogens would eliminate dormant forms of bacteria resulting in increase of antibiotic treatment efficiency.

609 Context specific action of macrolide antibiotics in causing programmed translation arrest

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Expression of several macrolide resistance genes is regulated by drug dependent translation arrest in their regulatory regions. Macrolide-dependent translation arrest depends on the amino acid sequence of the nascent peptide. For e.g., we previously determined by biochemical and bioinformatic methods that the Arg/Lys-X-Arg/Lys (+**X**+) motif encoded in the regulatory regions of several resistance genes as a problematic sequence for the drug-bound ribosome to synthesize. Consistent with this idea, recent ribosome profiling data of *E. coli* cells treated with macrolides showed that drug bound ribosomes have the propensity to stall when they encounter the +**X**+ sequence. However, we observed several instances both *in vitro* and *in vivo* where stalling does not occur at the +**X**+ motif in the presence of the drug, suggesting that, besides the presence of the stalling motif, additional elements of the peptide are necessary for drug-mediated translation arrest.

Our current study was focused on elucidating what causes the drug bound ribosome to either stop or continue translation when it encounters the +**X**+ motif. We used ErmDL peptide (MTHSM**RLR**), which regulates expression of resistance gene *ermD*, as a model arrest peptide in cell free translation reactions performed in the presence of macrolides. Detailed mutagenesis analysis of the peptide residues shows that integrity of the sequence THSM, rather than the identity of its individual amino acids, is critical to direct drug-dependent stalling at the RLR motif of ErmDL. Our study suggests that the context of the amino acid sequence preceding the site of arrest influences the positioning of the stalling domain residues at the catalytic center of the ribosome, thus determining interruption or continuation of protein synthesis. Therefore, the regulatory leader peptides likely evolved to contain not only the 'stalling sequence' but also the right 'context' in order to cause the translation arrest necessary to activate resistance genes.

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610 Cellular mRNA recruits the ribosome via eIF3-PABP bridge to initiate internal translation.

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Cap-dependent translation of cellular mRNAs is compromised under conditions of cellular stress such as hypoxia, DNA damage, and serum deprivation, conditions that often result in the induction of apoptosis. In contrast, the translation of the cellular mRNAs containing Internal Ribosome Entry Site (IRES) within their 5' untranslated regions is less sensitive to the repression of global translation. Importantly, translation of several pro- and anti-apoptotic proteins is mediated by IRES during cellular stress and serves to fine tune the cellular stress response. One such protein is the X chromosome-linked inhibitor of apoptosis protein (XIAP), which interacts directly with distinct caspases to inhibit apoptosis. We have previously described eIF5B dependent cell survival switch which promotes IRES mediated translation of XIAP. However, the mechanism by which the XIAP IRES, or in fact any cellular IRES recruits ribosome to support cap-independent translation remains unknown. Here we show that the structural integrity, particularly in the vicinity of AUG, is critical for ribosome recruitment on the XIAP IRES. The binding of eIF3 together with Poly A tail Binding Protein (PABP) potentiates ribosome recruitment to the IRES. Our data support a model in which eIF3 binds directly to the XIAP IRES RNA in a structure-dependent manner and acts as a scaffold for IRES RNA, PABP and the 40S ribosome.

611 Non-canonical activity of 4E-T is required for translational repression by the CCR4-NOT complex in *Xenopus* oocytes

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RNA regulatory factors bound to 3'UTRs control translation and stability. Repression often is associated with poly(A) removal. CAF1, a deadenylase in most organisms, is a core component of the CCR4-NOT complex. Our prior studies established that CAF1 represses translation independent of its deadenylation activity. We sought to determine how CAF1 carries out this activity in *Xenopus laevis* oocytes. Our data reveal a chain of interacting proteins that ultimately recruit 4E-T and exploit its ability to repress translation independent of eIF4E interaction.

Affinity purification-mass spectrometry and co-immunoprecipitation assays revealed that many proteins of the CCR4-NOT complex in oocytes were recruited by CAF1. Moreover, the ability of tethered CAF1 to repress a reporter mRNA required association with NOT1, which mediates the association of CAF1 with Xp54 and 4E-T. To identify components required for repression by NOT1 in oocytes, we analyzed a series of mutations that specifically disrupted interactions between NOT1 and CAF1, Xp54 and 4E-T. Expression of a truncated form of 4E-T, which still bound eIF4E, interfered with repression by tethered CAF1, NOT1, and Xp54. This activity was not observed with a mutant 4E-T that failed to bind eIF4E. We propose that RNA-binding proteins and perhaps miRNAs repress translation through an analogous chain of interactors and the non-canonical activity of 4E-T.

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612 Nanos expands the Pumilio target repertoire by enhancing RNA binding and mRNA repression

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Drosophila Pumilio and Nanos proteins function together to control diverse developmental processes, germline stem cell maintenance, and neurological functions including memory formation. How Pumilio and Nanos collaborate is not completely understood. Pumilio belongs to a conserved eukaryotic family of RNA binding proteins that bind with high affinity and specificity to Pumilio Response Elements (PREs), consequently inhibiting protein synthesis. Nanos belongs to a conserved family of tandem zinc finger proteins. Together they are implicated in mRNA localization, translational inhibition, and mRNA decay.

To investigate how Pumilio and Nanos work together, we developed cell-based and biochemical assays that measure their repression and RNA binding activities. Surprisingly, we found that Pumilio can repress protein and mRNA expression of PRE-containing mRNAs independent of Nanos. We characterized multiple mechanisms of Pumilio-mediated repression and identified three unique domains in Pumilio capable of inhibiting protein expression.

We then investigated the impact of Nanos on Pumilio and found that Nanos robustly stimulated Pumilio-mediated repression by two mechanisms. First, Nanos directly stimulates the RNA binding activity of Pumilio. We show that while recombinant Nanos does not bind to RNA alone, it binds to Pumilio and increases the affinity of Pumilio for PRE containing RNA. In fact, Nanos stimulates Pumilio binding to RNAs that are not normally bound by Pumilio. This effect is mirrored in cells; Nanos stimulates Pumilio-dependent repression of mRNAs bearing weak/degenerate PREs. Therefore, Nanos expands the repertoire of Pumilio target mRNAs.

Second, we discovered that Nanos possesses a repression domain that synergistically promotes repression with Pumilio. The Nanos repression domain (NRD) can function independently of Pumilio when tethered to a reporter mRNA. NRD activity does not require a poly(A) tail, nor previously proposed corepressors Brain Tumor, Ccr4-Not deadenylase, or Cup. Together, these data reveal a new mechanism of Nanos repression that contributes to combinatorial control by Pumilio.

We propose that Nanos augments the spatiotemporal control of mRNAs by Pumilio; Nanos specifies how tightly Pumilio binds mRNA and tunes the level of repression. These exciting findings imply that Nanos orthologs may expand the breadth of Pumilio targets and increase the magnitude of Pumilio repression throughout eukaryotes.

613 Identification of novel proteins involved in nuclear export of pre-tRNAs in *Saccharomyces cerevisiae*

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tRNAs perform essential role of delivering amino acids to the cytoplasmic protein synthesis machinery. To execute this role in protein production, eukaryotic tRNAs have to be escorted out of the nucleus, their site of synthesis, to the cytoplasm, their site of function. By the primary nuclear export, newly transcribed, end matured, partially modified tRNAs are shuttled to the cytoplasm, where, in yeast, but not vertebrate cells, the tRNA splicing machinery is located. tRNAs are also constitutively imported to the nucleus by a process known as retrograde tRNA import. These tRNAs are then shuttled back to the cytoplasm by tRNA re-export mechanisms. Genetic and biochemical studies have identified a set of RanGTPase binding proteins called β-importins, that are either directly or indirectly implicated in this bidirectional tRNA movement. Recent in vivo biochemical data in yeast have shown two such members of β-importin family, Los1 (Exportin-t in vertebrates) and Msn5 (Exportin 5), serve overlapping but distinct functions in tRNA export. Los1 forms complexes with RanGTP and both intron-less and intron containing tRNAs, regardless of their aminioacylation status. Hence Los1 is thought to participate in both primary export as well as re-export of tRNAs to the cytoplasm. In contrast, Msn5 preferentially binds only spliced aminoacylated tRNAs documenting its role to tRNA re-export. Although Los1 and Msn5 both participate in tRNA nuclear export, they cannot be the only nuclear exporters for tRNAs in yeast as los1Δ msn5Δ double mutant cells are viable. A comprehensive screening representing ~90% of the total yeast proteome conducted recently in our laboratory uncovered novel genes involved in tRNA subcellular localization. Two such proteins Mex67 and Mtr2 when inactivated, accumulate end-matured unspliced tRNAs. Interestingly, the Mex67:Mtr2 complex is the principal yeast nuclear export factor for bulk mRNA and also contributes to ribosomal subunit nuclear export. Hence it is possible that this complex can bind tRNAs as well and may constitute the missing third pathway for nuclear export. Genetic and biochemical studies are currently underway to elucidate the roles of this protein complex in tRNA subcellular dynamics and, if proven, will provide a novel Ran pathway-independent mechanism for tRNA export.

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614 Towards identification of human RNase MRP/P substrates

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RNase MRP and RNase P are two highly similar endoribonucleases with different substrate specificities, which are found in all eukaryotic cells. In humans, these two ribonucleoprotein complexes share all of their protein subunits, but have distinct RNA components. The RNA components are essential for their catalytic activities. Mutations in the RNA component of RNase MRP cause the severe autosomal disease cartilage hair hypoplasia (CHH). CHH is a pleiotropic disease characterized by a short stature and limbs.

