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	Keynote: Venki Ramakrishnan
Time:	Tuesday June 11, 15:00 - 15:45
Location:	Davos Ballroom
Abstract:	1
Chair(s):	Nenad Ban, *ETH Zürich*

1 Exceptions to Canonical Decoding by the Ribosome

Venki Ramakrishnan[1]

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The ribosome promotes the accuracy of selection of an aminoacyl tRNA that is cognate to the codon on mRNA as specified by the rules of the genetic code. Proper codon-anticodon pairing results in a series of conformational changes that results in GTP hydrolysis by elongation factor Tu, which allows the addition of the amino acid on the selected tRNA to the nascent peptide. However, there are notable exceptions to this rule. In one case, involving tmRNA, the role of both mRNA and tRNA is played by a protein. A second exception involves the read-through of stop codons that are modified to contain pseudouridine instead of uridine. These codons are read via a mechanism involving non-canonical base pairing previously thought to be forbidden by the genetic code. This talk will discuss how recent structures have shed light on both canonical decoding and exceptions to it.

Plenary 1: **Ribosome biogenesis and translation**

Time: Tuesday June 11, 16:00 - 18:00

Location: Davos Ballroom

Abstracts: 2 – 9, 397 B

Chair(s): Nenad Ban, *ETH Zürich*

2	**Structure of the eukaryotic 40S ribosomal subunit in complex with initiation factors eIF1 and eIF1A**
3	**Versatile binding of eukaryotic initiation factor 3 on the small ribosomal 40S subunit and the CSFV IRES**
4	**A novel strategy for protein synthesis initiation: 40S ribosomes bind to the 3' UTR of barley yellow dwarf virus (BYDV) mRNA**
5	**Probing the dynamics of Ribosome biogenesis in yeast**
6	**The casein kinase 1d homolog Hrr25 promotes dissociation of the ribosome assembly factor Ltv1 from nascent small ribosomal subunits to allow joining of large subunits.**
7	**Exonucleolytic processing of the 18S rRNA precursors during nuclear export in human cells**
8	**An mRNA-derived ncRNA targets and regulates the ribosome**
9	**Structural basis of translational regulation of msl2 mRNA by SXL and UNR during dosage compensation in Drosophila**
397 B	**Dom34-mediated dissociation of non-translating ribosomes allows efficient restart of translation after stress**

2 Structure of the eukaryotic 40S ribosomal subunit in complex with initiation factors eIF1 and eIF1A

Melanie Weisser[1], *Marc Leibundgut*[1], *Nenad Ban*[1]

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Bacterial and eukaryotic protein synthesis differ considerably at the level of initiation. The eukaryotic small ribosomal subunit (40S) binds a large number of initiation factors necessary for scanning of messenger RNAs for the correct start codon and for assembly of the translation-competent 80S ribosome. Although biochemical and genetic studies have revealed the roles of various initiation factors involved in this process, little structural information exists on their interaction with the 40S subunit in pre-initiation complexes, which would help us understand the underlying molecular mechanisms.

Following up on our studies of the complex between the 40S ribosomal subunit and eukaryotic initiation factor 1 (eIF1) [Rabl et al., 2011] we were recently able to solve the crystal structure of a larger pre-initiation complex comprising the 40S ribosomal subunit and eukaryotic translation initiation factors eIF1 and eIF1A at a resolution of 3.7 Å. Both factors are crucial for scanning for and recognition of the correct start codon and they interact with numerous other initiation factors (eIF5, eIF3, eIF2) in the context of the larger 43S pre-initiation complex.

Our structure reveals the interactions of the two initiation factors with the small ribosomal subunit and their positions relative to each other. The eukaryotic initiation factor eIF1A binds, as expected, in the vicinity of the ribosomal A-site, and its eukaryotic-specific C-terminal domain extends towards the mRNA channel. The structure offers a good starting point for understanding the structural interactions within the 43S pre-initiation complex and the role of the initiation factors in ribosomal scanning and start codon recognition.

Rabl J, Leibundgut M, Ataide SF, Haag A, Ban N. (2011) Science **331**, 730-6.

3 Versatile binding of eukaryotic initiation factor 3 on the small ribosomal 40S subunit and the CSFV IRES

Yaser Hashem[1], Amedee Des Georges[2], Vidya Dhote[3], Robert Langlois[2], Robert A. Grassucci[1], Tatyana V. Pestova[3], Christopher U.T. Hellen[3], Joachim Frank[1]

[1]Columbia University / HHMI; [2]Columbia University; [3]SUNY Downstate Medical Center

Protein translation initiation in most eukaryotes starts by the formation of the 43S preinitiation complex, comprising the Met-tRNAiMet, eukaryotic initiation factors 1, 1A, 2 and 3. The 43S is poised to attach the mRNA and start scanning for the start codon. Certain mRNAs possess internal ribosomal entry sites (IRESs), often at their 5' UTR, which allows end-independent initiation to take place, circumventing canonical initiation. These IRES-containing mRNAs don't follow the same regulatory pathway that supervises the recruitment of most mRNAs to the preinitiation complex. Examples of IRES-containing mRNAs can be found in many viruses such as the Hepatitis C Virus (HCV) and the Classical Swine Fever Virus (CSFV). The interaction of these IRESs with the 40S subunit has been studied for decades by various methods and few low-resolution cryo-electron microscopy (cryo-EM) structures are already available, however their low-resolution was insufficient for atomic modeling and many aspects related to their interaction with the 40S remain unknown.

Initiation on IRES-containing mRNAs requires eIF3 that was thought to interact in a complementary fashion with the IRES on the 40S subunit, allowing it to maintain the same binding site on the 40S in the presence or absence of the IRES. Here we present several cryo-EM structures of the CSFV IRES in interaction with the 40S subunit, eIF3 and DHX29, a DExH-box protein required for scanning on structured mRNAs and was found previously to stabilize the binding of eIF3 on the 40S. Our structures show that contrarily to the current model, eIF3 binds differently in presence of an IRES and its conserved core doesn't interact directly anymore with the 40S (figure 1 below). In addition, we were able to create a convincing atomic model of the CSFV IRES into a 10Å cryo-EM map (figure 2 below), which sheds more light on the IRES contacts with the 40S subunit. Our cryo-EM reconstructions also show the versatility of eIF3 binding and capture the latter in several close orientations on the CSFV IRES. Our study poses the first structural basis of eIF3 interaction with IRES-containing mRNAs and rectifies the erroneous eIF3•IRES•40S interaction model, which we anticipate to have tremendous implications on the field.

References:

Hellen et al. *Genes & Dev.* 2001

Hashem et al. *Cell* 2013, in press

Spahn et al. *Science* 2001

Siridechadilok et al. *Science* 2005

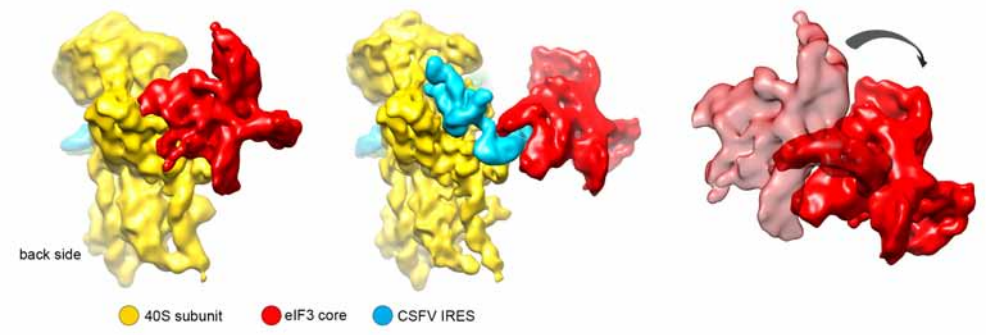

Figure 1

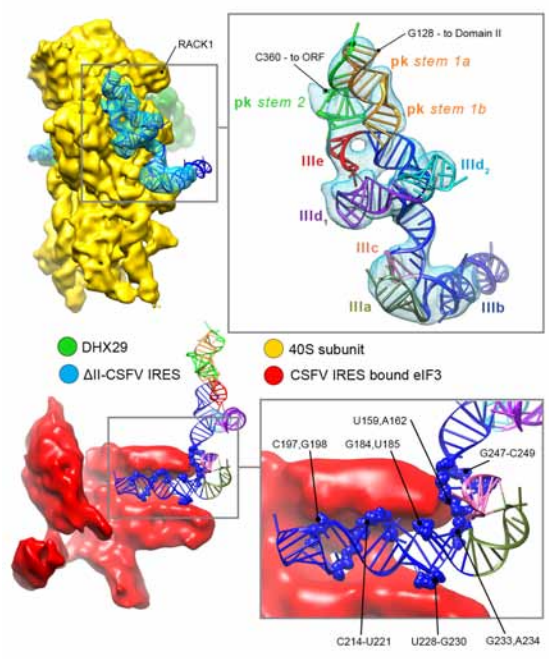

Figure 2

4 A novel strategy for protein synthesis initiation: 40S ribosomes bind to the 3' UTR of barley yellow dwarf virus (BYDV) mRNA

Sohani Das Sharma[1], Bidisha Banerjee[1], Jelena Kraft[2], W. Allen Miller[2], Dixie Goss[1]

[1]Hunter College CUNY; [2]Iowa State University

Most gene expression in uncapped RNA viruses is regulated by either an internal ribosomal entry site (IRES) or a cap independent translation element (CITE) that are located in the 5' and 3' UTR respectively of the viral mRNA. Barley yellow dwarf virus (BYDV) mRNA, which lacks both cap and poly (A) tail, has a translation element (BTE) present in the 3' UTR that is essential for efficient translation initiation at the 5' proximal AUG. The molecular mechanism of translation initiation is not well understood. Using fluorescence anisotropy, SHAPE analysis and toeprinting, we report: 1) eIF4F binds to the 3' UTR and the binding affinity correlates with the translational efficiency of the mutant BTEs; 2) 40S ribosomes bind to the BTE first; 3) RNA structural elements in the 3' and 5' UTRs interact to transfer ribosomes to the 5' UTR; 4) Sequence interactions between 18S rRNA and 3' viral RNA BTE are required for ribosome binding. We are currently identifying eIFs required for ribosome transfer. Taken together, these results suggest a novel mechanism for eIF4F binding to the 3' UTR to recruit the 43S ribosome followed by subsequent transfer of the ribosome complex to the 5' UTR, scanning to the AUG and initiation of protein synthesis.

Grant Support: NSF MCB1157632 (DJG) and NIH 2R01 GM067104 (WAM).

5 Probing the dynamics of Ribosome biogenesis in yeast

Ralph D Hector[1], Elena Burlacu[1], Stuart Aitken[2], Atlanta Cook[3], Sander Granneman[1]

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Ribosome synthesis in eukaryotes is an incredibly complex process that, besides ribosomal proteins (r-proteins), requires the activity of ~200 ribosome assembly factors. Many of these assembly factors contain enzymatic motifs and are presumed to play crucial roles in remodeling of pre-ribosomes and rRNA folding steps. Although we have a fairly complete picture of the stages at which ribosome assembly factors bind to intermediates, we still lack detailed knowledge of the RNA folding steps that take place and the role of the putative enzymes in this process.

Inspired by impressive chemical probing work done by many groups on bacterial ribosomes, we have developed protocols for purification and chemical modification of specific yeast ribosome assembly intermediates. By combining this with high-throughput sequencing we are able to quantitatively measure structural changes and remodeling steps during ribosome synthesis in a single chemical probing reaction. Using this methodology, dubbed ChemModSeq, we discovered that a large number of ribosomal proteins that interact with the head domain of the 18S rRNA r-proteins are not in their final conformation. Our results support the notion that many ribosome assembly factors can (also) function as r-protein placeholders. This rearrangement of ribosomal proteins correlates with the presence of specific 40S ribosome assembly factors and our results show that the head domain undergoes major remodeling just before the final 18S rRNA cleavage event in the cytoplasm. Our data provide the first insights at nucleotide resolution into how assembly factors modulate the assembly of ribosomal proteins, and provide a platform for studying the role of NTPases in restructuring/remodeling during ribosome synthesis.

6 The casein kinase 1d homolog Hrr25 promotes dissociation of the ribosome assembly factor Ltv1 from nascent small ribosomal subunits to allow joining of large subunits

Homa Ghalei[1], Katrin Karbstein[2]

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Cytoplasmic small (40S) ribosome assembly intermediates are protected from premature translation initiation by assembly factors, which block recruitment of translation factors, mRNA and large subunits. Dissociation of these assembly factors is somehow coupled to a translation-like cycle, in which mature large (60S) subunits join to proofread essential activities of the maturing 40S subunit. However, how entry into this cycle is regulated and how assembly factors dissociate, remains unknown. Here we show that dissociation of the assembly factor Ltv1, located at the beak structure, initiates the cytoplasmic maturation cascade for 40S ribosomal subunits. Hrr25, the yeast homolog for casein kinase 1d, involved in many cellular processes, and linked to numerous human diseases, phosphorylates Ltv1 leading to its dissociation. Failure to release Ltv1 blocks subsequent joining of 60S subunits and entry into the translation-like cycle. We are now studying the effects from Ltv1 release on the incorporation of nearby ribosomal proteins and the 40S structure.

7 Exonucleolytic processing of the 18S rRNA precursors during nuclear export in human cells

Marie-Francoise O'Donohue[1], Milena Preti[1], Nathalie Montel-Lehry[1], Marie-Line Bortolin-Cavaille[1], Hanna Gazda[2], Pierre-Emmanuel Gleizes[1]

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Pre-ribosomal RNA maturation has long been considered a highly conserved process in eukaryotes, but recent studies have revealed evolutionary divergence between the yeast and mammalian ribosome synthesis pathways. Understanding the specifics of human ribosome biogenesis is likely to be important for elucidating pathological mechanisms in cancer and ribosomal diseases, like Diamond-Blackfan anemia, the Treacher-Collins syndrome, or the Shwachman-Diamond syndrome. Defects in ribosome biogenesis trigger stress response pathways that perturb cell proliferation and differentiation. Investigating how these signaling pathways are activated requires further understanding of the mechanisms of pre-rRNA processing in human cells.

Mutations in Diamond-Blackfan anemia, a congenital erythroblastopenia associated to mutations in ribosomal protein genes, affect ITS1 processing in a large proportion of patients. Processing of the ITS1 within the pre-40S particles starts in the nucleolus and ends in the cytoplasm in human cells as in yeast. Using loss-of-function experiments and extensive RNA analysis, we have determined that endonucleolytic cleavage E in the ITS1 takes place 78 or 81 nucleotides downstream of the 18S rRNA 3'-end. Cleavage at this site generates the 18S-E pre-rRNA, the last precursor to the 18S rRNA. Unexpectedly, we found that this endonucleolytic cleavage is followed by exonucleolytic processing of the cleavage products in both orientation. The 3'-5' exonucleoytic trimming of the 18S-E pre-rRNA occurs during nuclear export of the pre-40S particles, as revealed by detailed 3'-RACE analysis and cell frationation. The exosome may play some role in this process, but other exonucleases seem to be involved. Knockdown of several ribosomal proteins and maturation factors required for formation of the 18S rRNA results in the accumulation in the cytoplasm of a short form of the 18S-E pre-rRNA, whose final conversion into 18S rRNA requires the PIN-domain containing NOB1.

The requirement of exonucleases in the maturation of the 18S rRNA 3'-end has never been described so far in other eukaryotes, and might indicate a higher level of quality control in mammalian ribosome biogenesis. These results not only deliver a more complex picture of pre-rRNA maturation mechanisms in mammalian cells, but they also provide a mechanistic framework to further study the interplay of DBA-linked ribosomal proteins in this process. We are currently characterizing the exonucleases involved in 18S rRNA maturation, and have found that mutation of a novel DBA-associated gene encoding a large ribosomal subunit protein also affects ITS1 processing.

8 An mRNA-derived ncRNA targets and regulates the ribosome

Andreas Pircher[1], *Kamilla Bakowska-Zywicka*[2], *Marek Zywicki*[3], *Norbert Polacek*[1]

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Small non-protein-coding RNA (ncRNA) molecules have been recognized recently as major contributors to regulatory networks in controlling gene expression in a highly efficient manner. While the list of validated ncRNAs that regulate crucial cellular processes grows steadily, not a single ncRNA has been identified that directly interacts and regulates the ribosome during protein biosynthesis (with the notable exceptions of 7SL RNA and tmRNA). All of the recently discovered regulatory ncRNAs that act on translation (e.g. microRNAs, siRNAs, antisense RNAs) target the mRNA rather than the ribosome. This is unexpected, given the central position the ribosome plays during gene expression.

To investigate whether such a class of regulatory ncRNAs does exist we performed genomic screens for small ribosome-associated RNAs in model organisms of all three domains [1,2]. Here we focus on the functional characterisation of an 18 nucleotide long ncRNA candidate derived from an open reading frame of an annotated *S. cerevisiae* gene, which encodes a tRNA methyltransferase. Yeast cells lacking this tRNA methyltransferase showed clear growth defects in high salt containing media. Genetic analysis showed that the absence of the mRNA-derived ncRNA rather than the absence of the tRNA methyltransferase activity is responsible for the observed phenotype. Since we performed a screen for small ribosome-associated RNAs we examined the regulatory potential of the synthetic 18mer during translation *in vitro* and *in* vivo. Metabolic labeling experiments in the presence of the synthetic 18mer RNA revealed an inhibitory potential on the global protein biosynthesis rate. *In vitro* translation and northern blot analysis further strengthen the hypothesis, that this RNA is a ribosome-associated regulatory ncRNA.

Our studies in pro- and eukaryotic model organisms reveal the ribosome as a novel target for small regulatory ncRNAs in all domains of life. Ribosome-bound ncRNAs are capable of fine tuning translation and might represent a so far largely unexplored class of regulatory ncRNAs.

[1]Zywicki, M., K. Bakowska-Zywicka, et al. (2012). "Revealing stable processing products from ribosome-associated small RNAs by deep-sequencing data analysis." Nucleic Acids Res **40**(9): 4013-4024.

[2]Gebetsberger, J., Zywicki, M., KÜnzi, A., Polacek, N. (2012). „tRNA-derived fragments target the ribosome and function as regulatory non-coding RNA in *Haloferax volcanii*" Archaea

9 Structural basis of translational regulation of msl2 mRNA by SXL and UNR during dosage compensation in Drosophila

Janosch Hennig[1], Cristina Militti[2], Grzegorz Popowicz[4], Iren Wang[1], Miriam Sonntag[1], Arie Geerlof[1], Fatima Gebauer[3], Michael Sattler[1]

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The protein Upstream of N-Ras (UNR) is a key regulator of gene expression at the translational level in both humans and *Drosophila*. In *Drosophila*, the role of UNR in dosage compensation is well characterized. UNR and the female-specific protein Sex-lethal (SXL) bind cooperatively to the 3' UTR of *msl2* mRNA, which encodes the rate-limiting subunit of the dosage compensation complex. This interaction represses the translation of *msl2* mRNA and allows female fly viability.

We have investigated the structural basis for the assembly of the SXL-UNR-*msl2* ribonucleoprotein complex and studied the minimal relevant regions required for ternary complex formation by complementary structural biology methods, biochemistry and functional analysis. We report the crystal structure of the ternary complex at 2.8 Å resolution, which was validated by complementary data from solution NMR, SAXS and SANS. The structure reveals unprecedented insight into how the tandem RNA recognition motif (RRM) domains of SXL and UNR cold shock domains cooperate for specific the recognition of the *msl-2* RNA. Structure-based mutational analysis of protein-RNA and protein-protein interfaces reveal molecular details for translational repression by UNR during development. Our structural and functional analysis provide novel insight for an important molecular mechanism of the regulation of X-chromosome dosage compensation.

397 Studies on structure-function relationships of the snoRNP assembly machinery

Christiane Branlant[1], Régis Back[12], Benjamin Rothé[13], Jonathan Bizarro[5], Magali Blaud[11], Marc Quinternet[4], Solange Morera[8], Philippe Meyer[7], Cyril Dominguez[2], Frédéric Alain[3], Edouard Bertrand[6], Xavier Manival[10], Bruno Charpentier[9]

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As well pre-rRNA processing as pre-mRNA splicing depends upon activity of ribonucleoprotein particles. These RNPs are generated by association of sets of proteins onto small non coding RNAs, such as snoRNAs, scaRNAs or UsnRNAs. Work from the recent years demonstrated that like spliceosomal UsnRNP assembly, the assembly of snoRNPs which are involved in pre-rRNA processing and that of scaRNPs that are involved in UsnRNA modification depend upon cellular factors required to increase the efficiency and accuracy of assembly. We and others have identified an important factor of the snoRNP assembly machinery, the Rsa1(yeast)/NUFIP(human) protein. This plateform protein associates with nascent RNPs by binding to the snoRNP RNA primary binding protein (Snu13 in yeast, 15.5K in human). Rsa1/NUFIP is able to bind to several other snoRNP core proteins and can recruit the R2TP complex a co-chaperon of the Hsp90 protein. The yeast R2TP complex contains proteins: Tah1, Pih1, Rvb1 and Rvb2. Tah1 forms a heterodimer with Pih1 and binds to Hsp90 by interaction with the Hsp90 C-terminal region. The idea is that by its numerous interactions with the core proteins and cellular factors, Rsa1 favors the specific recruitment of snoRNP proteins on the snoRNA and their remodeling, leading to stable mature snoRNPs. We focused our effort on understanding the mechanism of action and architecture of the C/D box snoRNP assembly machinery. We used yeast genetic approaches to determine the order of action of the cellular factors and identify their domains of interaction. In parallel, we developed structure-function analyses on components of the snoRNP assembly machinery based on NMR and other biophysical-chemical approaches. Studies on the R2TP-Hsp90 are as much important as this complex is involved in other assembly processes, in particular RNA polymerase assembly.

Although they play similar functions in snoRNP assembly, proteins Rsa1 and NUFIP show limited sequence homology. Only one 31 amino acid long sequence is strongly conserved. We showed that this peptide is crucial for the Rsa1p/NUFIP activity in box C/D snoRNP assembly. We identified an essential sub-fragment and solved its 3D structure by NMR. Then, by using the known 3D structure of yeast Snu13, we built a 3D model of the Rsa1-Snu13 interaction. We validated this model by biophysical methods and functional assays in yeast. In parallel, we purified Tah1 at a high degree of purity and established the 3D structure of free Tah1 and Tah1 bound to Hsp90. Our data explain how Tah1 specifically recognizes Hsp90. We characterized the interacting domains of Tah1p and Pih1p. We showed that Rsa1 bound to Snu13p can interact with another assembly factor Hit1 and also characterized the interacting domains. We will present the present stage of our functional and structural studies and their implication in terms of mechanism of action of the snoRNP assembly machinery.

Plenary 2A: Regulation by long non-coding RNAs

Time: Wednesday, June 12, 8:30 - 10:15
Location: Davos Ballroom
Abstracts: 10 - 16
Chair(s): V. Narry Kim, Seoul National University

10	**Circular RNAs function as efficient microRNA sponges**
11	**The regulatory circuits mediated by RNAs in Staphylococcus aureus and implication of the endoribonuclease III**
12	**Messenger and long non-coding RNAs: dressed for the occasion?**
13	**Non-coding RNAs prevent spreading of a repressive histone mark**
14	**Single cell and genome-wide analysis to dissect antisense RNA-mediated gene silencing and pervasive transcription in S. cerevisiae**
15	**Telomeric non-coding RNA acts as a scaffold for telomerase high-order organization at short telomeres**
16	**Human α satellite derived transcripts interact with the active site of RNApolII**

10 Circular RNAs function as efficient microRNA sponges

Jorgen Kjems[1], Thomas B. Hansen[2], Christian K Damgaard[2], Trine I. Jensen[2], Jesper B. Bramsen[2], Bettina H. Clausen[3], Bente Finsen[3]

[1]Department of Molecular Biology and Genetics, Interdisciplinary Nanoscience Center, Aarhus University, Aarhus, Denmark; [2]Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark; [3]Neurobiology Research, Institute of MolecularMedicine, University of Southern Denmark

Circular RNAs that derive from RNA splicing events across exons are abounded in mammalian cells but their function has until recently been illusive. We recently reported that the antisense transcript to the neuronally expressed CDR-1 gene is almost exclusive circular and positively regulates the expression of the CDR-1 mRNA (Hansen et al. 2011).

We have now discovered that this circular RNA, in addition, acts as a highly efficient microRNA-7 (miR-7) sponge and named it ciRS-7 (Circular RNA Sponge for miR-7; Hansen et al. In press). ciRS-7 harbours more than 70 selectively conserved putative miRNA target sites and it is highly and widely associated with Ago-proteins in a miR-7 dependent manner. While the circular RNA is completely resistant towards miRNA-mediated target destabilization, it strongly suppresses miR-7 activity resulting in elevated levels of miR-7 targets. In the mouse brain, we observe overlapping neuronal expression patterns of ciRS-7 and miR-7 in the neocortex and hippocampus, and in thalamus and substantia nigra suggesting a high degree of endogenous interaction. We also show that ciRS-7 can induce established miR-7 targets including SNCA, EGFR and IRS2, implicated in Parkinson disease, cancer and diabetes, respectively. The ciRS-7 is itself under the control of miR-671 that, unusually for a mammalian miR, cleaves the ciRS-7 and effectively removes it from the cell. Hence miR-671 may constitute a novel therapeutic reagent in a number of human diseases.

The function of circular RNAs as miR sponges appears to be a general phenomenon. We demonstrate that circular testis specific RNA, SRY, serves as a miR-138 sponge and that circular SRY expression can increase miR-138 targeted mRNAs. Thus, this serves as the first functional study of a naturally expressed circular RNA.

We are currently establishing ciRS as generic platform enabling sponging of any miR of chose by reprogramming the seed sequences.

Hansen, T. B., Wiklund, E. D., Bramsen, J. B., Villadsen, S. B., Statham, A. L., Clark, S. J., and Kjems, J. (2011) miRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA, *The EMBO journal 30*, 4414-4422.

Thomas B Hansen; TB, Jensen, TI, Clausen, BH, Bramsen, JB, Finsen, B, Damgaard, CK, Kjems, J. (2013) Natural RNA circles function as efficient miRNA sponges. *Nature* (Published online 27 February 2013)

11 The regulatory circuits mediated by RNAs in Staphylococcus aureus and implication of the endoribonuclease III

Efthimia Lioliou[1], Cédric Romilly[1], Isabelle Caldelari[3], Cynthia Sharma[4], Thomas Geissmann[2], François Vandenesch[2], Joerg Vogel[4], Pascale Romby[1]

[1]CNRS; [2]INSERM; [3]University of Strasbourg; [4]University of Wurzburg

Staphylococcus aureus is a remarkable versatile pathogen, able to cause a wide spectrum of human diseases, and is one of the main causes of community as well as hospital-acquired infections. The contribution of regulatory RNAs in the establishment of virulence in this pathogen is increasingly appreciated. Our previous data emphasize the multitude of regulatory steps affected by RNAIII in establishing a network of *S. aureus* virulence factors. We show that RNAIII and the endoribonuclease III coordinately repress the expression of numerous mRNAs that encode the transcriptional repressor of toxins, several virulence factors acting early in the infection process, and several enzymes involved in peptidoglycan metabolism. The repressor activity of RNAIII involves the formation of RNA-mRNA duplexes that results in the inhibition of translation initiation and concomitantly triggers endoribonuclease III attack. Identification of the RNA targets of the endoribonuclease III further illustrates the multiple functions of the enzyme in the regulation of RNA metabolism. Besides RNAIII, we demonstrated that the *S. aureus* genome likely encodes a high diversity of RNAs including *cis*-acting regulatory regions of mRNAs, *cis*-acting antisense RNAs, and small non-coding RNAs. We will illustrate how some of these novel non-coding RNAs have direct consequences on biofilm and capsule formation, and stress responses, and how they converged to the quorum-sensing system.