To better understand the biological function(s) of RNase MRP and RNase P and their involvement in pathophysiological processes, it is important to identify and characterize their substrate RNAs. Well known substrates for RNase P are the tRNA precursors. In contrast, not much is known about human RNase MRP substrates. Therefore, the aim of these studies is to obtain a comprehensive overview of RNase MRP (and RNase P) substrates in human cells in order to gain insight in both the biochemical and pathophysiological role of RNase MRP. Relatively instable and/or transient interactions between RNase MRP/P and putative substrate RNAs will be stabilized by UV-crosslinking and subsequently the associated RNAs will be converted to cDNAs and analysed by deep sequencing (CRAC technology). Cell lines (HEK293) expressing HTP (hexahistidine-Tev cleavage site-protein A)-tagged RNase MRP/P components, Rpp20, Rpp25 or Rpp30, have been generated and characterized and the procedures for crosslinking and the isolation of complexes have been optimized. Preliminary data on substrate identifications will be presented.

615 Examination of substrate specificity of yeast tRNA methyltransferase Trm140

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Modifications in and around the anticodon loop are usually highly conserved and have profound roles in translation. Residues 32 and 38 at the borders of the anticodon loop often form a non-canonical base pair, which can be crucial for maintenance of the anticodon loop structure, binding of tRNA to the ribosome, and fidelity of decoding. Modifications occurring at these two residues usually have important roles in cell. For example, lack of 2'-*O*-methycytidine (Cm) at residue 32 in combination of 2′-*O*-methylguanosine (Gm) at residue 34 in a *Saccharomyces cerevisiae trm7Δ* mutant results in a severe growth defect and reduced translation; and absence of pseudouridine at U_{38} or U_{39} in a *pus3Δ* mutant results in poor growth, and particularly affects $tRNA^{Gln(UUG)}$ at high temperature due to the additional loss of 2-thiouridine at U_{34}.

We are investigating the m^3C_{32} modification in yeast, catalyzed by Trm140, which is found on three species of $tRNA^{Thr}$ and three species of $tRNA^{Ser}$. The Ado-Met binding domain of Trm140 that is necessary and sufficient for m^3C modification is highly conserved in eukaryotes, and the modification is found in all four characterized cytoplasmic $tRNA^{Thr}$ species from fungi or animals, in 14 of the 15 characterized cytoplasmic $tRNA^{Ser}$ species from plants and animals, and in four of five characterized fungal cytoplasmic $tRNA^{Ser}$ species with C_{32}. Furthermore, the m^3C_{32} modification has a distinct role in translation since a *trm140Δ trm1Δ* mutant (also lacking $m^{2,2}G_{26}$) is sensitive to low concentrations of the translation inhibitor cycloheximide.

We are focusing on the specificity of Trm140 for m^3C_{32} modification, since the determinants are not known, and it is not obvious why certain tRNAs are modified to m^3C_{32}, while others are modified to Cm_{32}, and others remain unmodified. We are investigating the determinants for m^3C_{32} modification by Trm140 with a modified pull-down assay. We will present results showing that this approach can be adopted for specificity studies, since binding observed with this assay corresponds to activity for Trm140, and we will describe initial results on substrate specificity.

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616 *In vivo* biochemical analyses reveal distinct roles of β-importins and eEF1A in tRNA subcellular traffic

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In yeast, there is bi-directional movement of tRNA between the nucleus and the cytoplasm. In the primary tRNA nuclear export step, newly transcribed, end-processed intron-containing tRNAs are exported to the cytoplasm where the splicing machinery located. Spliced, mature cytoplasmic tRNAs are constitutively imported into the nucleus via retrograde tRNA nuclear import. Then, these tRNAs can again be exported back to the cytoplasm via tRNA re-export. This tRNA retrograde pathway serves multiple biological functions and is conserved from yeast to vertebrates. To gain a biochemical understanding of the mechanisms for tRNA nuclear-cytoplasmic dynamics, we developed *in vivo* native b-importin complex co-IP assays employing budding yeast. Our studies provide the first *in vivo* biochemical evidence that two b-importin family members, Los1 (exportin-t) and Msn5 (exportin-5), serve partially overlapping but distinct roles in tRNA nuclear export. Los1 assembles an export complex with RanGTP and tRNA. Both intron-containing pre-tRNAs and spliced tRNAs, regardless of whether they are aminoacylated, assemble into Los1-RanGTP complexes. Our data document that Los1 participates in both primary nuclear export and re-export of tRNAs to the cytoplasm. In contrast, b-importin Msn5 preferentially assembles with RanGTP and spliced, aminoacylated tRNAs, documenting its role in tRNA nuclear re-export and thereby providing a quality control step that assures that tRNAs capable of being aminoacylated are delivered to the cytoplasm. Tef1/2 (eEF1A), which only binds aminoacylated tRNAs, aids the *in vivo* specificity of Msn5 for aminoacylated tRNAs. Msn5 assembles a quaternary complex with RanGTP, aminoacyl-tRNA, and Tef1/2. Assembly and/or stability of this quaternary complex requires Tef1/2 thereby facilitating efficient re-export of aminoacylated tRNAs to the cytoplasm.

617 Pyrolysin is a protease that specifically cleaves the C-terminal domain of the RtcB RNA ligase and represses its tRNA ligation activity in the hyperthermophilic archaeon *Pyrococcus furiosus*

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RNA ligase is an enzyme that catalyzes the ligation of RNA molecules through the formation of phosphodiester bonds. So far, three heat-stable RNA ligases have been identified in the hyperthermophilic archaeon, *Pyrococcus furiosus*: (1) a putative 2'-5' RNA ligase (PF0027 protein), (2) an RtcB RNA ligase (PF1615 protein), and (3) a T4-type RNA ligase (PF0353 protein). The RtcB RNA ligase ligates spliced tRNA halves into mature-sized tRNAs (Englert *et al.* 2011 *PNAS* 108(4): 1290–1295). Using the purified recombinant RtcB RNA ligase as the substrate, we previously found a protease activity in a *Pyrococcus* whole-cell extract, which specifically cleaves the C-terminal domain (approximately 5 kDa) of the RtcB ligase (RNA2013, Davos, Switzerland). No cleavage of the other two RNA ligases was observed.

We have now identified the protease responsible. Initially, the protease was partially purified with ammonium sulfate fractionation, followed by RESOURCE Q ion-exchange column chromatography and Superdex 200 Increase gel-filtration column chromatography (GE Healthcare Life Sciences). A nanoLC–MS/MS analysis of the partially purified fractions revealed several interesting proteins, including a pyrolysin, a cell-envelope-associated hyperthermostable serine protease with a molecular mass of approximately 100 kDa. We confirmed that the recombinant pyrolysin showed the previously observed proteolytic activity against the RtcB RNA ligase. The RtcB RNA ligase lacking the C-terminal domain showed no detectable tRNA ligation activity. These results demonstrate that the pyrolysin specifically cleaves the C-terminal domain of the RtcB RNA ligase and represses its tRNA ligation activity. A possible biological function of the proteolytic system will be discussed in the meeting.

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618 Stepwise evolution of ribosomal RNA gene cluster in Archaea

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Functionally related genes are often located closely to each other in the genome. Such characteristics could be derived through evolutionary processes. As a model case, we focused on the evolution of ribosomal RNA (rRNA) gene clusters (consisting of 16S, 23S and 5S rRNAs) in Archaea. The fact that the number of archaeal rRNA gene clusters is relatively restrictive (up to four) compared to other domains of life allows us to conduct comprehensive analyses to understand the evolution of rRNA gene clusters. Here, we performed a computational analysis of the patterns, localization and organization of rRNA gene clusters in archaeal complete genomes.

Through bioinformatic analyses of 144 archaeal genomes collected from GenBank database, 219 rRNA gene clusters were extracted by calculating distances between rRNA genes and by considering the order of each rRNA gene in the genome. Based on the analysis of the predicted rRNA gene clusters mapped on the archaeal phylogenetic tree, the evolutionary processes that shaped the formation of rRNA gene clusters are revealed. In Crenarchaeota, 16S-23S is the first to be formed, and the 5S gene is isolated in the genome. Next, in early-diverged Euryarchaeota, a tRNA gene is assembled to form 16S-tRNA-23S, and the 5S gene is duplicated. Then, another cluster formation by assembling a 5S gene and/or duplication of either cluster (16S-tRNA-23S or 16S-tRNA-23S-5S) is caused in more recently diverged euryarchaeal species. Moreover, our data showed a significant enrichment of the specific tRNA gene(s) in euryarchaeal clusters (e.g., $tRNA^{Ala}$ between 16S and 23S genes, and $tRNA^{Cys}$ between 5S genes). The type of tRNA anticodon included in these clusters is found to be unique, such as TGC for $tRNA^{Ala}$ and GCA for $tRNA^{Cys}$. In summary, rRNA gene clusters have evolved in a stepwise manner *via* the assembly of each rRNA gene and/or specific tRNA gene(s), as well as the duplication of the entire cluster.

619 Characterization of docetaxel and carboplatin-induced ribosomal RNA disruption in ovarian and breast cancer cells

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Recently, the NCIC-CTG-MA.22 phase I/II clinical trial involving locally advanced breast cancer patients undergoing treatment with epirubicin and docetaxel reported significant, dose-dependent reductions in tumor RNA integrity (RIN) values in response to treatment. The results from this clinical trial suggested the possible utility of RNA integrity as a tool to measure clinical response to chemotherapy.

The purpose of the present study was to characterize the effects of two chemotherapy drugs frequently administered to breast or ovarian cancer patients, on tumour RNA integrity in ovarian and breast cancer cells in culture. Two ovarian (A2780 , CAOV-3) and two breast (MCF-7, MDA-MD-231) cancer cell lines were treated with various docetaxel and carboplatin concentrations in order to determine the effect of these drugs on cellular RNA content and RNA integrity. We report that chemotherapy treatment induced dose- and time-dependent changes in the RNA banding pattern, as assessed by capillary gel electrophoresis. This included the formation of novel discrete bands on the electropherogram distinct from the 28s and 18s rRNA bands (a phenomenon we call "RNA disruption"). Furthermore, we describe the lack of RNA disruption in cells selected for resistance to the same agent being assessed.