12 Messenger and long non-coding RNAs: dressed for the occasion

Alex Tuck[1], *David Tollervey*[2]

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In yeast, pervasive transcription generates a heterogeneous ensemble of long non-coding RNAs (lncRNAs) as well as mRNAs and stable, structural RNAs. The mRNAs are exported to the cytoplasm for translation, whereas characterized lncRNAs perform distinct functions, such as directing chromatin modifications. LncRNAs and mRNAs share many properties including a 5' cap, poly(A) tail and transcription by Pol II, raising the question of why they have such different fates. Throughout their maturation, export and decay, mRNAs interact with a defined series of protein factors. We reasoned that analysis of the interactions of these proteins with other transcripts, such as lncRNAs, would reveal the point at which their maturation separates from that of mRNAs. We therefore determined the in vivo, transcriptome-wide targets of key protein factors in this pathway. This revealed distinct classes of lncRNAs and mRNAs, with RNP compositions tailored to the regulation and functions of transcripts within each class. Therefore, rather than undergoing a "standard" maturation process, mRNAs and lncRNAs are assembled into purpose-built RNPs. LncRNAs were abundant targets of the nuclear surveillance machinery, so are predominantly retained and degraded in the nucleus. However, further analyses revealed a subclass of stable lncRNAs that undergo cleavage and polyadenylation and are exported to the cytoplasm. In contrast, the unstable lncRNAs are subject to a distinct mode of termination. Therefore, 3' end processing is a key step in RNP biogenesis at which transcript fate is determined, and differences here explain the origin of the marked heterogeneity amongst mRNAs and lncRNAs. In support of this model, we identified dual roles for two proteins in both cleavage and polyadenylation and surveillance of lncRNAs. Unexpectedly, we also observed "lncRNA-like" mRNAs, subject to post-transcriptional regulation in the nucleus, which involves early transcription termination, upstream lncRNAs, or surveillance by the nuclear poly(A) binding protein Nab2. In yeast subjected to a short nutrient downshift there was extensive retargeting of the nuclear surveillance factor Mtr4 amongst these "lncRNA-like" mRNAs. Changes in lncRNA expression are therefore rapid and may play a widespread role in reprogramming gene expression. In conclusion, our comprehensive atlas of RNP composition effectively captures the diversity within the transcriptome and has unearthed several prominent mechanisms of post-transcriptional regulation in the nucleus.

13 Non-coding RNAs prevent spreading of a repressive histone mark

Marc Bühler[1], *Claudia Keller*[1], *Raghavendran Kulasegaran-Shylini*[1], *Yukiko Shimada*[1], *Hans-Rudolf Hotz*[1]

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Besides messenger RNAs (mRNAs), eukaryotic cells produce a plethora of RNAs that appear to be non-protein coding (ncRNAs). Whereas substantial progress has been made in cataloging ncRNAs, the extent of their involvement in regulatory circuits and the mechanisms through which they might act remain to be explored further. In the fission yeast *Schizosaccharomyces pombe*, ncRNAs are known to play a prominent role in the assembly and maintenance of heterochromatin. Transcription of centromeric repeat sequences results in the production of long ncRNAs (lncRNAs) that are processed by Dicer (Dcr1) into short ncRNAs (siRNAs). These are found in Argonaute (Ago1) and target the Ago1-containing RNA-induced transcriptional silencing complex (RITS) to nascent chromatin-bound lncRNAs. Subsequently, RITS recruits the histone methyltransferase (HMTase) Clr4 to methylate histone H3 at Lys 9 (H3K9), a hallmark of heterochromatin. Thus, long and short ncRNAs cooperate in the targeting of a histone modifying activity to the appropriate location in the *S. pombe* genome. In contrast to this mode of ncRNA action, we have now discovered a novel class of ncRNAs that counteract H3K9 methylation. We have identified a lncRNA residing in centromeric heterochromatin, termed BORDERLINE, which prevents the spreading of heterochromatin beyond the pericentromeric repeat region. Our results demonstrate that the production of RNA is sufficient to demarcate an epigenetically distinct chromosomal domain, irrespective of the underlying DNA sequence. In contrast to the recurring theme that ncRNAs function to recruit or guide proteins to chromatin, we show that RNA can also counteract chromatin association.

14 Single cell and genome-wide analysis to dissect antisense RNA-mediated gene silencing and pervasive transcription in S. cerevisiae

Manuele Castelnuovo[1], Samir Rahman[4], Judith Zaugg[2], Elisa Guffanti[1], Zhenyu Xu[3], Jurgi Camblong[1], Nick Luscombe[2], Lars Steinmetz[3], Daniel Zenklusen[4], Françoise Stutz[1]

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The *S. cerevisiae* genome codes for a myriad of intergenic and antisense (AS) transcripts, some of which are unstable and degraded by the exosome component Rrp6 [1, 2]. Loss of Rrp6 results in the accumulation of long *PHO84* AS RNAs and repression of sense transcription through a process that involves *PHO84* promoter deacetylation by the Hda1/2/3 histone deacetylase (HDAC) complex [3]. Here, we use single molecule resolution fluorescent in situ hybridization (smFISH) [4] to investigate the mechanism of PHO84 antisense-mediated transcription regulation in single cells. We show that PHO84 AS acts as a bimodal switch, where continuous low frequency PHO84 AS transcription represses sense transcription within individual cells. Surprisingly, AS RNAs do not accumulate at the *PHO84* gene but are exported to the cytoplasm. Furthermore, loss of Rrp6, rather than stabilizing *PHO84* AS RNA, promotes AS elongation by reducing its early transcription termination by Nrd1/Nab3/Sen1. These observations suggest that PHO84 silencing results from low frequency but constant AS transcription through the promoter rather than its static accumulation at the repressed gene. To investigate the generality of this regulation we profiled various histone modification mutants in a D*rrp6* strain using high-density tiling arrays. We confirm a widespread occurrence of antisense-dependent gene regulation and identify three classes of genes that accumulate asRNA in the absence of Rrp6, which differ in whether their genes are silenced by the asRNA and whether the repression involves HDACs and HMT. Distinguishing features between functional and non-functional antisense RNAs include sensitivity to early termination, extension into the promoter region, or the promoter structure of the repressed gene. The data indicate that histone-modifying enzymes are particularly important for antisense-mediated silencing of highly regulated genes subjected to extensive chromatin remodeling.

[1] Neil H, et al., Widespread bidirectional promoters are the major source of cryptic transcripts in yeast. Nature. 2009 Feb 19;457(7232):1038-42.

[2] Xu Z, et al., Bidirectional promoters generate pervasive transcription in yeast. Nature. 2009 Feb 19;457(7232):1033-7.

[3] Camblong J, et al., Antisense RNA stabilization induces transcriptional gene silencing via histone deacetylation in S. cerevisiae. Cell 2007 131:706-17

[4] Zenklusen D, et al., . Analyzing mRNA expression using single mRNA resolution fluorescent in situ hybridization. Methods Enzymol. 2010;470:641-59.

15 Telomeric non-coding RNA acts as a scaffold for telomerase high-order organization at short telomeres

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Telomerase, which is composed of both protein and RNA, maintains genome stability by replenishing telomeric repeats at the ends of chromosomes. On short telomeres, several molecules of telomerase are recruited, leading to the formation of telomerase foci or clusters, which reflects the distributive extension of short telomeres by this enzyme. How these telomerase clusters are formed on short telomeres is still unknown. Herein, we show that telomeric non-coding RNA is involved in the nucleation of telomerase clusters at short telomeres.

Telomeres are transcribed in a strand specific manner, giving rise to a G-rich telomeric-repeat containing RNA (TERRA). In mammalian cells, TERRA is nuclear and accumulates to some extent at telomeres. In yeast, TERRA expression is negatively regulated by the 5'-3' exonuclease Rat1, which actively degrades TERRA transcripts. Although several functions for TERRA have been proposed in mammalian cells, direct evidence for a specific role for TERRA in yeast is still missing. We developed a live-cell imaging assay based on the MS2-GFP system to study endogenous TERRA expression from a unique telomere at the single cell level in yeast. We show that TERRA expression is induced when its telomere shortens, leading to the accumulation of TERRA molecules into a single perinuclear focus. Live-cell imaging of a GFP-labeled TERRA and its RFP-labeled telomere revealed that a TERRA focus associates specifically but transiently with its telomere of origin in S phase, which was confirmed by chromatin immunoprecipitation.

Furthermore, an interaction between TERRA and the yeast telomerase RNA (*TLC1*) was detected *in vivo* by co-immunoprecipitation. Surprisingly, by simultaneously imaging TERRA-GFP and *TLC1* RNA-CFP, we captured spontaneous events of nucleation of *TLC1* RNA molecules on TERRA foci in S phase, suggesting that a TERRA focus acts as a scaffold for the recruitment of telomerase and triggers the formation of a telomerase cluster. Simultaneous imaging of telomere 6R- or telomere 1L-TERRA, *TLC1* RNA and telomere 6R revealed that a TERRA-*TLC1* RNA cluster forms in early S phase, and is later recruited preferentially to the telomere from which TERRA molecules originate. This association depends on factors involved in the recruitment of telomerase at short telomeres, such as Mre11, Tel1 and yKu70. We propose that a short telomere expresses non-coding RNA to nucleate telomerase molecules into a cluster in order to promote the simultaneous recruitment of several telomerase molecules on this telomere.

16 Human a satellite derived transcripts interact with the active site of RNApolII

Katarzyna Matylla-Kulinska[1], *Renee Schroeder*[1]

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Whether repetitive regions of the human genome have a function is a very intriguing question arising from two recently reported facts, firstly 80 % of the human DNA is transcribed into RNA, and secondly two-thirds of the genome is repetitive or repeat-derived. Human a satellites consist of 171 bp monomers arranged tandemly in a head-to-tail manner, organized into arrays of higher order repeats spanning hundreds of kilobases to megabases. They are predominantly located near centromeres on every human chromosome, where they assure the proper chromosome segregation being a site for the spindle attachment.

Here, we present that a satellite arrays are transcribed into large transcripts (more than 8 kb) in both orientations (direct and reverse complement) to the consensus a satellite sequence. Their expression is more pronounced in cellular stress conditions and restrained to the S phase of the cell cycle. Transcription of a satellites is sensitive to a amanitin treatment, indicating that they are RNApolII transcripts. Unlike most RNApolII transcripts, they are not polyadenylated and remain in the nucleus. Intriguingly, analysis of the 5' termini of RNAs reveals that only transcripts synthesized in the direct orientation possess cap structure while reverse complement are not capped. Driven by this finding we investigated a satellites interaction with RNApolII. In genomic SELEX against RNApolII, we had isolated several aptamers derived from a satellite repeats. Moreover, we found that a satellite RNAs interact with the active core of RNApolII resulting in DRB (5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole) sensitive 3' labeling of the RNA or/and to the second strand synthesis, what in turn implies that RNApolII might act as RNA dependent RNA polymerase (RdRP) on endogenous RNAs. Using HeLa cells nucleofection with chimeric transcripts combining a satellite with an artificial sequence, we observed RNA dependent RNA synthesis activity on genomic derived RNA template *in vivo*.

Plenary 2B: **RNA modification**

Time: Wednesday, June 12, 10:45 - 12.30

Location: Davos Ballroom

Abstracts: 17 - 24

Chair(s): Juan Alfonzo, *Ohio State University*

17	**Methylated mRNA recognition by the YTH domain**
18	**Determination of N6-methyladenosine RNA modification status at single nucleotide resolution and the application to a long non-coding RNA-protein interaction**
19	**Inosine-mediated modulation of RNA sensing by innate immune sensors**
20	**Impact of ADARs on abundance and sequence of miRNAs and other non-coding RNAs.**
21	**ADAR proteins suppress activation of antiviral signaling by cellular RNA.**
22	**Mechanism of gRNA Biogenesis in Trypanosome Mitochondria**
23	**Biogenesis and function of cyclic N6-threonylcarbamoyladenosine (ct6A) as a widely distributed tRNA hypermodification**
24	**Unusual non-canonical editing important for tRNA processing in Trypanosomes as revealed by shallow sequencing.**

17 Methylated mRNA recognition by the YTH domain

Dominik Theler[1], *Cyril Dominguez*[2], *Frederic Allain*[1]

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Methylation of adenosine at the N6 position is considered the most abundant messenger RNA modification and known since nearly 40 years. Methylation is assumed to be important based on the severe phenotypes of methylase knockouts in several organisms. Recently also the demethylase has been postulated to be a disease risk gene. In 2012 two reports [1,2] were published investigating with high throughput sequencing this process and expanded the repertoire of known sites and lead to a better definition of the consensus sequence for methylation. One of these recent studies [1] investigated, which proteins bind preferentially to the methylated form of a RNA compared to the unmodified form. Two of the three hits in the top confidence category were YTH domain containing proteins (Ydf2,Ydf3).