The origin of the novel RNA fragments arising in the A2780 cell line upon treatment with docetaxel for 48 hours was investigated using end-labeled oligonucleotide probes targeted to various regions in the 28s and 18s rRNA sequences. Northern blots using 28S specific probes demonstrated that two fragments of about 3012 and 1630 nt were derived from 28s rRNA, while no fragments were shown to be derived from 18s rRNA.

These findings support the view that chemotherapy-dependent changes in tumor cell RNA content and integrity, especially the formation of discrete bands of RNA, could effectively be used to monitor cellular response to chemotherapy agents and to differentiate between drug sensitivity and drug resistance in vitro. The evidence of discrete banding patterns of RNA was observed in 4 cancer cell lines (2 breast, 2 ovarian) with docetaxel and carboplatin, suggesting that RNA disruption can occur in response to a variety of chemotherapy agents in tumour cells from varying tissue types.

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620 A new approach to *in vitro* analysis of 16S rRNA methyltransferases interaction with small ribosomal subunit

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Methyltransferases play important role in ribosome biogenesis and decorate rRNA with methyl groups during different stages of ribosome subunits maturation. However, rRNA methyltransferases can also have critical importance for antibiotic resistance, and one the emerging mechanisms of resistance to aminoglycoside antibiotics is the methylation of 16S rRNA in the decoding center of small ribosomal subunit. 16S rRNA methyltransferases conferring resistance to aminoglycoside antibiotics can be found in antibiotic producers, as well as resistant pathogenic strains found in clinical isolates, and represent a major health concern in the fight against aminoglycoside antibiotic resistance.

In vitro study of 16S rRNA methyltransferases is hampered by the fact that 16S rRNA methyltransferases responsible for resistance against aminoglycoside antibiotics act upon mature, fully assembled 30S ribosomal subunits as the substrate. Their activity cannot be assayed on naked 16S rRNA or oligonucleotides, because metyltransferases make intricate network of interactions with fully folded 16S rRNA in the assembled 30S ribosomal subunits. We were interested in developing an assay for direct *in vitro* detection and analysis of macromolecular interactions between these small methylating enzymes and their large, complex macromolecular interacting partner - the 30S ribosomal subunits. First, a simple and qualitative assay for detection of the macromolecular complex between 16S rRNA methyltransferases and small ribosomal subunit has been developed, based on pull-down assay, revealing for the first time that the macromolecular complexes between methyltransferases and 30S ribosomal subunit are stable enough to be physically isolated. Furthermore, the binding of several methyltransferases from both antibiotic producing (Sgm, KamB) and resistant pathogenic strains (RmtA, RmtC, and NpmA) has been quantitatively analyzed by a novel technique, called microscale thermophoresis. The dissociation constants for the methyltransferase complexes with small ribosomal subunits have been determined, and they were in micromolar range. Thus, we have shown that the *in vitro* analysis of methyltransferase binding to ribonucleoprotein particles, such as small ribosomal subunits, is feasible. The results of the *in vitro* analysis of 16S rRNA methyltransferase binding to immature small ribosomal subunits will be presented and the timing of 16S rRNA methylation during the late phases of 30S ribosomal subunit maturation will be discussed.

621 Chemotherapy dependent RNA disruption in tumour cells: Role of apoptosis and other possible mechanisms

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Ribosomal RNA (rRNA) degradation can be induced in many different cell types by a variety of cytotoxic agents, including glucocorticoids, TNF, FAS, cycloheximide and okadaic acid, in the presence or absence of apoptosis. We have recently shown that rRNA degradation is also induced by chemotherapy agents and that this is distinct from autolytic degradation of RNA---a phenomenon we call "RNA disruption". In addition, RNA disruption was demonstrated in a recent clinical trial (CAN-NCIC-MA.22) involving locally advanced breast cancer patients treated with epirubicin and docetaxel. The researchers in this study observed a significant dose-dependent reduction in tumor RNA integrity values, which correlated with response to treatment.

The purpose of the present study was to investigate if the phenomenon of chemotherapy-induced RNA disruption is associated with an apoptotic response in taxane treated A2780 cells. RNA disruption was quantified using a proprietary "RNA Disruption Assay" (RDA) to generate a RNA Disruption Index (RDI). Apoptosis induction was shown to correlate temporally with the onset of RNA disruption using annexin-V/propidium iodide (PI) staining. Cell cycle analysis using PI staining of docetaxel-treated cells at various time points showed cell cycle arrest at the G2-M phase, followed by an increase in the number of cells with a sub G1 level of DNA content at later time points. Furthermore, immunoblots for apoptotic proteins, along with a caspase activity assay showed that RNA disruption was temporally correlated with the induction of apoptosis. Using a caspase-3 inhibitor, we were also able to demonstrate a significant reduction in RNA disruption, suggesting that RNA disruption by taxanes may be associated with caspase activation. RNA disruption became increasingly evident with time, while all the apoptotic markers investigated waned.

Our findings indicate that chemotherapy agents significantly impact tumour RNA quality, as seen by altered RNA banding profiles and an increase in RDI. These novel findings temporally correlated with apoptosis activation. Inhibition of an apoptosis-initiating caspase leads to reduction in RNA disruption, demonstrating a possible link between taxane - induced apoptosis and the initiation of RNA disruption, the latter of which may be persisting longer than apoptotic markers.

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622 Nop17 is a key R2TP factor for the assembly and maturation of box C/D snoRNP complex

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Box C/D snoRNPs are responsible for rRNA methylation and processing, and are formed by snoRNAs and four conserved proteins, Nop1, Nop56, Nop58 and Snu13. The snoRNP assembly is a stepwise process, involving other protein complexes, among which the R2TP and Hsp90 chaperone. Nop17, also known as Pih1, has been shown to be a constituent of the R2TP (Rvb1, Rvb2, Tah1, Pih1) and to participate in box C/D snoRNP assembly by its interaction with Nop58. The molecular function of Nop17, however, has not yet been described. To shed light on the role played by Nop17 in the maturation of snoRNP, here we analyzed the interactions domains of Nop58 - Nop17 - Tah1 and the importance of ATP to the interaction between Nop17 and the ATPase Rvb1/2. Based on the results shown here, we propose a model for the assembly of box C/D snoRNP, according to which R2TP complex is important for reducing the affinity of Nop58 for snoRNA, and for the binding of the other snoRNP subunits.

623 The Rapid tRNA Decay (RTD) Pathway Holistically Monitors tRNAs

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tRNAs are crucial for translation in cells, and defects in tRNA stability and modification are associated with mitochondrial diseases and neurological defects. In the yeast Saccharomyces cerevisiae, mature tRNAs are monitored by the rapid tRNA decay (RTD) pathway in which the 5'-3' exonucleases Rat1 and Xrn1 degrade certain hypomodified or destabilized tRNAs. The RTD pathway is suppressed by a *met22*Δ mutation likely through increased pAp, which inhibits Rat1 and Xrn1 in vitro. Previous work has shown that predicted instability of the acceptor stem and T-stem was highly correlated with RTD susceptibility for variants of the $tRNA^{Ser}$ family. However, our recent high-throughput experiments with $tRNA^{Tyr}$ (using $SUP4_{oc}$ to evaluate nonsense suppression) extend the scope of RTD to mutations in all regions, implying that cells broadly monitor the integrity of tRNA. In fact, 38 of the 80 scoreable single mutants were identified as RTD substrates by our high-throughput studies.

RTD variants with mutations in the anticodon stem-loop (ASL) are of particular interest since the ASL is remote from where the 5' exonucleases attack during RTD. We found that tRNA levels were increased in a *met22*Δ strain (compared to WT) for each of 10 RTD substrates examined, including 6 in the ASL. Furthermore, we infer that the degradation of ASL substrates is still likely mediated by an exonuclease based on epistasis studies showing that stabilization of the acceptor stem can rescue degradation of RTD substrates in the anticodon stem. We will describe these and other results analyzing RTD of ASL variants, which suggest that degradation of these variants may require other factors not yet implicated in the RTD pathway.

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624 Studies of proteins required for proper function and localization of tRNA splicing endonuclease in *Saccharomyces cerevisiae*

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tRNAs function to bring amino acids to ribosomes during protein synthesis. In yeast, splicing of pre-tRNAs is essential for the production of 10 families of tRNAs. Intron removal is catalyzed by the heterotetrameric tRNA splicing endonuclease (SEN) complex, which is located on the cytoplasmic surface of mitochondria. However, how and why SEN subunits (Sen2, Sen15, Sen34, and Sen54) assemble on the surface of mitochondria is unknown. Recently our lab completed a genome-wide screen to search for all yeast gene products involved in tRNA biology. We identified three interesting mitochondrial outer membrane proteins Tom70, Sam37, and Mdm10. Deletion of TOM70, SAM37, or MDM10 causes pre-tRNA splicing defects and the accumulation of end-matured, intron-containing tRNAs. We first hypothesized that TOM70, SAM37 or MDM10 mutations may cause impaired oxidative phosphorylation by mitochondria which results in pre-tRNA splicing defect. To test this idea, we isolated petites by ethidium bromide treatment of wild-type cells and by use of mip1Δ cells that are defective in mitochondrial DNA replication. Both methods generated petites that spliced pre-tRNAs as well as wild-type cells, showing that loss of oxidative phosphorylation has no affect upon SEN activity. As Tom70, Sam37, and Mdm10 function in targeting proteins to the mitochondrial outer membrane and initial import steps of proteins into mitochondria, we then hypothesized that deletion of TOM70, SAM37 or MDM10 causes mislocalization of SEN subunits, preventing formation of the heterotetramer on the mitochondrial surface. To test this, each SEN subunit was tagged with GFP at its endogenous locus in wild-type, tom70Δ, sam37Δ and mdm10Δ strains. Employing live cell confocal microscopy, we learned that the distribution of SEN subunits on mitochondria was reduced and the cytoplasmic pools were increased in the mutant cells compared with wild-type cells. We will conduct cell fractionation to determine the ratio of SEN subunits localizing between mitochondria surface and cytoplasm. Thus far our data indicate that via direct or indirect interactions, Tom70, Sam37 and Mdm10 are required for the proper localization, assembly, and function of the SEN subunits on mitochondria and that appropriate assembly of the SEN complex proteins on mitochondria is necessary for efficient pre-tRNA splicing.