We have previously shown that the YTH domain of YT521-B is a RNA binding domain [3] and performed NMR titrations using partially randomized nucleotides to better define the binding preferences of this domain, since the SELEX derived sequences were quite degenerate. This approach yielded a better defined binding sequence and a preliminary structure of this complex could be obtained.

Both studies [1,2] on N6-methylation of adenosine reported a consensus sequence for an A to get methylated, which resembles our NMR derived sequence. The methylated adenosine is mostly flanked 5' by a G and 3' by a C. This prompted us to perform a titration experiment using the NMR derived sequence containing a N6-methyladenosine. The addition of this methyl group shifted the exchange regime of this complex from fast/intermediate to slow indicating much higher affinity. We determined the structure with methylated RNA, which represents the first structure of this domain in complex with RNA.

We were able to obtain 14 InterNOEs between the protein and the methyl group, which is contacted by two tryptophan residues via CH-π interactions. This resembles the recognition of methylated arginines and lysines of histones by their respective binding domains [4]. The two tryptophan residues are strictly conserved in all YTH domains [5]. Furthermore our experiments were carried out with the domain of YT521-B and not the domains of YDF2 or YDF3.

The structure reveals, how the YTH domain is able to recognize a methylated adenosine and it is tempting to assign the YTH domain, which is on a molecular and functional level not well characterized, the role of recognizing mRNAs containing N6-methylated adenosines. YT521-B regulates alternative splicing and the presence of the YTH domain is crucial for this [3]. Our results might therefore hint at a role of mRNA methylation in the control of splicing.

[1] Dominissini, D. et al. (2012), Nature 485:201-206

[2] Meyer, KD. et al. (2012), Cell 149:1635-1646

[3] Zhang Z. et al. (2010), JBC 285:14701-14710

[4] Taverna SD. et al. (2007), NSMB 14(11):1025-1040

[5] Stoilov, P. et al. (2002), Trends in Biochem Sci 27(10):495-497

18 Determination of N6-methyladenosine RNA modification status at single nucleotide resolution and the application to a long non-coding RNA-protein interaction

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N^6-methyladenosine (m^{6}A) is the most abundant modification in eukaryotic mRNA and long non-coding RNA, but m^{6}A studies have been hindered by the lack of methods for its precise identification. Here we develop a method, named Site-specific Cleavage And Radioactively-labeling followed by Ligation-assisted Extraction and TLC (SCARLET), that identifies m^{6}A and its modification fraction in mRNA/lncRNA at single nucleotide resolution. We applied SCARLET to locate four m^{6}A sites on the nuclear-localized MALAT1 (metastasis associated lung adenocarcinoma transcript), an abundant lncRNA known to regulate alternative splicing and chromatin remodeling.

We further identified nuclear-localized proteins that selectively bind to two m^{6}A modified sites in MALAT1. Gel shift using the synthetic m^{6}A-containing RNA oligos from MALAT1 and the HeLa nuclear extract showed that m^{6}A-containing RNA oligos can recruit 2-5 fold more proteins compared with the unmodified RNA. Further, RNA pull down followed by LC-MS/MS show that heteronuclear ribonucleoprotein G (hnRNP-G) and hnRNP-C selectively bind to one of the two m^{6}A-modified MALAT1 sites; both hnRNP proteins are known to be involved in RNA splicing and transport. We also demonstrate that hnRNP-G and hnRNP-C prefer m^{6}A modified sites *in vivo*. The identification of m^{6}A-selective proteins indicates that a primary role of m^{6}A modification is to recruit specific proteins or protein complexes to the modification site to modulate the function or activity associated with these RNA-protein complexes.

This work is supported by a NIH EUREKA award (GM088599).

19 Inosine-mediated modulation of RNA sensing by innate immune sensors

Michael Gantier[1], *Soroush Sarvestani*[1], *Bryan Williams*[1]

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RNA-specific adenosine deaminase (ADAR)-mediated adenosine to inosine (A-to-I) editing is a critical arm of the antiviral response. However, mechanistic insights into how A-to-I RNA editing affects viral infection are lacking. We posited that inosine incorporation into RNA facilitates sensing of non-self RNA by innate immune sensors, and accordingly investigated the impact of inosine-modified RNA on Toll Like Receptor (TLR) 7/8 sensing. Inosine incorporation into synthetic ssRNA potentiated TNF-a or IFN-a production in human PBMCs, in a sequence-dependent manner, indicative of TLR7/8 recruitment. The effect of inosine incorporation on TLR7/8 sensing was restricted to immunostimulatory ssRNAs, and was not seen with inosine-containing short dsRNAs, nor with a deoxy-inosine-modified ssRNA. Inosine-mediated increase of self-secondary structure of an ssRNA resulted in potentiated mouse Tlr7 activation, as established through the use of *Tlr7*-deficient cells. There was a correlation between hyper-editing of influenza A viral ssRNA and its ability to stimulate TNF-a, independent of 5'-triphosphate residues. Furthermore, A-to-I editing of viral ssRNA directly enhanced mouse Tlr7 sensing, when present in proportions reproducing biologically relevant levels of RNA editing. Thus we demonstrate for the first time that inosine incorporation into immunostimulatory ssRNA can potentiate TLR7/8 activation. Our results suggest a novel function of A-to-I RNA editing, which is to facilitate sensing of phagocytosed viral RNA by innate immune sensors TLR7/8.

20 Impact of ADARs on abundance and sequence of miRNAs and other non-coding RNAs.

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Adenosine deaminases that act on RNA (ADARs) bind double-stranded RNAs and deaminate adenosines to inosines. Inosines are recognized as guanosines and thus editing changes sequence information but also the structure of RNAs. A to I editing is required for normal life and development. Besides mRNAs and repetitive elements, ADARs also target miRNA precursors and affect their processing efficiency or alter their target specificity. Previous studies on the impact of editing of miRNAs only focused on few specific miRNAs. However, the genome wide effect of editing on miRNAs had not been firmly established.

Using transgenic mouse embryos lacking the editing enzymes ADAR, ADARB1, or both we compared relative abundance and sequence composition of mature miRNAs to wild type mice by NextGen sequencing. Deficiency of ADARB1 leads to a reproducible change in abundance of several miRNAs, which seem to be unrelated to editing events. The additional lack of ADAR has little impact on the deregulation of mature miRNAs, indicating that proper miRNA processing is primarily dependent on ADARB1. A to G transitions reflecting A to I editing events can be detected at few sites and at low frequency during the early embryonic stage investigated. Again, most editing events are ADARB1 dependent with only few editing sites being specifically edited by ADAR.

Due to the strong impact of ADARB1 on miRNA abundance and the known higher editing levels in adult mice we applied the same experimental setup to miRNAs from adult mouse brain in wild type and ADARB1 mutant mice. ADARB1 deficient mice show reproducible changes in abundance of specific miRNAs, of which again many are unrelated to A to I editing events. Thus, binding of miRNA precursors by ADARB1, without editing, influences their abundance. Unlike in the embryo, A to G transitions are found in many sites and with a frequency up to 80% in the mature miRNA sequence. More than half of the editing events are primarily caused by ADARB1. Moreover, also previously unrecognized editing sites in miRNAs were identified. Interestingly, 64% of editing events occur in the seed region of miRNAs, allowing a retargeting of the edited miRNA.

Lack of ADARs also affects other non coding RNAs such as snoRNAs, snRNAs, and transcripts of repetitive elements. Understanding the consequences of these changes is a current focus of our studies.

21 ADAR proteins suppress activation of antiviral signaling by cellular RNA.

Sam Greenwood[1], Niamh Mannion[1], Xianghua Li[1], Liam Keegan[1], Robert Young[1], Simona Paro[1], Sarah Cox[1], Leeanne McGurk[1], Marion Hogg[1], James Brindle[1], David Read[1], Rui Zhang[2], Christoffer Nellåker[4], Chris Ponting[4], Jin-Billy Li[2], Matthew Ronshaugen[3], Julia Dorin[1], Ian Adams[1], Mary O'Connell[1]

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Mutations in the gene encoding the RNA editing enzyme ADAR1 have recently been shown to cause the rare autoimmune disorder Aicardi-Goutières syndrome (AGS). AGS is a clinical mimic of in utero-acquired infection and is associated with increased production of the antiviral cytokine interferon (IFN) alpha. *Adar1*-null mice also have aberrant expression of Type-1 IFN, interferon-stimulated genes (ISGs) and pro-inflammatory cytokines, yet the phenotype is more severe with embryonic lethality around E12.5. Therefore it has been proposed that ADAR1 is required to suppress antiviral responses.

Genetic crosses revealed that mice lacking both ADAR1 and the interferon receptor can survive to E16.5, suggesting the effect of the *Adar1* mutation is cell autonomous. Moreover, embryonic lethality in *Adar1*-null mice is rescued by crossing with a mutant lacking a key protein involved in antiviral signaling. These double homozygous mice have decreased IFN production, ISG expression and pro-inflammatory cytokine levels. Analysis of cellular RNA from *Adar1*-null mice revealed increased expression of transcripts encoding specific classes of repetitive elements. Surprisingly, increased expression of immune-response genes and altered levels of repetitive element transcripts are also observed in *Adar*-null *Drosophila*, supporting a conserved role for ADAR proteins as suppressors of innate immunity.

22 Mechanism of gRNA Biogenesis in Trypanosome Mitochondria

Ruslan Afasizhev[1], Takuma Suematsu[1], Inna Afasizheva[1]

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The U-insertion/deletion mRNA editing reactions are directed by guide RNAs (gRNAs). Mature 50-60 nt gRNAs are generated from ~800 nt precursors by 3' end nucleolytic processing and subsequent RET1-catalyzed uridylation. We previously demonstrated that TbRET1 repression in T. brucei leads to a loss of 3' oligo(U) tails and accumulation of gRNA precursors. The former observation is consistent with TbRET1's uridylyl transferase activity; the latter finding remained puzzling because TbRET1 lacks nucleolytic activity. Here, we show that TbRET1 forms a stoichiometric complex with a 3'-5' exonuclease TbDSS1 and several proteins without any discernible motifs, which we named TbRDS (TbRET1-TbDSS1) complex. In S. cerevisiae, DSS1 interacts with SUV3 helicase to form a mitochondrial degradosome. However, in T. brucei we found no evidence of stable DSS1-SUV3 interaction. Repression of TbDSS1 led to a loss of mature gRNAs and accumulation of ~800 nt gRNA precursors indicating that TbRET1 and TbDSS1 function in the same processing pathway. Furthermore, overexpression of enzymatically inactive DSS1 triggered gRNA processing defects. In addition to minicircle-encoded gRNA precursors, TbRDS complex also targets maxicirlce-encoded messenger and ribosomal RNA precursors leading to generation of mature molecules. In vitro, TbRDS complex processively degrades single-stranded RNA to 5-6 nt oligonucleotides whereas in vivo degradation stops precisely at the 3' ends of gRNAs, mRNAs and rRNAs. Deep sequencing of small mitochondrial RNA fraction indicated that guide RNA-sized antisense transcripts may define the 3' boundary of mature mitochondrial RNAs. Collectively, our data indicate that nucleolytic processing of gRNA precursors by TbDSS1 and uridylation of mature gRNAs by TbRET1 are coupled by virtue of both enzymes being assembled into a stable TbRDS complex. The degradation activity of TbRDS is likely controlled by antisense RNAs thereby the 5' end of the antisense determines the 3' end of the sense strand.

23 Biogenesis and function of cyclic N6-threonylcarbamoyladenosine (ct6A) as a widely distributed tRNA hypermodification

Tsutomu Suzuki[1], Kenjyo Miyauchi[1], Takuya Sakashita[1], Satoshi Kimura[1], Tomoyuki Numata[2]

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N^6-threonylcarbamoyladenosine (t^{6}A) has been recognized as a universally conserved, essential modified nucleoside found in tRNAs responsible for ANN codons in all three domains of life. t^{6}A plays a crucial role in maintaining decoding accuracy during protein synthesis. The presence of t^{6}A in cellular tRNAs has been well documented for more than four decades. However, under conditions optimized for nucleoside preparation, we detected little t^{6}A in tRNAs from *Escherichia coli*. Instead, we identified a novel modified base named "cyclic t^{6}A" (ct^{6}A) (ref1), which is a cyclized active ester with an oxazolone ring. ct^{6}A is widely distributed in many bacteria, fungi, protists and plants. ct^{6}A has a supportive role in promoting decoding efficiency of tRNA^{Lys}. Structural modeling indicated that ct^{6}A recognizes the first adenine base of ANN codon at the ribosomal A-site.

We also identified an E1-like enzyme named tRNA threonylcarbamoyladenosine dehydratase A (TcdA) which catalyzes ATP-dependent dehydration of t^{6}A to form ct^{6}A. Detailed catalytic mechanism of t^{6}A dehydration has been investigated by structural and biochemical approaches. In addition, we identified a cysteine desulfurase (CsdA) and a sulfur carrier protein (CsdE) to be required for efficient ct^{6}A formation, indicating that sulfur relay system is involved in this reaction.