625 Perturbations of the Peptidyl Transferase Center of the Ribosome Affect mRNA Translation Differently

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Protein synthesis is a major metabolic activity in all organisms. Dys-regulation in this process is closely associated with a wide range of human diseases. All proteins in a cell are synthesized in a spectacular molecular machine called ribosome, which translates all the messenger RNAs (mRNAs) expressed in the genome into proteins. Maturation of the ribosome is a complex process in the cell. The primary transcript of ribosomal RNA (pre-rRNA) undergoes extensive, site-specific covalent modifications, mainly 2'-O-ribose-methylations and replacement of uridines by pseudouridines (Ψ). In archaea and eukaryotes, these modifications are promoted by small nucleolar ribonucleoprotein (snoRNP) complexes. The protein components confer a generalized methylation and pseudouridylation activity for snoRNP complexes, and the RNA components, the small nucleolar RNAs (snoRNAs), account for the sequence specificities of snoRNPs. SnoRNAs serve as modification-guide molecules by base-pairing with pre-rRNA, and they direct the snoRNP machinery to the appropriate target site for functions.

rRNA modifications have long been of great interest because of their abundance and their conservation. Importantly, most of the modifications are clustered in the functionally important regions of the ribosome. Here we explore connections between snoRNA expression and the translational activity at a genome-wide scale using ribosome profiling, thereby associating the biogenesis with the translational function of the ribosome in the cell. Our results demonstrate perturbations in the peptidyl transferase center of the ribosome affect mRNA translation differently. Work is ongoing to verify such a connection at molecular level.

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626 Tobramycin derivatives with enhanced ribosome-targeting activity

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With the increased evolution of aminoglycoside-resistant bacterial strains, the need to develop aminoglycosides with (i) enhanced antimicrobial activity, (ii) the ability to evade resistance mechanisms, and (iii) the capability of targeting the ribosome with higher efficiency, is more and more pressing. The chemical derivatization of the naturally occurring tobramycin (TOB) by attachment of 37 different thioethers groups at the 6"-position led to the identification of generally poorer substrates of TOB-targeting aminoglycoside-modifying enzymes. Thirteen of these displayed better antibacterial activity than the parental TOB while retaining ribosome-targeting specificity. Analysis of these compounds in vitro sheds light on the mechanism by which they act and reveals three with clearly enhanced ribosome-targeting activity.

627 Links between HIV-1 genomic RNA trafficking and the efficiency of virus particle assembly

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Human immunodeficiency virus type 1 (HIV-1) virion assembly is a stepwise process driven by the structural polyproteins Gag and Gag-Pol that multimerize at the plasma membrane to form enveloped, immature capsids. Assembly is a cooperative process with Gag-Gag interactions promoted both through the binding of Gag's N-terminal Matrix (MA) domain to plasma membrane phospholipids, and also Gag's C-terminal Nucleocapsid (NC) domain binding to RNA molecules in the cytoplasm. NC's core RNA-binding function is to ensure encapsidation of two copies of the viral positive-strand genomic RNA (gRNA). gRNAs also serve as the viral mRNAs encoding Gag and Gag-Pol, and thus Gag translation and genome packaging may be integrated processes. However, in the absence of package-able gRNAs Gag is still able to drive formation of non-infectious virus-like particles through promiscuous interactions between NC and cellular mRNAs. Thus, how HIV-1 coordinates and guarantees the specific packaging of gRNA molecules despite the abundance of other cellular and viral RNAs remains a puzzle. We have hypothesized that crosstalk between gRNAs and Gag's MA domain may govern at least part of the mechanism because gRNA nucleocytoplasmic transport pathways can markedly impact the efficiency of Gag's MA-dependent targeting plasma membrane assembly sites in diverse cell systems (e.g., murine cells; Sherer et al. J. Virol, 83(17):8525-35, 2009). However, it has been difficult to distinguish between contributions of Gag expression kinetics vs. the presence of one or more gRNA-intrinsic "assembly" signals. Here, we established a tractable cell based system to compare gRNA/Gag interactions in cis and in trans using a combination of live cell imaging and functional assembly assays. We show that full-length HIV-1 constructs mutated to no longer express Gag/Gag-Pol can markedly enhance HIV-1 particle assembly when provided in trans, such that non-Gag genes are capable of stimulating efficient virion production. Ongoing studies also indicate that gRNA-MA interactions play a role in regulating the integrity of gRNA encapsidation. Taken together, these results emphasize the importance of functional crosstalk between MA and gRNAs in regulating both efficient virion assembly and the specificity of gRNA encapsidation.

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628 Post-transcriptional modifications of RNA regulate RIG-I activation and anti-viral signaling

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Innate immune pattern recognition receptors in mammalian cells must distinguish the molecular patterns of ‘self' versus ‘non-self' molecules. For retinoic acid inducible gene I (RIG-I), a cytosolic RNA helicase, the 5' triphosphate group of ssRNA and dsRNA is one key chemical feature of ‘non-self' RNA, because most mammalian RNAs have a 5' cap or monophosphate. Recent observations in our lab and others indicate that post-transcriptional modifications of RNA - such as the methylation and pseudouridylation observed in tRNA and rRNA - can regulate the activation of nucleic acid-sensing Toll-like receptors (TLRs) and RIG-I like receptors (RLRs). These modifications of the internal regions of RNA generally decrease the innate immune response to the RNA ligand, as compared to an identical sequence of canonical (non-modified) nucleotides. Therefore, we and others postulate that some post-transcriptional modification patterns may serve as a mark of ‘self' in mammalian cells.

When RIG-I binds ‘non-self' RNA, the protein changes conformation, undergoes activating K63-linked ubiquitination, and forms multi-protein signaling complexes on the mitochondrial outer membrane. These protein complexes activate transcription factors, such as interferon regulatory factor 3 (IRF3), that induce transcription of interferons and other anti-viral response genes. The present study determines the mechanism by which certain ribonucleotide modifications reduce RIG-I activation when incorporated into an otherwise stimulatory ssRNA ligand - derived from the 3' untranslated region of the hepatitis C virus genomic RNA. The methyl-6-adenosine (m6A), pseudouridine (Ψ), and 2-fluoro-deoxyuridine (2FdU) modifications are introduced by substitution of canonical ribonucleotide triphosphates during in vitro transcription. While previous reports have focused on dsRNA ligands, less is known about RIG-I interaction with ssRNA ligands. The present study employs rigorous purification and characterization techniques to ensure ssRNA inputs for both cell culture transfection experiments and for biochemical assays. Current data suggest that these RNA modifications block the RIG-I signaling cascade between the initial step of RIG-I:RNA binding and the downstream steps of IRF3 activation. Understanding the mechanism by which some ribonucleotide modifications evade RIG-I signaling will advance the design of RNA interference tools and RNA therapeutics to alternately maximize or minimize the immuno-stimulatory potential of the RNA.

629 Influenza A virus preferentially snatches non-coding capped RNAs in A549 cells

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Influenza A virus lacks an enzyme for adding a 5' cap to its own RNAs. Instead it snatches and uses the ~12nt 5' ends of host capped RNAs to prime transcription. Neither the preference of the host RNA sequences snatched, nor the effect of the "snatching" on host processes has been well defined. Previous studies have either used polyA-selected RNA from infected cells or relied solely on annotated host protein-coding genes to define which host mRNAs are selected by the virus. We used an enzymatic technique, CapSeq, to identify the host and viral capped RNAs in the same samples and interrogate the substrate-product correlation between all the host RNAs including coding and non-coding RNAs, and the viral RNAs. We find that the virus predominantly snatches caps from non-coding host RNAs, particularly U1 and U2 snRNAs. The promoter associated capped small RNAs are generated during Pol II-dependent transcription initiation in a bidirectional way. Interestingly, our data strongly indicates that at least the anti-sense promoter associated capped small RNAs (relative to the promoter direction), are also snatched by influenza virus. The sense promoter associated capped small RNAs and the corresponding long transcripts, such as mRNAs, initiate at the same transcription start sites. It is likely that these sense capped small RNAs can also serve as cap snatching substrates. And the previous observation of mRNAs as cap snatching substrates did not consider such an alternative explanation. Further analyses are needed to address if cap snatching by influenza virus could affect U1 and U2 dependent RNA splicing and if the promoter associated capped small RNAs are regulated during the virus infection.

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630 A novel combined RNA-protein interaction analysis distinguishes HIV-1 Gag protein binding sites involved in genomic RNA encapsidation from associated RNA structural change in the viral RNA leader.