(ref1) Miyauchi et al., *Nat. Chem. Biol*., 9, 105-111 (2013)

24 Unusual non-canonical editing important for tRNA processing in Trypanosomes as revealed by shallow sequencing.

Mary Anne T. Rubio[1], Christopher R. Trotta[2], Juan D. Alfonzo[1]

[1]Department of Microbiology; Center for RNA Biology; The Ohio State University; [2]PTC Therapeutics

In eukaryotes, precursor tRNAs contain introns whose cleavage is mediated by an evolutionarily conserved endonuclease complex that generates fully functional mature tRNAs. The *Trypanosoma brucei* genome encodes a single intron-containing tRNA (tRNATyr), responsible for decoding all tyrosine codons; therefore, intron removal is essential for protein synthesis and consequently cell viability. In this organism, little is known about the mechanism of intron processing, but owing to its early evolutionary divergence from other eukaryotes, *T. brucei* often reveals unexpected peculiarities. Bioinformatic analyses revealed only one homolog of the four conserved canonical subunits of the multi-protein complex endonucleases required for tRNA splicing in other eukaryotes. Here we show an unprecedented number of nucleotide differences within the intron-containing pre-tRNATyr and its genomic copy. Significantly, these differences occur at the RNA level and cannot be ascribed to canonical deamination-type editing. Intron editing is required for proper pre-tRNA processing, establishing its functional significance for production of the full complement of tRNAs needed for translation. The demonstration of a novel editing mechanism required for proper function of a highly divergent splicing endonuclease in kinetoplastids has great implications to our understanding of the evolution of tRNA processing across eukaryotes.

Concurrent 1: Splicing mechanisms

Time: Wednesday, June 12, 14:00 - 16.30
Location: Davos Ballroom
Abstracts: 25 - 33
Chair(s): Soo-chen Cheng, *Academia Sinica*

25	**Structural insights into the assembly of spliceosomal U snRNPs**
26	**Crystal structure of human spliceosomal U1 snRNP at 3.3 Å resolution.**
27	**Crystal structure of Prp5p reveals intra-molecular interactions that impact splicing fidelity**
28	**Sequential contacts of DExD/H-box protein Prp28p with Prp8p, Brr2p, and Snu114p during splicing as captured by a chemical cross-linking approach**
29	**Functional spliceosome assembly without stable U4/U6 snRNA pairing**
30	**A group II intron-like catalytic triplex in the U6 snRNA forms during spliceosome activation**
31	**3D Cryo-EM structure of the yeast activated spliceosome (Bact) and localisation of functionally important regions**
32	**A conformational switch in PRP8 mediates metal ion coordination that promotes pre-mRNA exon ligation**
33	**Versatile reaction catalyzed by the Spliceosome in a competitive manner**

25 Structural insights into the assembly of spliceosomal U snRNPs

Clemens Grimm[1], *Jann Pelz*[1], *Utz Fischer*[1]

[1]**University of Wuerzburg**

Splicing is the process in which non-coding introns are excised from the primary transcript (pre-mRNA) and the coding exons are joined. Splicing is catalyzed by the spliceosome, which consists of the four small nuclear ribonucleoproteins (snRNPs) U1, U2, U4/6 and U5 as well as a large number of other proteins. Assembly of snRNPs from RNA and protein is an essential pre-requisite for splicesome formation. *In vivo*, this is facilitated in a highly complex biogenesis pathway. Initially, the snRNA is transported from its site of transcription to the cytoplasm. Within this compartment, the common (Sm) proteins as well as some proteins specific for the respective U snRNP associate with the snRNA. Finally, the mature particle is transported to the nucleus and eventually incorporated into the spliceosome. Our group has identified a unique machinery that assists the assembly of spliceosomal U snRNPs (Chari et al., 2008, Cell 135, 497-509). This machinery consists of two cooperating units termed SMN-complex and PRMT5-complex, respectively. While the SMN-complex is an RNP assembler and loads Sm proteins onto the snRNA, the PRMT5 complex functions upstream in this pathway. It acts as an assembly chaperone by forcing Sm proteins into a higher order structure (termed 6S complex) required for the subsequent transfer onto the SMN-complex. We will report on the atomic structure of two key intermediates of the snRNP assembly machinery. The structure of the early 6S intermediate identifies pICln as an Sm-protein mimic, which enables the topological organization of five Sm proteins in a closed ring and prevents premature RNA binding. A second structure of the 6S assembly intermediate bound to the SMN-complex components SMN and Gemin2 (see figure below) uncovers the likely mechanism of pICln elimination and Sm protein activation for snRNA binding. These structural studies along with biochemical experiments reveal how assembly factors facilitate formation of RNA-protein complexes *in vivo*.

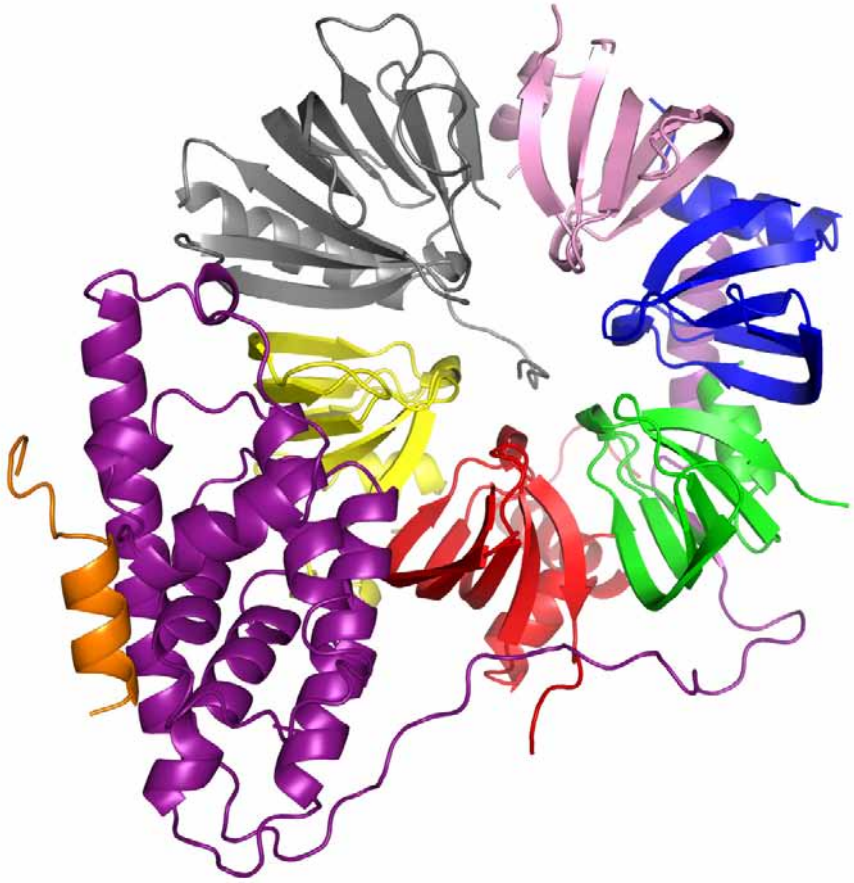

Figure 1

26 Crystal structure of human spliceosomal U1 snRNP at 3.3 Å resolution.

Yasushi Kondo[1], *Chris Oubridge*[1], *Marike van Roon*[1], *Kiyoshi Nagai*[1]

[1]MRC Laboratory of Molecular Biology

Human spliceosomal U1 snRNP consists of U1 snRNA, seven Sm proteins and three U1-specific proteins; U1-A, U1-C, and U1-70k. The binding of U1 snRNP to the 5' splice site (5'ss) within the mRNA precursor (pre-mRNA) initiates spliceosome assembly. Numerous biochemical and genetic studies showed that U1 snRNP recognizes the 5'ss of pre-mRNA through base-pairing with the 5' end of U1 snRNA and that U1-C is important for the efficiency of the binding (1). Our previously-reported 5.5 Å crystal structure revealed that the U1-C zinc finger domain locates next to U1 snRNA 5'-end: 5'ss duplex, but we could not study this interaction in detail due to the limited resolution (2). Although the sequence of the 5'-end of U1 snRNA is completely conserved from yeast to human, the 5' splice site sequence of the pre-mRNA to which it binds shows different degrees of conservation in different species. The 5'ss is stringently conserved in *Saccharomyces cerevisiae* whereas in human only the GU di-nucleotide is strictly conserved.

In order to gain further insights into the recognition of the 5'ss by U1 snRNP, using previous structures as a guide, we designed a minimal sub-structure of U1 snRNP containing all the components necessary for 5'ss recognition. We reconstituted and crystallised this sub-structure with the seven Sm proteins, U1-C, the N-terminal domain of U1-70k, and a U1 snRNA lacking stem/loop I, II and III,.

Here we report a 3.3 Å resolution crystal structure of a U1 snRNP sub-structure to which an RNA oligonucleotide containing a 5'ss sequence is bound. The crystal structure reveals how the U1-70k N-terminal peptide stabilizes the binding of U1-C protein and how U1-C in turn facilitates the binding of the 5' splice site. The structure also shows the differences between the U1 and U4 core domain structures and provides insight into the specificity of the N-terminal peptide of U1-70k for the U1, and not the U4 core domain.

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27 Crystal structure of Prp5p reveals intra-molecular interactions that impact splicing fidelity

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Prp5p, a DEAD-box ATPase, is essential for spliceosome assembly by facilitating U2 snRNP binding to the intron branch site (BS) region and proofreads splicing. However, the structural basis for the function of Prp5p remains unclear. Here, we report crystal structures of *S. cerevisiae* Prp5p alone and in complex with ADP at resolutions of 2.12 Å and 1.95 Å, respectively. The structures reveal that two RecA-like subdomains (D1 and D2) of Prp5p adopt an unusual twisted 'open state' conformation by extensive intra-molecular interactions with flanking sequences and the subdomain linker. Further in vivo mutagenesis in *S. cerevisiae* demonstrated that a number of *prp5* alleles, which destabilized the intra-molecular interactions, specifically inhibit splicing of suboptimal BS substrates. The mutant Prp5p proteins possess faster-than-WT ATPase activities in vitro. These observations suggest a mechanism in which the twisted 'open state' conformation of Prp5p must be remodeled into a 'closed state' conformation during branch site recognition in order to activate its ATPase activity and to commit the intron to the splicing pathway. Changes in the relative stabilities of the 'open' and 'closed' forms of Prp5p result in alteration of splicing fidelity.

28 Sequential contacts of DExD/H-box protein Prp28p with Prp8p, Brr2p, and Snu114p during splicing as captured by a chemical cross-linking approach

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Although DExD/H-box proteins are known to unwind RNA duplexes and/or modulate RNA structures *in vitro*, it seems plausible that, *in vivo*, some may function as RNPases to dissociate proteins from RNA or to remodel RNA-protein complexes. Precisely how the latter can be achieved remains unknown. We have been trying to approach this issue by using yeast Prp28p as a model system. Prp28p is an evolutionarily conserved DExD/H-box splicing factor that facilitates the U1/U6 switch at the 5' splice site (5'ss) during spliceosomal assembly. We have previously shown that Prp28p can be made dispensable in the presence of specific mutations that alter U1C, Prp42p, Snu71p, Cbp80p, and Ynl187p. These data suggest a model that Prp28p counteracts the stabilizing effect by those proteins on U1 snRNP/5'ss interaction. To probe how Prp28p contacts its targets in a splicing-dependent manner, we strategically placed a chemical cross-linker, benzoyl-phenylalanine (BPA), along the length of Prp28p *in vivo* using a nonsense-suppressor-mediated approach. Extracts prepared from these strains were then used for splicing at various ATP concentrations and for UV-activated cross-linking reactions. Prp28p appears to transiently interact with the spliceosome at low ATP concentration, which is known to accumulate A2-1 (or B; mammalian system) complex. Under such a condition, we observed that Prp28p cross-links with a small number of proteins and some of these cross-linked products are dependent on the presence of UV, ATP, RNA, and, importantly, functional 5'ss and branch site. Furthermore, the cross-linked products are resistant to RNase treatment. Using mass-spec and Western blotting analysis, we found that Prp28p-K136^{BPA} cross-links to Prp8p and Prp28p-K82^{BPA} contacts both Prp8p and Snu114p. Furthermore, Prp28p-K27^{BPA} and Prp28p-K41^{BPA} were cross-linked to Brr2p and Snu114p, respectively. These data are consistent with the structural study of the U5–U4/U6 tri-snRNP, which placed Snu114p next to Prp8p, which is in turn adjacent to Brr2p. ChIP analysis revealed that alanine substitutions at Prp28p-K136 region reduce the binding of Prp28p to spliceosome and delay the release of U1 snRNP from spliceosome, consistent with our genetic analysis suggesting that Prp28p works in concert with Prp8p. Finally, mixing experiments suggested that Prp28p makes contact with Prp8p and then with Brr2p. Taken together, our data suggest a working model that Prp28p approaches the complex spliceosomal milieu by making prior contact with Prp8p to effect U1 snRNP dissociation and then with Brr2p (and perhaps Snu114p), which may transmit the signal to couple U1 dissociation with the U4/U6 unwinding, a key step in spliceosomal remodeling.

29 Functional spliceosome assembly without stable U4/U6 snRNA pairing

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The cycle of spliceosome assembly, intron excision, and disassembly involves large-scale snRNA structural rearrangements. U6 snRNA toggles between a U2 snRNA-bound state in the catalytically active spliceosome and a U4 snRNA-bound state during spliceosome assembly. The function of U4/U6 pairing is not clear, but is presumed to be essential since it occurs in all eukaryotes examined. Here we report the surprising finding that stable U4/U6 pairing is dispensable for splicing in yeast. Single point mutations in U4 and U6 that disrupt their pairing are lethal in combination, but are rescued by a second mutation in U6 that extends U2/U6 Helix II and stabilizes the U2/U6 complex. The resulting triple mutant strain lacks detectable U4/U6 snRNP, and accumulates a novel U2/U6 snRNP. NMR analysis of wild-type and mutant U2/U6 RNA constructs reveals that either single mutation in U6 disrupts the wild-type three-helix junction structure, but the two mutations together restore and further stabilize the U2/U6 three-helix junction. We conclude that, in the presence of a stabilized U2/U6 snRNP, stable U4/U6 pairing is not essential for spliceosome assembly and function. Nevertheless, U4 snRNA is still required for cell viability, and a U4.U5.U6 tri-snRNP accumulates even in the absence of U4/U6 pairing. We propose that, in the triple-mutant strain, the U2 snRNP acts as a chaperone for assembly of the tri-snRNP, as has previously been suggested for the human U4/U6 snRNP.

30 A group II intron-like catalytic triplex in the U6 snRNA forms during spliceosome activation

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Catalysis of pre-mRNA splicing and group II intronic RNA self-splicing has been proposed to occur by a two-metal ion mechanism. The crystal structure of a group II self-splicing intron shows the catalytic domain V in a configuration where five non-bridging oxygens of the phosphate backbone coordinate two metal ions. We have recently provided evidence that the corresponding five residues in the spliceosomal U6 snRNA are bona fide metal ion ligands and one of these ligands - the bulged U80 in the U6 ISL - plays a direct catalytic role by binding metal ions that stabilize the leaving groups during both steps of splicing. In the group II intron, the metal binding configuration of domain V depends on formation of three RNA base-triple interactions that function during both steps of splicing, and it has been suggested that similar interactions may occur between within the U6 snRNA between the ACAGAGA, the AGC triad, and U80. Consistent with this prediction we found that mutations in the AGC triad are suppressed in vivo by mutations that restore the base-triple interactions. Furthermore, we show by in vitro molecular genetics that the U6 base triples function at the catalytic stage and are required for efficient exon ligation.

Unexpectedly, by site-directed cross-linking, we found that U6 base triples form before Prp2-dependent catalytic activation of the spliceosome and require the NTC complex, suggesting that the catalytic core forms already at the pre-catalytic stage. Preliminary evidence also suggests that Cwc2 promotes formation of the base triples, supporting previous proposals that Cwc2 may stabilize formation of catalytic structures in U6 by substituting in the spliceosome for RNA structures that in the group II intron induce the catalytic configuration of domain V.

Our findings indicate that the U6 snRNA adopts a group II intron-like tertiary conformation to catalyze pre-mRNA splicing, providing further evidence that the spliceosome and group II introns share common catalytic mechanisms and evolutionary origins.

31 3D Cryo-EM structure of the yeast activated spliceosome (Bact) and localisation of functionally important regions

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The spliceosome, which catalyses the removal of the intron from nuclear pre-mRNAs, assembles initially into a pre-catalytic ensemble, termed complex B, which contains the snRNPs U1, U2 and the U4/U6.U5 tri-snRNP and numerous non-snRNP proteins. For catalytic activation the spliceosome undergoes a major structural rearrangement; this yields the B^{act} complex and entails the dissociation of U1 and U4 RNPs and the formation of a new RNA–RNA interaction network involving U6, U2 and the pre-mRNA, which lies at the heart of the emerging catalytic centre. At the same time the Prp19 complex proteins (NTC) and ca. 10 NTC-related proteins are stably integrated into the B^{act} complex.

We have used single-particle electron cryomicroscopy to reconstruct the 3D structure of purified yeast B^{act} complexes at a resolution of 20–25 Å. Consistently with the dramatic change in biochemical composition that accompanies its formation, the structure of the B^{act} complex differs greatly from that of the pre-catalytic B complex. The reconstituted 3D structure of the B^{act} complex from *S. cerevisiae* has a mushroom-like appearance. The "mushroom cap" (the main body) consists of a relatively compact, prolate body about 37 nm long and 28 nm wide. From it, the "mushroom stalk" is seen to emerge as a slightly tapering, 15-nm-long protuberance. At the two ends of the main body large clefts and tunnels are seen, while its centre is clearly more solid. The protuberance is roughly perpendicular to the ellipsoid's main axis at its base, but is slightly curved.

We have begun to map the position of selected proteins such as Cwc2, Prp2, Prp19, Brr2 and Prp8 and some U2 proteins. These data provide initial insights into the location of the emerging catalytic centre and other functionally important sites in the B^{act} complex.

Finally, we have located certain U5- and U4/U6-specific proteins at the surface of purified pre-catalytic B complexes, which – in combination with our earlier electron-microscopic localisation studies of proteins in the purified U4/U6.U5 tri-snRNP (NSMB 2008; 15: 1206) – allows us to fit the tri-snRNP structure into the B complex at an unambiguously defined position.

32 A conformational switch in PRP8 mediates metal ion coordination that promotes pre-mRNA exon ligation

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The spliceosome is a large and complex RNA-protein machine that removes introns during maturation of pre-mRNAs by a mechanism involving two sequential phosphotransfer reactions. A wealth of evidence shows that the spliceosome is a dynamic structure undergoing a series of conformational changes as it assembles on a pre-mRNA substrate, catalyzes two the two splicing reactions, and disassembles/recycles for subsequent rounds of catalysis.

The catalytic center of the splicing machinery includes an RNA structure comprised of the U2 and U6 spliceosomal snRNAs with divalent metal binding by U6 snRNA implicated in catalysis of both steps of splicing. A wealth of genetic and biochemical evidence places the protein PRP8 at the heart of the splicing machinery during spliceosome assembly through to catalysis, and suggests that it undergoes a conformational change between the two steps of splicing.

We have obtained structural and functional evidence that the RNase H domain of PRP8 undergoes a conformational change between the two transesterification steps of splicing rationalizing yeast *prp8* alleles that promote either the first or second step. We have further discovered that this conformational switch unmasks a metal-binding site involved in the second transesterification reaction (see Figure 1 below). Together these data establish that PRP8 is a metalloprotein that promotes exon ligation within the spliceosome.

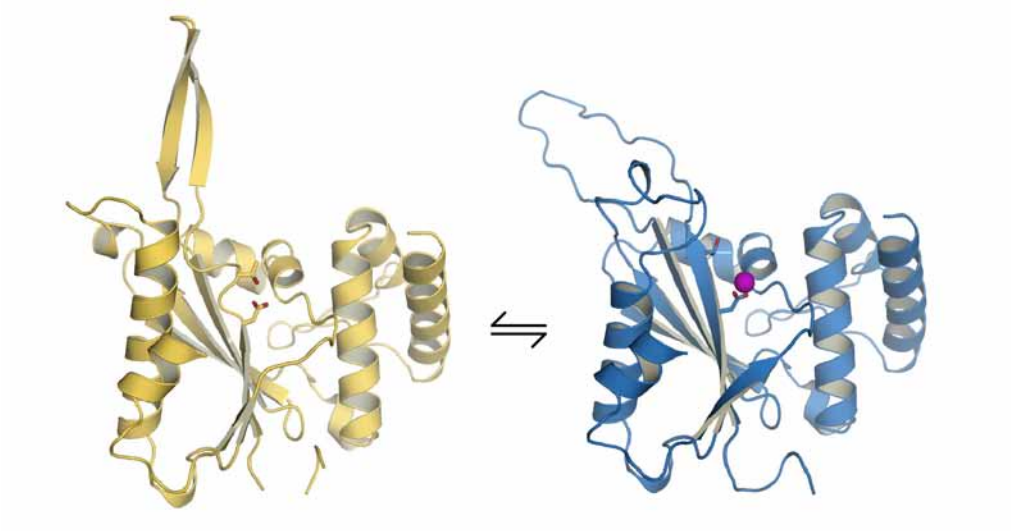

Figure 1

33 Versatile reaction catalyzed by the Spliceosome in a competitive manner

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The spliceosome is a large and dynamic ribonucleoprotein complex, which undergoes components exchange and structural rearrangements throughout the entire splicing pathway. During catalytic steps, Prp16 is required to facilitate conformational change from step one to step two by destabilization of Yju2 and Cwc25, which are required for the first catalytic reaction. Despite of the energy requirement of such structural changes for the progression of the reaction, both steps of the splicing reaction can reverse on the purified spliceosome under appropriate conditions without having to replace splicing factors. In addition, the spliceosome can catalyze hydrolytic spliced-exon reopening (SER) reaction when arrested in step-two conformation, and debranching of lariat-intron-exon 2 (DBR) when arrested in step-one conformation. The SER reaction is in competition with the reverse reaction of step-two splicing (R2), whereas DBR is in competition with the reverse reaction of step-one splicing (R1). Recently we have found that changes in the N-terminal structure of Yju2 and Cwc25 biased the reaction toward R1 and strongly inhibited DBR. Cwc25 with epitope tagged at the N-terminus was destabilized from the spliceosome after the first catalytic reaction. Such spliceosomes could promote high efficiency of R1 and also promote the second reaction (F2) at a low level. Adding back Cwc25 inhibits the F2 reaction. These results indicate that the spliceosome can bypass the requirement of Prp16 and other step-two factors to promote the second reaction under the condition of Prp16-independent Cwc25 destabilization. The F2 reaction that occurs in the absence of step-two factors requires KCl and is inhibited by the presence of Cwc25, which instead promotes the debranching reaction. Our studies provide novel mechanistic insights into the molecular mechanism of the splicing reaction.

Concurrent 2: RNA localization

Time: Wednesday, June 12, 14:00 - 16.30
Location: Aspen
Abstracts: 34 - 42
Chair(s): Ralf Jansen, *Univ. Tübingen*

34	**An unexpected role of the nuclear periphery for mRNA export in yeast**
35	**NMD3 regulates mRNA nuclear export via an Xpo1-linked mechanism**
36	**RNA recognition and architectural activity of Zipcode Binding Protein 14**
37	**Identification and analysis of Staufen2 target RNAs from rat brain**
38	**The order of assembly and disassembly of nuclear ASH1-mRNPs**
39	**Novel players and novel mRNAs transported by the Bic-D / Egl / Dynein RNA localization machinery**
40	**An RNA biosensor for imaging translation of single mRNAs in living cells.**
41	**Single molecule systems biology of RNA silencing**
42	**GRSF1 regulates RNA processing in mitochondrial RNA granules**

34 An unexpected role of the nuclear periphery for mRNA export in yeast

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The export of mRNAs from the nucleus to the cytoplasm is one of the many steps along the gene expression pathway and reflects only a short time period within the lifetime of an mRNA. However, mRNA export cannot be seen as an isolated process, as it has been functionally linked to different upstream and downstream processes, in particular the localization of the gene within the nucleus, transcription, mRNA processing and quality control. In the yeast *S cerevisiae*, many active genes are tethered to the nuclear periphery through interactions with the nuclear pore complex; the role of this perinuclear localization however is not fully understood. As many genes showing perinuclear localization are regulated by stress, NPC anchoring has been hypothesized to ensure fast and efficient export of newly synthetized RNAs under these conditions.

The NPC itself has to fulfill a dual function in mRNA transport, as it not only has to allow for fast export of mRNAs, but also ensure that only mature mRNPs are exported to the cytoplasm. Indeed, quality control steps have been suggested to occur at the NPC, such as the retention of unspliced pre-mRNAs. To better understand how the interconnection of gene localization, RNA processing and quality control affects the efficient export of mRNAs, we use single molecule resolution real-time microscopy to follow individual mRNAs from their site of transcription on their path towards to cytoplasm. We show that, different to current models, gene tethering to the periphery does not lead to the export of mRNAs through an adjacent nuclear pore complexes, as mRNAs are released into the nucleoplasm where they reach the nuclear periphery by diffusion within less then a second. Once reaching the nuclear periphery, they are not immediately exported but start scanning along the nuclear periphery, likely to find a nuclear pore that allows export. Interestingly, deleting specific components of the nuclear basket significantly reduces the time mRNPs scan the nuclear periphery and result in frequent release of mRNPs back into the nucleoplasm. This suggests that the nuclear basket not only acts as a landing platform for mRNPs at the nuclear pore but also has the capacity to maintain mRNAs at the periphery, possibly to allow sufficient time for quality control steps to occur without releasing the mRNPs back into the nucleoplasm.

35 NMD3 regulates mRNA nuclear export via an XpoI-linked mechanism

Pegine Walrad[1], Melanie Bühlmann[2], Pegine Walrad[1], Paul Capewell[2], Arunasalam Naguleswaran[4], Isabel Roditi[4], Elisabetta Ullu[3], Keith R. Matthews[2]

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The RBP NMD3 (Upf3) has been implicated in mRNA Nonsense Mediated Decay and rRNA processing and export. Interestingly, we have also discovered that NMD3 RNAi generates a strong upregulation of a specific subset of transcripts. RNAseq transcriptome analysis of NMD3-depleted cells show that this effect is highly specific whereas reporter assays demonstrate that this effect is *UTR*-dependent and restricted to the mRNA level (no protein elevation occurs). ChIP analysis confirmed the transcript upregulation is not mediated by enhanced locus-specific transcription, nor is it a consequence of translational inhibition resulting from the effects of NMD3 depletion on rRNA maturation. Importantly, depletion of the 7SL RNA nuclear export factor, XPO1, recapitulates the effects of NMD3 depletion on specific mRNAs. Moreover, *in situ* hybridisation analyses demonstrate a bulk nuclear accumulation of all mRNAs in response to NMD3 depletion. This invokes a novel mRNA regulatory mechanism involving the NMD3- and XPO1-dependent nuclear export of mRNA cargos, with specific mRNA accumulation resulting from nuclear sequestration and hence protection from rapid cytoplasmic turnover. This novel function for NMD3 may be conserved in other systems.