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RNA-protein interactions govern many viral and host cell processes. Conventional 'footprinting' to examine RNA-protein complex formation often cannot distinguish sites of RNA-protein interaction from sites of RNA structural remodelling. We have developed a novel technique combining photo crosslinking with RNA 2' hydroxyl reactivity ('SHAPE') that achieves rapid and hitherto unachievable resolution of both RNA structural changes and the sites of protein interaction within an RNA-protein complex. 'XL-SHAPE' was validated using well-characterized viral RNA-protein interactions: HIV-1 Tat/TAR and bacteriophage MS2 RNA/Coat Binding Protein. It was then used to model the HIV-1 packaging process.

The HIV-1 genome is selected for packaging by the viral structural protein, Gag. This highly specific interaction allows Gag to recognise the genomic viral RNA amongst the wealth of other RNA species in the cytoplasm. The details of this interaction, however, remain unclear. One high-affinity binding site has been observed (SL3) but packaging appears to be a multi-step process and is likely to involve structural changes in both genomic RNA and Gag protein. We used XL-SHAPE to map HIV-1 Gag protein interactions on 2D and 3D models of the viral RNA leader. Distinct Gag binding sites were identified on exposed RNA surfaces corresponding to regions identified by mutagenesis as important for genome packaging. This widely applicable technique has revealed a first view of the stoichiometry and structure of the initial complex formed when HIV captures its genome.

631 High-resolution structural profiling of the Chikungunya viral genome

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Chikungunya virus is a positive-sense single-stranded alphavirus transmitted by mosquitos but also able to infect humans. We used SHAPE-MaP, a high-throughput chemical probing technique, to characterize the structure of the Chikungunya viral genome at 28° and 37°C. These reactivities can be used to inform secondary structure prediction algorithms to find functionally important structures in the genome. Since the virus can replicate in both a mosquito vector and a human host, we are particularly interested in understanding the elements of RNA structure that fold to different conformations in the vector and the host.

With SHAPE-MaP, accessible or unpaired nucleotides are preferentially modified and fixed as mutations through reverse transcription. These mutations are then identified by analysis of high-throughput sequencing data. In conjunction with this analysis, we also used the reactivities found by SHAPE-MaP to inform secondary structure prediction for the genome. From these data, we incorporate additional metrics such as Shannon entropy to identify robust structural regions of the Chikungunya genome. These findings provide new insight into structurally important features of the Chikungunya virus and other alphaviruses, as well as represent innovative approaches to examining and understanding RNA viral structure.

Although RNA secondary structure prediction remains a complex and computationally intensive problem, experimental data can inform and greatly improve the accuracy of secondary structure predictions. The structure of an RNA molecule, especially for RNA viruses such as Chikungunya virus, determines its function. Thus, knowledge of the structural features of an RNA virus is critical for understanding its mechanisms and developing treatment.

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632 Nonsense-mediated mRNA decay (NMD) restricts replication of mammalian RNA viruses

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A genome-wide siRNA screen for host factors that inhibit the replication of Semliki Forest virus (SFV), a positive-strand (+)RNA virus, revealed that components of the nonsense-mediated mRNA decay (NMD) pathway restrict early post-entry steps of the infection cycle (Balistreri et al., 2014). In HeLa cells and primary human fibroblasts, knockdown of UPF1, SMG5 and SMG7 leads to increased levels of viral proteins and RNA, and to higher titers of released virus. The inhibitory effect of NMD was more pronounced when the efficiency of virus replication was impaired by mutations or deletions in the replicase proteins. Consequently, knockdown of UPF1 resulted in a more than 20-fold increased production of these attenuated viruses. Single molecule FISH showed that UPF1 depletion increased the half-life of the SFV genomic (g) RNA, indicating that the gRNA, which serves as the mRNA for the non-structural proteins, is targeted by NMD. It seemed likely that the 4000 nucleotide long 3' UTR of the gRNA might render it susceptible to NMD, but a deletion of most of the 3'UTR sequence still resulted in a UPF1-dependent reduction of non-structural proteins. We are currently trying to identify the NMD-inducing feature of the SFV gRNA. The fact that SFV replication is entirely cytoplasmic strongly suggests that degradation of the viral RNA occurs through the exon junction complex (EJC)-independent mode of NMD. Collectively, our findings uncover a new biological function for NMD as an intrinsic barrier to the translation of early viral proteins and the amplification of (+)RNA viruses. Notably, replication of potato virus X (PVX) in *Arabidopsis thaliana* is also restricted by NMD (Garcia et al., 2014). Thus, in addition to its role in mRNA surveillance and post-transcriptional gene regulation, NMD also contributes to protect cells against different RNA viruses.

633 Partitioning of the influenza A virus cap-snatching process

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Influenza A Virus causes yearly epidemics and significant worldwide mortality. This virus has a segmented genome composed of eight single-stranded negative sense viral RNAs (vRNAs). At the earliest step of infection, a complex formed by the viral RNA-dependent RNA polymerase (RdRp) and any of the vRNAs interacts with host RNAP II, cleaves the 5' end of host pre-mRNAs, and uses these capped RNA fragments as primers for viral mRNA synthesis, using a process called cap-snatching. Because the capped RNA fragments also contain 10-15 nucleotides downstream of the cap, sequence heterogeneity is found at the host-derived 5' ends of viral mRNAs.

To investigate whether RNA selection occurs during cap-snatching, we performed high-throughput sequencing of the host primers found at the 5' ends of the eight viral mRNAs, following 5'-RACE on IAV transcripts from human (A549) or mouse (M-1) cells infected with either A/Hong Kong/1/1968 (H3N2) or A/Puerto Rico/8/1934 (H1N1). While the virus-encoded sequence is conserved, our results indicate that the host primers are divergent between the eight viral transcripts, and this in both human and mouse -derived samples. We observed noticeable differences in the length distributions, the nucleotides motifs and the identity of the host primers between the eight viral mRNAs. Mapping the reads to known transcription start sites indicates that the virus targets the most abundant host mRNAs, which is likely caused by the higher expression of these genes. Because RdRp complexes containing any of eight vRNAs do not target the same host mRNAs, our findings suggest negligible competition amongst RdRp:vRNA complexes for individual host mRNA templates during cap-snatching, and indicate a new layer within the IAV cap-snatching mechanism, wherein each RdRp:vRNA complex recruit different cellular proteins to specifically target sets of genes/pre-mRNAs. Our results provide a better understanding of the molecular mechanism governing the first step of transcription of this virus.

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634 Do viroid-induced 'mutant swarms' encode viroid-essential signals?

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Pathogenic subviral particles, which are small and basic molecules, harbor devastating potentials as determined e. g. for viroids that infect plants and cause significant crop losses with their single-stranded, circular, unencapsulated, non-coding RNA genomes of about 250-400 nucleotide lengths. The RNA of *Potato spindle tuber viroid* (PSTVd), type strain of *Pospiviroidae*, is replicated by the host DNA-dependent-RNA-polymerase II in an asymmetric rolling circle mechanism. Viroid infections are associated with the appearance of viroid-specific small RNAs (vsRNAs) of both polarities. Recently, vsRNAs were identified to target a host transcript and cause symptoms in case of *Peach latent mosaic viroid*, member of *Avsunviroidae*. Here, we investigate the combination of an error-prone viroid replication and a restricted sequence space based on the small viroid genome size. We computationally investigated vsRNAs to infer the populated sequence space of viroid genomes. We identified PSTVd variant-specific sequence variations that deviate from the sequence of the primary infecting RNA. The frequency of observed variation patterns ranged from dominant to barley detectable on the background of sequencing errors. Strand-specific frequencies and patterns in distinct genomic regions suggest that strands are differentially prone to transcription errors, potentially pointing to distinct roles. We speculate that specific sequence variations are incompatible with critical primary and secondary structure motifs but form essential signals on which the genome is dependent. Hence, we hypothesize that viroids rely on a stochastic process that generates a ‘mutant swarm' including variants required for the viroid's "life" cycle. Thus, the error-prone replication might extend the plasticity and adaptability of viroid's genome to exploit host resources, to implement evasion of host adaptive defensive systems, and to establish a diverse population.

635 ORF57 exploits the sequence bias of Kaposi sarcoma herpesviral RNAs to enhance their expression

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The KSHV post-transcriptional regulator protein, ORF57 is involved in nuclear export and stability of lytic, intron-less viral transcripts. The mechanism for how ORF57 recognizes its target RNAs remains elusive. Most of the KSHV RNAs regulated by ORF57 are devoid of a concrete binding or response element. Here, we investigate which features of viral RNAs provide the target specificity for ORF57. As starting point we examined the multiple intron-containing K15 gene. We could sort out that the cDNA is important for ORF57-dependency. The sequence bias of K15 revealed an unusual high AT content. Raising the frequency of GC nucleotides by sequence optimization we yielded an ORF57-independent K15 version. Based on a bioinformatic approach we observed a reduction of motifs recognized by hnRNPs in the optimized K15 variant. To further prove the importance of the sequence bias of ORF57-dependent RNAs, we grouped KSHV mRNAs according to their AT content and found a correlation between AT-richness and ORF57-dependency. Importantly, latent genes, which are expressed in the absence ORF57 show a lower AT content and are ORF57 independent. The nucleotide composition of K15 resembles that of HIV gag. Interestingly, ORF57 can partially rescue HIV Gag expression. In combination with a constitutive transport element (CTE) we observed an additive effect, since both ORF57 and CTE use the Tap/NXT1 pathway. The HCMV homologue of ORF57, pUL69 also enhances KSHV K15 expression. Preliminary experiments using a UL69 deletion mutant defective in RNA-binding may suggest a different mechanism for target recognition. Our findings demonstrate that ORF57 does not recognize a single response element in the majority of KSHV, but rather employs their unusual nucleotide bias instead. We hypothesize that this sequence bias constitutes a distinct signature after recognition by RNA-binding proteins, which is then read by ORF57, which harbors only a weak affinity for nucleic acids. This strategy could both target ORF57 to viral RNAs and also favour viral over cellular RNAs. Thus, we speculate that a cellular RNA-binding protein provides the sequence preference for ORF57.

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636 Decapping activator Sbp1 promotes PAB1 mRNA translation

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Sbp1, an RNA-binding protein, was reported to associate with decapping enzymes and affects translational repression in yeast *Saccharomyces cerevisiae* (**1**). It has also been demonstrated that sbp1 directly binds translation initiation factor eIF4G1 via its RGG motif, thereby blocking binding of the ribosome, suggesting that eIF4G1 is the major target of sbp1 for repressing the translation (**2**). Here we show, while repressing the general translation in the cell, sbp1 promotes the translation of PAB1 mRNA both *in vivo* and *in vitro*. Based on our results, we propose a dual role of sbp1 in translation regulation: In addition to acting as a general translation repressor, sbp1 promotes the translation of subset mRNAs. Work is ongoing to establish the molecular mechanism of the translation activation of PAB1 mRNA by sbp1.

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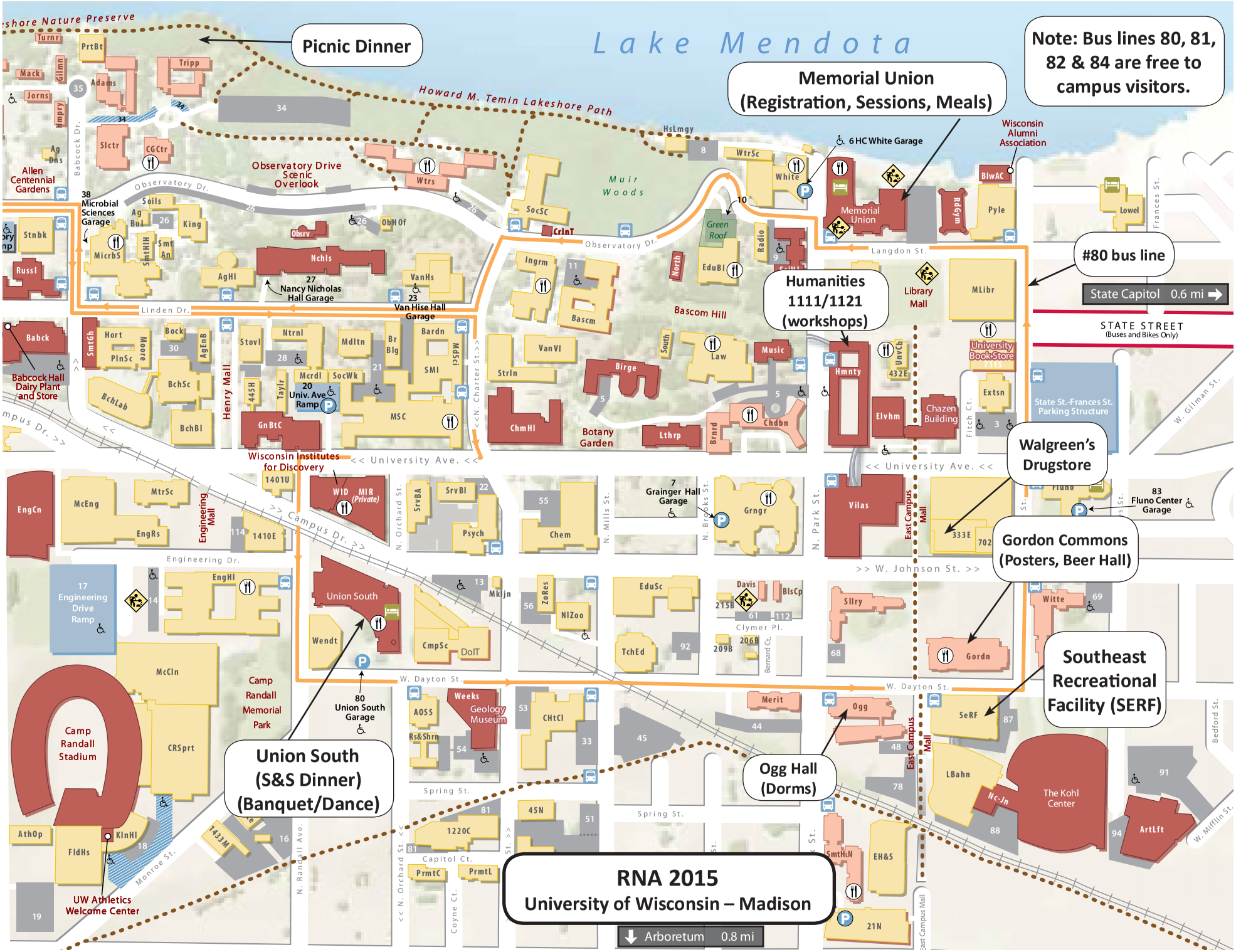
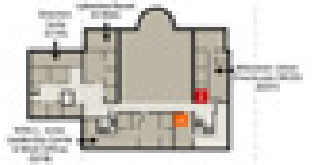

Lake Mendota
Picnic Dinner
Note: Bus lines 80, 81, 82 & 84 are free to campus visitors.
Memorial Union (Registration, Sessions, Meals)
Howard M. Temin Lakeshore Path
Observatory Drive Scenic Overlook
Muir Woods
Allen Centennial Gardens
38 Microbial Sciences Garage
27 Nancy Nicholas Hall Garage
23 Van Hise Hall Garage
6 HC White Garage
Wisconsin Alumni Association
Memorial Union
Green Roof
Observatory Dr.
Linden Dr.
Langdon St.
#80 bus line
State Capitol 0.6 mi
STATE STREET
(Buses and Bikes Only)
Humanities 1111/1121 (workshops)
Library Mall
Bascom Hill
Babcock Hall Dairy Plant and Store
Henry Mall
Wisconsin Institutes for Discovery
<< University Ave. <<
Walgreen's Drugstore
State St.-Frances St. Parking Structure
Gordon Commons (Posters, Beer Hall)
>> Campus Dr. >>
Engineering Mall
Engineering Dr.
>> W. Johnson St. >>
Union South
Wendt
80 Union South Garage
Union South (S&S Dinner) (Banquet/Dance)
Southeast Recreational Facility (SERF)
Ogg Hall (Dorms)
W. Dayton St.
Camp Randall Stadium
Camp Randall Memorial Park
Spring St.
Capitol Ct.
Monroe St.
N. Randall Ave.
N. Orchard St.
N. Mills St.
N. Brooks St.
N. Park St.
East Campus Mall
Fitch Ct.
Clymer Pl.
Bernard Ct.
Coyne Ct.
Bedford St.
W. Mifflin St.
W. Gilman St.
The Kohl Center
UW Athletics Welcome Center
RNA 2015
University of Wisconsin – Madison
Arboretum 0.8 mi

MEMORIAL UNION DIRECTORY

WEST WING CENTRAL CORE EAST WING

(Alphabetical)

Accounting (4187) ..... 4th Floor West Wing
Annex (2214) ..... 2nd Floor Central Core
Artful Restroom ..... L1 West Wing
ATM ..... 1st Floor East Wing
Beefeaters (3320) ..... 3rd Floor East Wing
Board Room (3306) ..... 3rd Floor East Wing
Box Office (1182) ..... 1st Floor West Wing
Bradley (Harold C.) Lounge (B176) ..... L1 West Wing
Brat Stand (seasonal) ..... Terrace
Browsing Library (Hamel Family) (2356) ..... 2nd Floor Central Core
Building Manager Office (1109) ..... 1st Floor East Wing
Campus & Community Information (1186) ..... 1st Floor West Wing
Campus Event Services Office (2224) ..... 2nd Floor Central Core
Capitol View (4070) ..... 4th Floor Central Core
Chark Room (B147) ..... L1 West Wing
Class of '24 Reception Room (4209) ..... 4th Floor East Wing
Class of 1925 Gallery (2195) ..... 2nd Floor West Wing
Council Room (4199) ..... 4th Floor East Wing
Daily Scoop (1314) ..... 1st Floor East Wing
Der Rathskeller (1301) ..... 1st Floor Central Core
Der Schkeller (1305) ..... 1st Floor Central Core
Dining Services Offices (1352/1356) ..... 1st Floor East Wing
Directors Suite (5195) ..... 5th Floor West Wing Wing
Elevators ..... All Floors
Essentials Supervisor (1328) ..... 1st Floor East Wing
Essentials/Wisconsin Union Store (1339) ..... 1st Floor East Wing
Fan Taylor Lobby (1130) ..... 1st Floor West Wing
Festival Room (B196) ..... L1 West Wing
Flounders Room (2210) ..... 2nd Floor Central Core
Fredric March Play Circle (2190) ..... 2nd Floor West Wing
Green Hall (4214) ..... 4th Floor Central Core
Guestrooms ..... 4th Floor East Wing
Guestrooms Check-in/Essentials (1128) ..... 1st Floor East Wing
Hamel Family Browsing Library (2356) ..... 2nd Floor Central Core
Human Resources, Training & Payroll (4186) ..... 4th Floor West Wing
Inn Wisconsin (2312) ..... 2nd Floor East Wing
Jones Leadership Center (5218) ..... 5th Floor Central Core
Lakefront on Langdon (1355) ..... 1st Floor East Wing
Lakefront on Langdon Gallery ..... 1st Floor East Wing
Lakeview Room (5192) ..... 5th Floor West Wing
Langdon Room (4206) ..... 4th Floor Central Core
Main Lounge (2218) ..... 2nd Floor Central Core
Main Lounge Deck ..... 2nd Floor Central Core
Marketing (3188) ..... 3rd Floor East Wing
Mendota Lodge (B104) ..... L1 West Wing
Old Madison (3312) ..... 3rd Floor East Wing
Outdoor UW Program Office (B135) ..... L1 West Wing
Outdoor UW Service Desk (B101) ..... L1 West Wing
Paul Bunyan Room (1304) ..... 1st Floor Central Core
Peet's Coffee and Tea (1319) ..... 1st Floor Central Core
Play Circle (Fredric March) (2190) ..... 2nd Floor West Wing
Porter Butts Gallery (2225) ..... 2nd Floor Central Core
Profile Room (2216) ..... 2nd Floor East Wing
Rathskeller (1301) ..... 1st Floor Central Core
Restrooms ..... All Floors
Men's Women's Unisex
Shannon Hall (1130) ..... 1st Floor West Wing
Sunset Deck ..... 1st Floor West Wing
Sunset Deck - Upper ..... 2nd Floor West Wing
Shannon Sunset Lounge (1133) ..... 1st Floor West Wing
Stiftskeller (1355) ..... 1st Floor Central Core
Terrace ..... 1st Floor Central Core
Theater Offices (3192) ..... 3rd Floor West Wing
Tripp Commons (2324) ..... 2nd Floor West Wing
Tripp Deck ..... 2nd Floor East Wing
Trophy Room (1214) ..... 1st Floor Central Core
Wheelhouse Studios (B196) ..... L1 West Wing
Willis L. Jones Leadership Center (5218) ..... 5th Floor Central Core
Winkler Lounge (B152) ..... L1 West Wing
Wisconsin Union Directorate (5218) ..... 5th Floor Central Core