36 RNA recognition and architectural activity of Zipcode Binding Protein 1

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IGF2 mRNA binding protein 1 (IMP1)/Zipcode binding protein 1 (ZBP1) is an oncofetal protein expressed at very low level in most adult tissues. ZBP1 is important for cell motility and its expression in cancer cells has been linked to tumor dissemination. ZBP1 is an RNA-binding protein that mediates the transport of a set of mRNAs from the nucleus to the cell periphery. It contains two RRM and 4 KH domains organised in three di-domain units. An important and well studied target of ZBP1 is the β-actin mRNA, and it has been shown that ZBP1 binds to β-actin mRNA in the nucleus and mediates its transport to the cell edge in a translationally repressed state. Here ZBP1 is phosphorylated by Src and the mRNA is released and translated. ZBP1-mediated transport takes place in large protein-RNA particles and the structure of the KH3 and KH4 domains, the key domains for the recognition of the β-actin RNA, suggest that ZBP1 may play an architectural role. We have solved the high resolution structure of ZBP1 KH3-KH4 in complex with the β-actin RNA target. We discuss a novel mode of KH-RNA binding and a mechanistic model for ZBP1 recruitment and ZBP1 architectural activity.

37 Identification and analysis of Staufen2 target RNAs from rat brain

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In neurons, RNA-binding proteins (RBPs) play an important role in directing RNA translation to subregions of the cell, including synapses. This process is known to critically contribute to synaptic plasticity. The double-stranded RBP, Staufen2 (Stau2), has been implicated in cell fate determination as well as in dendritic RNA localization and synaptic plasticity in mammalian neurons.

In order to better elucidate the role of Stau2 in neurons and its contribution to RNA localization, we have identified Stau2 target RNAs in the rat brain by immunoprecipitation of endogenous soluble Stau2 particles. Many of the Stau2 target RNAs encode functionally related proteins involved in G-protein-coupled receptor (GPCR) and small GTPase-mediated signaling pathways. Additionally, 77% of the Stau2 targets that are expressed in the hippocampus overlap with a new dataset of neuronal process-localized mRNAs (Cajigas *et al.*, Neuron, 2012) indicating most Stau2 RNPs can localize away from the cell body. Interestingly, the activity of extracellular signal-regulated kinases-1 and -2 (ERK1/2) is reduced upon Stau2 knockdown, suggesting Stau2 affects intracellular signaling pathways in the brain. Furthermore, we present evidence that Stau2 stabilizes the expression of one of the GPCR pathway components, the Regulator of G-protein Signaling 4 (*Rgs4*) mRNA. This effect is mediated via the *Rgs4* 3'-UTR since downregulation of Stau2 in neurons leads to reduced expression of a luciferase reporter. We hypothesize that the regulation of ERK1/2 and Rgs4 may underlie the involvement of Stau2 in dendritic spine morphogenesis and synaptic plasticity. Taken together, our data provide new insight into the role of Stau2 in neurons.

38 The order of assembly and disassembly of nuclear ASH1-mRNPs

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mRNA localization is a universal feature in eukaryotes, requiring the assembly of motor-dependent transport particles. The cytoplasmic transport of mRNAs is preceded by the nuclear assembly of pre-mRNPs. A transit of such nuclear pre-mRNPs through the nucleolus has been reported. One example is the *ASH1* mRNA, where the nucleolar protein Loc1p is required for efficient cytoplasmic *ASH1* mRNA localization, most likely by remodeling the nuclear mRNP. Although immunoprecipitation experiments already revealed that Loc1p forms co-complexes with other nuclear components of the transport complex, it remains unclear how Loc1p is recruited into this mRNP. Because genomic deletion of *LOC1* also results in impaired ribosome biogenesis, it has been suggested that impaired *ASH1* mRNA localization might be only an indirect effect.

Here we show that Loc1p interacts directly and specifically with the *ASH1* mRNA-binding protein She2p, indicating a direct role of Loc1p in mRNA localization. Our data suggest that the Loc1p interaction with She2p and RNA results in the formation of a stable ternary complex, in which both proteins directly contact the RNA. A second nucleolar factor co-immunoprecipitated with She2p and *ASH1* mRNA is Puf6p. We do not find a direct interaction of Puf6p with Loc1p and She2p, suggesting that these factors do not form a joint protein complex. After nuclear export, myosin-bound She3p joins the complex of ASH1 mRNA, She2p, and Puf6p. Since Loc1p remains in the nucleus, it must be removed from the complex prior or during export. Our competition experiments show that She3p is able to displace Loc1p from the *ASH1* complex. Together these findings imply an ordered process of nuclear assembly and disassembly, and suggest that Loc1p and She3p encounter each other in the vicinity of the nuclear pore. Furthermore our data indicate that the mutual recruitment of Loc1p and She2p to the *ASH1* mRNA enables She2p to escort the *ASH1* mRNA through the nucle(ol)us. It confirms an integral role of Loc1p in *ASH1* mRNP biogenesis.

39 Novel players and novel mRNAs transported by the Bic-D / Egl / Dynein RNA localization machinery

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Many key cellular processes in eukaryotes depend on mRNA localization. In *Drosophila*, the mRNA transport machinery consisting of Bic-D, Egl and the dynein motor is used at many different developmental stages and in many cell types to localize key mRNAs required for oocyte differentiation, embryonic axis formation and patterning. Through the dynein / dynactin motor the mRNA cargo is moved on microtubules to distinct cellular compartments. While Egl links these motors and Bic-D with mRNA cargo, it is unclear, which additional proteins are required to confer mRNA target specificity and translational control during the transport. Using immunopurification of Bic-D and Egl complexes we have identified several novel components of the Bic-D / Egl transport machinery. Amongst them are the poly(A) binding protein (Pabp) and the insulin growth factor mRNA binding protein (Imp). Through a polyA independent activity, Pabp localizes *osk* mRNA in the female germ line, while *Drosophila* Imp is found to co-localize with the Bic-D/*hairy* mRNA–RNP complexes in embryos. Despite this, our functional analysis revealed that *imp* plays at most a redundant role in localizing this mRNA. Instead, it seems to function in repressing translation of *hairy* mRNA during transport. Two additional proteins were shown to interact with Bic-D and novel findings regarding them will be presented at the meeting. Encouraged by the success of the isolation of protein partners, we also screened for novel mRNAs present in complexes with Bic-D and Egl. We immunoprecipitated these proteins, purified the bound mRNAs and quantified the transcripts using high-throughput cDNA sequencing. The *in situ* localization patterns of the IP-enriched mRNAs, the presence of the known targets of the Bic-D localization machinery in these complexes and the dependence of their localization patterns on *Bic-D* revealed a rewarding success rate for this approach. Such targets and their analyses will be presented in Davos, too.

40 An RNA biosensor for imaging translation of single mRNAs in living cells.

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After transcription, an mRNA's fate is determined by an orchestrated series of events that is regulated both temporally and spatially within the cell. The regulation of translation is crucial for control of gene expression. While measurements of ribosome occupancy of mRNAs have provided a genome-wide view of translation, the cellular context has been lost. Fluorescent microscopy can complement these global approaches because it allows interrogation of gene expression with single-molecule resolution in intact cells. We have developed an RNA biosensor that allows individual untranslated mRNAs to be distinguished from mRNAs that have already been translated in living cells.

Most approaches for imaging translation have relied on either detecting the newly synthesized polypeptide or identifying actively translating ribosomes within the cell, which are inherently limited by low signal-to-noise. We have chosen to consider translation from the perspective of the mRNA because imaging single-molecules of RNA has been well established. We have utilized the orthogonal MS2 and PP7 RNA-protein complexes to label an mRNA in the coding sequence (PP7-GFP) and 3' UTR (MS2-RFP) with spectrally distinct fluorescent proteins. When this mRNA is untranslated it will be labeled with both fluorescent proteins, however, once the mRNA encounters the ribosome for the first time, the PP7-GFP signal will be displaced from the transcript resulting in only MS2-RFP labeled mRNAs. This simple strategy allows untranslated mRNAs (yellow) to be readily distinguished from translated ones (red). We refer to this technique as translating mRNA imaging by coat protein knock-off (TRICK).

The TRICK assay has been used to characterize the translation of a reporter mRNA in U2 O-S cells. We find no evidence for translation of the reporter mRNA in the nucleus. In the cytoplasm, however, almost every mRNA has been translated at least once. By combining fixed and live cell measurements, our data suggests that the average mRNA takes approximately two minutes once exported to the cytoplasm to be bound, scanned and have translation initiated by the ribosome.

41 Single molecule systems biology of RNA silencing

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Imagine we could use eukaryotic cells as reaction vessels in which we directly "see" physiologically low numbers of RNA molecules that individually go about their biological functions within their natural "habitat", under the constant influence of molecular crowding amongst all known and unknown interaction partners. This arguably ultimate goal of a quantitative biology for the 21st century has only just come within reach, opening entirely new avenues towards understanding cellular life and disease – after all, "seeing is believing". To realize this vision, we recently developed iSHiRLoC, or intracellular Single molecule, High-Resolution Localization and Counting, as an innovative probe concept optimized for detecting single, biologically functional RNA molecules inside live human cells. For iSHiRLoC, we microinject an RNA of interest, inconspicuously labeled with a single fluorophore, into cultured human cells. Microinjection allows us to keep the number of molecules low so as not to overwhelm the cellular RNA processing machinery, while enabling intracellular single RNA molecule detection during low-background highly inclined laminar optical sheet (HILO) microscopy. At defined time points after microinjection, we then perform one of two experiments – single particle tracking in the live cell or, upon formaldehyde fixation, counting of the number of photobleaching steps per particle as a fingerprint of RNA assembly – giving us the unique opportunity to directly watch and quantify the dynamics of intracellular RNA pathways. In its first successful implementation, we applied iSHiRLoC to gene regulation by microRNAs (miRNAs)[1], key effectors of the evolutionarily conserved RNA silencing pathway, with profound significance for human health as a master regulator of gene expression. We showed that microinjected, 3' fluorophore labeled microRNAs retain full functionality in RNA silencing and display two kinetically distinct assembly pathways that we propose are sequential translational inhibition and degradation of the targeted messenger RNAs (mRNAs). We have recently extended iSHiRLoC to two colors to co-track and count pairwise combinations of miRNAs and their target mRNA and protein interaction partners. To further interpret these data, we are performing systems biology modeling through Monte-Carlo simulations in the molecular environment of the cell, using our experimental iSHiRLoC data as input to predict the cellular output. To deeply integrate our experimental and computational approaches into the envisioned Single Molecule Systems Biology, we experimentally measure the extent of gene repression by miRNAs, then compare the computational prediction with experimental reality iteratively to derive a fully converged mechanistic description of RNA silencing.

(1) Pitchiaya, S.; Androsavich, J. R.; Walter, N. G. *EMBO rep* **2012**, *13*, 709-715.

42 GRSF1 regulates RNA processing in mitochondrial RNA granules

Alexis A. Jourdain[1], *Jean-Claude Martinou*[1]

[1]University of Geneva

RNA molecules sometimes assemble into macromolecular structures called RNA granules. These large ribonucleoparticles, known as "stress granules", "P-bodies" or "neuronal RNA granules" have many RNA-related functions such RNA storage, protection, degradation or transport.

We will describe the existence of RNA granules within mitochondria[1]. These mitochondrial RNA granules contain nascent RNA molecules together with specifically associated proteins including the G-rich RNA sequence binding factor 1 (GRSF1), the Fas-Activated Serine/Threonine kinase (FASTK), RNAse P, and the helicase hSuv3p. We found that GRSF1 is required for the processing of long polycistronic mitochondrial transcripts into mature mRNAs, tRNAs and rRNAs, which is an essential aspect of mitochondrial gene expression that remains poorly understood. Impairment of RNA processing delays the release of newly synthesized RNA from mitochondrial RNA granules, indicating that these structures may correspond to factories where primary RNA transcripts converge and are processed before release.

Based on these results, we hypothesize that mitochondrial RNA granules are involved in several aspects of post-transcriptional regulation of mitochondrial gene expression and that dysfunction of key components of these RNA granules may lead to severe pathologies.

1. *Alexis A. Jourdain, Mirko Koppen, Mateusz Wydro, Chris D. Rodley, Robert N. Lightowlers, Zofia M. Chrzanowska-Lightowlers, Jean-Claude Martinou. GRSF1 regulates RNA processing in mitochondrial RNA granules. Cell Metabolism. In press.*

Concurrent 3: Viral RNAs

Time: Wednesday, June 12, 14:00 - 16.30
Location: Sanada
Abstracts: 43 - 51
Chair(s): Karen Beemon, Johns Hopkins University

43	**Virus-Induced Dysregulation of Cellular mRNA Decay and Alternative Polyadenylaton – Implications for Pathogenesis**
44	**A dengue virus 2 non-coding RNA downregulates translation of antiviral interferon-stimulated mRNAs through interaction with host RNA binding proteins.**
45	**RNA/RNA interactions govern selective packaging of influenza A genomic segments**
46	**Interplay between retroviral genomic RNA packaging and mRNA translation**
47	**HIV1, Antisense RNA and ADAR editing**
48	**The Identification of a novel posttranscriptional regulatory element in gamma retroviruses**
49	**Shunting revisited.**
50	**Inquiry into the variability of HCV IRES and its impact on function by developing and evaluation of a large-scale mutation database that also unfolds potential of some new nucleotides.**
51	**Novel Insights from Structural Analysis of Lentiviral and Gammaretroviral Reverse Transcriptases in Complex with RNA/DNA Hybrids.**

43 Virus-Induced Dysregulation of Cellular mRNA Decay and Alternative Polyadenylaton – Implications for Pathogenesis

Stephanie L. Moon[1], Michael D. Barnhart[1], Liang Liu[2], Carol J. Wilusz[1], Bin Tian[2], Jeffrey Wilusz[1]

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RNA viruses must successfully interface with the cellular RNA decay machinery in order to maintain the stability of their transcripts and promote a productive infection. We have determined that alphaviruses interact with the cellular HuR protein through high affinity binding sites in their 3' UTR to stabilize viral transcripts. Furthermore, alphaviruses cause a redistribution of the cellular HuR protein from the nucleus to the cytoplasm during infection. Transfection studies have revealed that this is due to the abundant cytoplasmic viral transcripts acting as molecular sponges for the protein. This decrease of HuR protein in the nucleus causes alterations in patterns of alternative polyadenylation in the cell. To our knowledge this is the first time that a cytoplasmic RNA virus has been shown to influence patterns of nuclear poly(A) site choice. The sequestration of HuR protein by alphaviruses also dramatically reduces the stability of cellular mRNAs that normally depend on the protein for regulated stability or to prevent miRNA targeting. Many of these mRNAs encode mediators of innate immune functions, cytokines or apoptosis regulators that can directly impact on the outcome of a virus infection. A similar story is also evolving from our flavivirus studies. All arthropod-borne flaviviruses produce a small non-coding sfRNA from their 3' UTR as a decay intermediate from Xrn1 exoribonuclease digestion. Xrn1 remains associated with sfRNA and becomes functionally repressed during infection. This effectively shuts down a major pathway of cellular mRNA decay during flavivirus infection, resulting in stability of viral transcripts and significant dysregulation of the stability of cellular mRNAs. Thus we believe that disruption of regulated mRNA stability during infections with either alphaviruses or flaviviruses likely plays an important and underappreciated role in cytopathology and viral pathogenesis.