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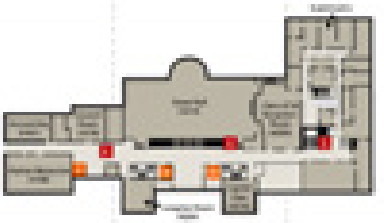
Directors Suite (5195)
Elevators
Lakeview Room (5192)
Restrooms
Wisconsin Union Directorate
Willis L. Jones Leadership Center (5218)



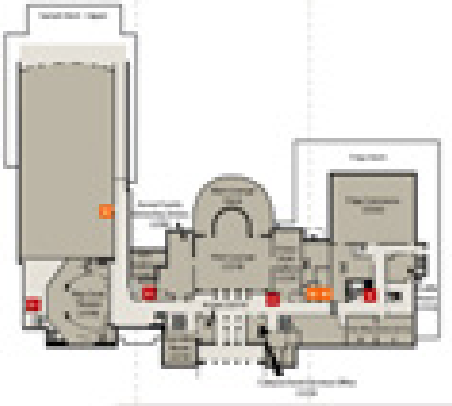
4

Accounting (4187)
Capitol View (4070)
Class of '24 Reception Room (4209)
Council Room (4199)
Elevators
Great Hall (4214)
Guestrooms
Human Resources, Training & Payroll (4186)
Langdon Room (4206)
Restrooms



3

Beefeaters (3320)
Board Room (3306)
Elevators
Marketing (3188)
Old Madison (3312)
Restroom
Theater Offices (3192)



2

Annex (2214)
Campus Event Services Office (2224)
Class of 1925 Gallery (2195)
Elevators
Flounders Room (2210)
Fredric March Play Circle (2190)
Hamel Family Browsing Library (2356)
Inn Wisconsin (2312)
Main Lounge (2218)
Main Lounge Deck
Porter Butts Gallery (2225)
Profile Room (2216)
Restrooms
Sunset Deck - Upper
Tripp Commons (2324)
Tripp Deck



1

ATM
Box Office (1182)
Building Manager Office (1109)
Campus & Community Information (1186)
Daily Scoop (1314)
Der Rathskeller (1301)
Der Schkeller (1305)
Dining Services Offices (1352/1356)
Elevators
Essentials Supervisor (1328)
Essentials/Wisconsin Union Store (1339)
Fan Taylor Lobby (1130)
Guestrooms Check-in (Essentials) (1128)
Lakefront on Langdon (1355)
Lakefront on Langdon Gallery
Paul Bunyan Room (1304)
Peet's Coffee and Tea (1319)
Restrooms
Shannon Hall (1130)
Shannon Sunset Lounge (1133)
Sunset Deck
Terrace
Trophy Room (1214)

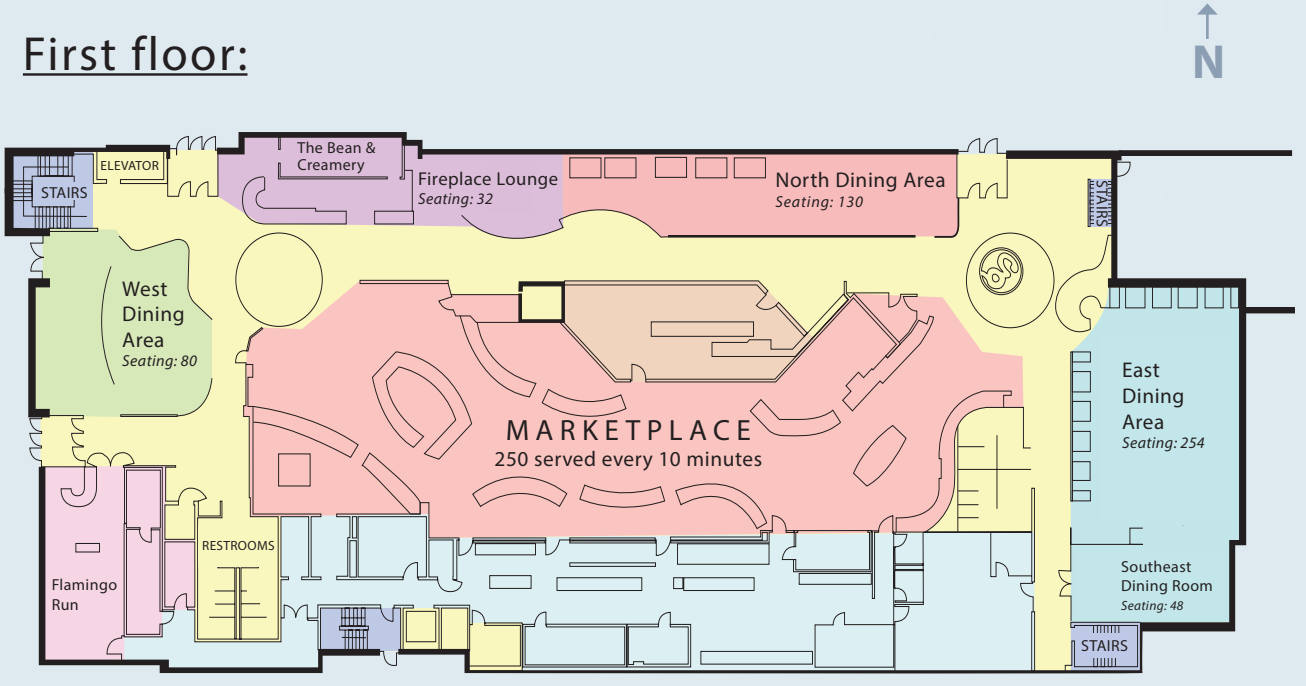
UNION SOUTH FLOOR PLANS



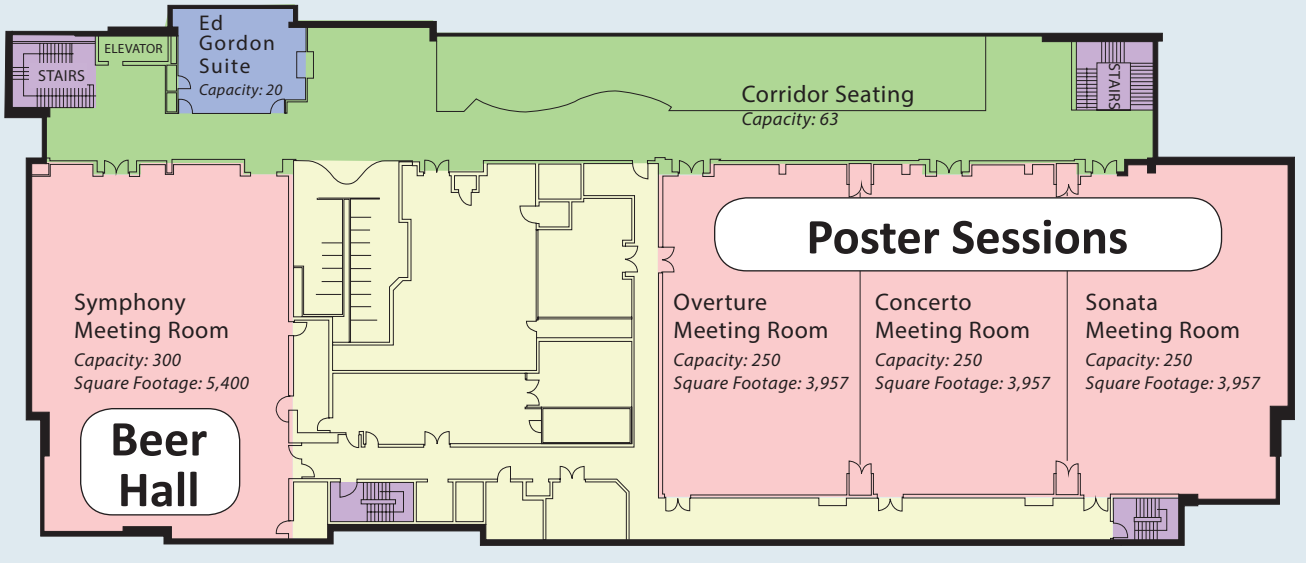


GORDON DINING AND EVENT CENTER FLOOR PLANS

First floor:



Second floor:



TUESDAY MAY 26

1:00 – 7:30 pm	Registration	Main Lounge
5:00 – 7:15 pm	Opening reception/light dinner	Tripp Commons/Tripp Deck
7:30 – 7:45 pm	Welcome and meeting overview	Shannon Hall
7:45 – 10:00 pm	Keynote talks	Shannon Hall

WEDNESDAY MAY 27

7:30 am – 8:30 pm	Poster installation	Gordon Dining and Event Center
8:00 am – 6:30 pm	Registration continues	Annex
9:00 am – 12:30 pm	Plenary session 1: Ribozymes, riboswitches & RNA structure (1-12) [Rob Batey]	Shannon Hall
12:30 – 2:00 pm	Lunch	Inn Wisconsin/Main Lounge
12:30 – 2:00 pm	Meetings Committee lunch/meeting	Class of '24 Reception Room
2:00 – 3:30 pm	Concurrent session 2A: Ribosome assembly and function (13-19) [Gloria Culver]	Shannon Hall
	Concurrent session 2B: RNA transport and localization (20-25, 616) [Samie Jaffrey]	Great Hall
4:00 – 5:30 pm	Workshops:	
	W1: RNA and disease [Chonghui Cheng]	Shannon Hall
	W2: Deciphering the mRNP code [Niels Gehring and Utz Fischer]	Great Hall
	W3: Careers in RNA science [Allison Didychuk]	Play Circle Theater
	W4: RNA structure prediction [Tamar Schlick]	Humanities 1111
	W5: RNA synthetic biology [Hans-Joachim Wieden]	Humanities 1121
6:30 – 8:00 pm	Science and Society Dinner [Speaker: Jon Lorsch]	Varsity Hall/Union South
8:30 – 11:00 pm	Poster session 1 and beer hall (even numbers)	Gordon Dining and Event Center

THURSDAY MAY 28

8:00 am – 6:00 pm	Registration continues	Annex
9:00 am – 12:30 pm	Plenary session 3: Pre-mRNA splicing (26-37) [Charles Query]	Shannon Hall
12:30 – 2:00 pm	Mentor-Mentee lunch [Coordinator: Beth Tran]	Inn Wisconsin/Main Lounge
12:30 – 2:00 pm	Conference lunch (anyone not attending M/M lunch)	Gordon Dining and Event Center
2:00 – 3:30 pm	Concurrent session 4A: Alternative splicing (38-44) [Jernej Ule]	Shannon Hall
	Concurrent session 4B: Interconnections between RNA processes (45-51) [Melissa Moore]	Great Hall
4:00 – 5:30 pm	History of RNA research panel discussion [Marv Wickens]	Shannon Hall
5:30 – 6:30 pm	Junior Scientist Social [Coordinated by the Junior Scientist Committee]	Tripp Deck
6:00 – 8:00 pm	Picnic dinner	Lakeshore dorms
	Free evening (posters and beer hall open 8:00 – 10:30 pm)	Gordon Dining and Event Center

FRIDAY MAY 29

8:00 am – 6:00 pm	Registration continues	Annex
9:00 am – 12:30 pm	Plenary session 5: Short non-coding RNAs (52-64) [Erik Sontheimer]	Shannon Hall
12.30 – 2:00 pm	Lunch	Inn Wisconsin/Main Lounge
2:00 – 3:30 pm	Concurrent session 6A: Long non-coding RNAs (65-70) [Howard Chang]	Shannon Hall
	Concurrent session 6B: RNA processing/3' end formation (71-76, 453) [Elmar Wahle]	Great Hall
	Concurrent session 6C: RNA modification and editing (77-82) [Jane Jackman]	Play Circle Theater
4:00 – 5:30 pm	Workshops:	
	W6: Emerging techniques [Marv Wickens]	Shannon Hall
	W7: Interface of theory and experiments in functional RNAs [Darrin York and Phil Bevilacqua]	Great Hall
	W8: RNA therapeutics [Brett Monia]	Play Circle Theater
	W9: La and related proteins [Rich Maraia]	Humanities 1111
	W10: Splicing structure and mechanism [Stephen Rader]	Humanities 1121
6:00 – 7:30 pm	Dinner	Inn Wisconsin/Main Lounge
6:00 – 7:30 pm	Board of Directors dinner/meeting	Class of '24 Reception Room
8:00 – 10:30 pm	Poster session 2 and beer hall (odd numbers)	Gordon Dining and Event Center
10:30 – 11:00 pm	Remove posters	

SATURDAY MAY 30

8:00 am – 6:00 pm	Registration continues	Annex
7:30 – 9 am	Remove posters	Gordon Dining and Event Center
9:00 – 12:30 pm	Plenary session 7: mRNA turnover and translational control (83-94) [Ambro van Hoof]	Shannon Hall
12:30 – 2:00 pm	Lunch	Inn Wisconsin/Main Lounge
2:00 – 4:30 pm	Plenary session 8: RNP biogenesis, structure and function (95-102) [Reinhard Lührmann]	Shannon Hall
5:00 – 6:00 pm	Awards ceremony	Shannon Hall
6:30 – 7:30 pm	Reception	Varsity Hall Lobby & Terrace/Union South
7:30 – 9:00 pm	Dinner	Varsity Hall/Union South
9:00 – 11:30 pm	Dance and "Gomeroke"with music by the Gomers	The Sett/Union South

SUNDAY MAY 31

Conference concludes

5:00 – 11:00 am	Shuttle bus runs from Ogg Hall to Dane County Airport

Cell Research

*2013 Journal Citation Report, Science Edition (Thomson Reuters,2014)

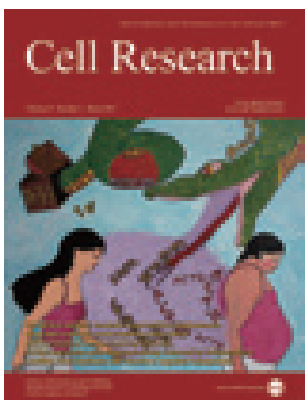


Introducing some of RNA-related articles recently published in *Cell Research*

Honeysuckle-encoded atypical microRNA2911 directly targets influenza A viruses **(OPEN)**
Zhen Zhou, Xihan Li, Jinxiong Liu, Lei Dong, Qun Chen, Jialing Liu, Huihui Kong, Qianyi Zhang, Xian Qi, Dongxia Hou, Lin Zhang, Guoquan Zhang, Yuchen Liu, Yujing Zhang, Jing Li, Jin Wang, Xi Chen, Hua Wang, Junfeng Zhang, Hualan Chen, Ke Zen and Chen-Yu Zhang
Cell Res ***25***: 39-49; advance online publication, October 7, 2014; doi:10.1038/cr.2014.130

FTO-dependent demethylation of N6-methyladenosine regulates mRNA splicing and is required for adipogenesis **(OPEN)**
Xu Zhao, Ying Yang, Bao-Fa Sun, Yue Shi, Xin Yang, Wen Xiao, Ya-Juan Hao, Xiao-Li Ping, Yu-Sheng Chen, Wen-Jia Wang, Kang-Xuan Jin, Xing Wang, Chun-Min Huang, Yu Fu, Xiao-Meng Ge, Shu-Hui Song, Hyun Seok Jeong, Hiroyuki Yanagisawa, Yamei Niu, Gui-Fang Jia, Wei Wu, Wei-Min Tong, Akimitsu Okamoto, Chuan He, Jannie M Rendtlew Danielsen, Xiu-Jie Wang and Yun-Gui Yang
Cell Res ***24***: 1403-1419; advance online publication, November 21, 2014; doi:10.1038/cr.2014.151

Pachytene piRNAs instruct massive mRNA elimination during late spermiogenesis **(OPEN)**
Lan-Tao Gou, Peng Dai, Jian-Hua Yang, Yuanchao Xue, Yun-Ping Hu, Yu Zhou, Jun-Yan Kang, Xin Wang, Hairi Li, Min-Min Hua, Shuang Zhao, Si-Da Hu, Li-Gang Wu, Hui-Juan Shi, Yong Li, Xiang-Dong Fu, Liang-Hu Qu, En-Duo Wang and Mo-Fang Liu
Cell Res ***24***: 680-700; advance online publication, May 2, 2014; doi:10.1038/cr.2014.41



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The best microRNA qPCR system

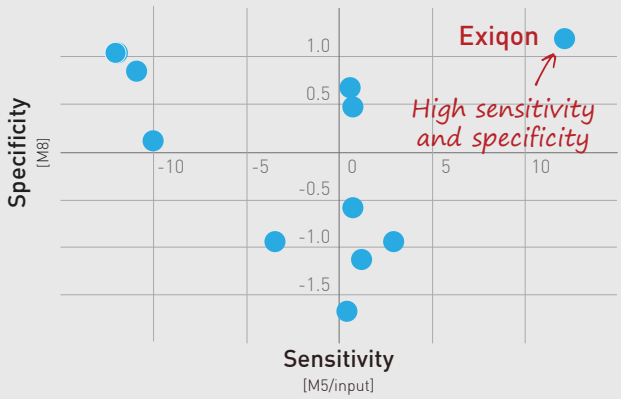
In a recent study published in Nature Methods*, Pieter Mestdagh and colleagues performed the largest independent comparative study of commercially available microRNA expression platforms to date.

The authors evaluate 12 platforms in key areas: specificity, sensitivity, reproducibility and accuracy. Exiqon's qPCR system is the overall best performing microRNA profiling platform, offering the best balance between the four key parameters.

Get the best sensitivity and specificity

In the study, Exiqon's qPCR platform is the only system that provides perfect specificity for both of the tested microRNA families. Specificity data is taken from the M8 measurements of the study*.

Combined with the sensitivity data, this figure shows that Exiqon provides the best combination of sensitivity and specificity, which results in high call rates with low numbers of false positives.



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*] Mestdagh *et al.* Nature Methods 2014, 11: 809-15

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