44 A dengue virus 2 non-coding RNA downregulates translation of antiviral interferon-stimulated mRNAs through interaction with host RNA binding proteins.

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Flaviviruses, as other viruses, has evolved powerful strategies to counteract the antiviral action of type I interferon (IFN). In particular, it was recently shown that a short, abundant, highly structured non-coding RNA termed sfRNA was required for evasion of the IFN response. Although the mechanism of action of the sfRNA remains to be elucidated, several non-coding RNAs of viral origin have been proposed to act as sponges for host RNA-binding proteins (RBPs) to modulate host cell function. To gain insight in the role of sfRNA we used dengue virus 2 (DENV-2) sfRNA, whose interactome was recently characterized in our laboratory, as a model. We found that DENV-2 sfRNA specifically downregulated translation of IFN-stimulated genes (ISGs) mRNAs, interfering with the establishment of the antiviral state. DENV-2 sfRNA was able to interact with host RBPs G3BP1, G3BP2 and CAPRIN1, which we showed were required for antiviral action of IFNs. Strikingly, this activity was independent from their previously described role in cellular stress response but they were required for translation of ISG mRNAs. Finally, we established that the ability of DENV-2 sfRNA to decrease ISG induction was conditional on G3BP1, G3BP2 and CAPRIN1 binding, suggesting that the sfRNA sequesters the host RBPs to counteract their activity. Taken together, this study identifies G3BP1, G3BP2 and CAPRIN1 as novel cellular regulators of the IFN response, highlight translation as a previously underappreciated, important layer of regulation of immune responses and provide the first mechanism of action for DENV-2 sfRNA.

45 RNA/RNA interactions govern selective packaging of influenza A genomic segments

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The genome of influenza A viruses (IAV) is split into eight viral RNAs (vRNAs) that are encapsidated as viral ribonucleoproteins (vRNPs). The existence of a segment-specific packaging mechanism is well established, but the molecular basis of this mechanism remains to be deciphered. Selective packaging could be mediated by direct interactions between the vRNA packaging regions, but such interactions have never been demonstrated in virions. We showed that the eight vRNAs of a human H3N2 IAV (MO) form a single interaction network *in vitro* that involves regions of the vRNAs known to contain packaging signals in the most studied human H1N1 IAV strains. When we applied the same strategy to an avian H5N2 IAV (EN), we observed that the eight EN vRNAs of ENG also form a single network of interactions *in vitro*, but the interactions and the regions of the vRNAs they involve differed compared to the MO virus. Electron tomography also revealed significant differences in the interactions between vRNPs in ENG and MO viral particles. All together, these data suggest that the packaging signals are not conserved between IAVs. We identified at the nucleotide level the sequences involved in several interactions, and in a few cases, we confirmed the interactions using silent compensatory mutations in the interacting sequences. Then, we focused on the interaction between the PB1 and NS EN segments, which is inhibited *in vitro* by silent mutations in either vRNA, and restored when combining the two mutated segments. Using reverse genetics, we produced the wild type, the two single mutants, and the double mutant viruses. Quantification of the vRNAs by Q-RT-PCR and observation of the viral particles by electron microscopy indicated that this interaction also exists in infected cells and/or in viral particles and revealed a global defect in vRNA packaging in single mutant viruses. In addition, competition experiments between wild type and mutant vRNAs for packaging supported a role of this interaction in the selective packaging of these genomic RNA segments. To our knowledge, these results constitute the first direct evidence of an interaction between two vRNAs involved in the selective packaging of IAV vRNPs.

46 Interplay between retroviral genomic RNA packaging and mRNA translation

Katarzyna J. Purzycka[1], Mastooreh Chamanian[4], Katarzyna J. Purzycka[1], Paul Wille[3], Janice S. Ha[3], David McDonald[4], Yong Gao[2], Stuart F.J. Le Grice[5], Eric J. Arts[2], Ryszard W. Adamiak[1]

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During retroviral RNA encapsidation, two full-length genomic (g) RNAs are selectively incorporated into assembling virions. Packaging involves a cis-acting packaging element (?) within the 5' untranslated region of unspliced HIV-1 RNA genome. However, the mechanism(s) that selects and limits gRNAs for packaging remains uncertain. Using a dual complementation system involving bipartite HIV-1 gRNA, we observed that gRNA packaging is additionally dependent on a cis-acting RNA element, the genomic RNA packaging enhancer (GRPE), found within the gag p1-p6 domain and overlapping the Gag-Pol ribosomal frameshift signal (1). Deleting or disrupting the structure of the two conserved GRPE stem loops diminished gRNA packaging and infectivity >50-fold, while deleting gag sequences between ? and GRPE had no effect. Our studies allowed us to conclude that only the HIV-1 RNAs employed for Gag-Pol translation may be specifically selected for encapsidation, possibly explaining the limitation of two gRNAs per virion. The basis for this mechanism will be presented.

1. M. Chamanian, K. J. Purzycka, P. T. Wille, J. S. Ha, D. McDonald, Y. Gao, S. F.J. Le Grice, E. J. Arts (2013) A cis-Acting Element in Retroviral Genomic RNA Links Gag-Pol Ribosomal Frameshifting to Selective Viral RNA Encapsidation, Cell Host and Microbe, **13**, 181-192.

47 HIV1, Antisense RNA and ADAR editing

Siripong Tongjai[1], Keanan McGonigle[1], Yeou-Cherng Bor[1], David Rekosh[1], Marie-Louise Hammarskjöld[1]

[1]University of Virginia

The HIV1 genome consists of a 9kB "single stranded" mRNA. Structure analysis using SHAPE and other methods have shown a complex HIV1 genome secondary structure with several regions that are potential targets for ADAR editing. It has previously been reported that HIV1 can be edited by ADAR1, as well as by ADAR2. Several recent studies have also demonstrated the presence of natural antisense HIV1 RNA. We have studied HIV1 inhibition mediated by a therapeutic 937nt long antisense (AS) HIV1 RNA as a model system for the potential effects of HIV1 AS RNA . We have previously shown that the surprisingly potent inhibition that is observed with this therapeutic AS RNA, requires that it traffics on the CRM1 export pathway through Rev and the HIV1-RRE. If the RNA is redirected to the NXF1 pathway (using RevM10Tap), efficient AS inhibition is abolished. To further investigate the mechanism, we sequenced the AS target region in individual RT-PCR clones from cells expressing HIV1 +/- RRE-AS RNA. When the RRE-AS RNA was present, we observed several A to G changes, consistent with ADAR editing. However, in most clones, only a few residues were altered, with only 1-8 percent of the A residues modified, rather than the 50% that would be expected in a long perfect sense/antisense RNA region. The changes were present throughout the AS target region, with different A residues changed in the different clones. These results suggest that the RNA had been subject to regulated ADAR editing. In the absence of AS RNA, or when the AS RNA was redirected to the NXF1 pathway, most clones contained zero or one modified A, demonstrating that the regulation was specific for the Rev/RRE pathway.

When ADAR2 was over-expressed, AS inhibition was significantly relieved and even less inhibition was seen with a cytoplasmic mutant of ADAR2 (with aa 1-72 deleted). RT-PCR analysis of the AS target region did not show any increase in editing when ADAR2 was expressed. However, catalytic and ds-RNA binding domain mutants of ADAR2 failed to relieve inhibition, indicating a role for editing. These results suggest a complex role for ADAR in the regulation of AS inhibition at the cytoplasmic level. Interestingly, expression of ADAR2 led to significant editing in other regions of the HIV1 genome with or without the therapeutic AS-RNA (in TAR and the RRE). Taken together, our results indicate that HIV1 is regulated by ADAR at many levels. Editing of specific residues in TAR and the RRE is likely to be involved in post-transcriptional RNA regulation of HIV1, whereas expression of natural HIV1 AS RNA could serve to regulate HIV1 at the translational level, potentially through miRNAs generated from ADAR edited AS RNA. Most intriguingly, natural AS RNA expression and regulated ADAR editing may also be a strategy used by the virus to increase the rate of HIV1 evolution to help the virus evade the immune system (in conjunction with editing by ApoBec3G regulated by the viral Vif protein).

48 The Identification of a novel posttranscriptional regulatory element in gamma retroviruses

Guy Pilkington[1], Katarzyna Purzycka[2], Jenifer Bear[1], Stuart Le Grice[2], Barbara Felber[1]

[1]Vaccine Branch, National Cancer Institute; [2]Drug Resistance Program, National Cancer Institute

Posttranscriptional regulatory mechanisms of several simple retroviruses and retroelements have been elucidated with the only exception being the gamma retrovirus family, e.g. MuLV. We identified a novel ~1500 nt PostTranscriptional regulatory Element (PTE) located in *pro-pol* (nt 3184-4652) of MuLV and XMRV, being transferrable and promoting potent expression of an HIV *gag* reporter and the cat DM128 reporter transcript. Deletion of *pro-pol* and *env* from the provirus failed to produce Gag particles, whereas *env* deletion produced Gag particles, pointing to a critical role of the *pro-pol* sequence. We showed that the *pro-pol* sequences could be replaced by heterologous RNA export elements e.g. CTE. We found that the RNA encoding G-?retroviral *gag* only has a reduced half-life and this defect can be counteracted upon insertion of a *pro-pol* or CTE elements acting in cis. SHAPE technology was applied to the *pro-pol* RNA, with particular emphasis on the PTE, which revealed a very complex stem-loop structure. Comparisons of *pro-pol* sequences from MuLV, XMRV and related G-retroviruses showed that despite sequence divergence in the 3' end of pol, their RNA structures show little variability, indicating that the RNA structure is invariant in PTE. We conclude that complex structural components are critical for function, in addition to sequences representing interaction sites for export factors. With our discovery of the PTE, we now have a complete picture of posttranscriptional regulation of simple retroviruses. Collectively, these viruses contain negative-acting sequences embedded in *gag* mediating RNA instability, hence impair expression. These viruses also contain positive-acting RNA elements, essential for promoting Gag expression. These RNA elements do not share sequence or structural features, their location within the full-length retroviral RNA can vary, but they depend on the cellular NXF1 export machinery.

49 Shunting revisited.

Thomas Hohn[1]

[1]Basel University

Viruses must manage their genome sustainably and efficient. As a consequence they developed ways to control their replication, transcription, splicing, and translation. For efficient translation, viruses use various types of polycistronic translation, alternative translation starts and stops, internal ribosome entries and bypass of sequences by shunting.

During shunting caulimovirus leaders of about 600nt are bypassed by the scanning ribosome, as originally observed with *Cauliflower mosaic virus* (CaMV). The CaMV leader is highly structured and contains several small open reading frames. Of importance is just the first one, which ends six to nine nucleotides in front of the root of a strong stem structure. After translation of this sORF the ribosome is not dissociated but jumps across the base of the stem structure, continues scanning and reinitiates at the main ORF. The positioning of the sORF and the strength of the stem-structure, but not their primary sequences, are important for successful shunting. Interestingly, this shunt structure is preserved in the in the six genera of plant pararetroviruses (caulimoviridae). One of these, *Rice tungro bacilliform virus* (RTBV), infects rice plants together with the *Rice tungro spherical (Waika)virus* (RTSV), a RNA virus. Interestingly, not only RTBV, but also the accompanying RTSV have a related shunt structure. It is an interesting question whether this double occurrence is coincidence or of mutual benefit for both of the viruses.

It seems puzzling that the long leader, which seemingly constitutes a basic hindrance for translation, has evolved. One reason might be that it harbours a series of signals, i.e. for enhancement, polyadenylation, splicing, packaging, but those signals could cope with much less space. Recently, we found another function. The leader spawns huge amounts of siRNAs of all size classes. They make up half of the total load of sRNAs present in the infected plant. The coding region of the CaMV genome is represented by a much lower amount of siRNAs.

The RNA-form of the CaMV genome is reverse transcribed within viral particles to yield open circular dsDNA. Since repair- and ligase enzymes are missing within particles and cytoplasm, the termini of the DNA strands are not joined, until the DNA enters the nucleus. The DNA minus strand is just interrupted between leader and coding region and if transcription is initiated before repair and ligation, the RNA falls off and is consequently not polyadenylated. This aberrant ("8S") RNA, which covers just the leader gives rise to the production of dsRNA, the substrate for dicers and consequently the source of the massive amounts of leader derived siRNAs.

Due to its strong secondary structure the CaMV leader itself is a poor substrate for the set of leader-derived siRNAs, rsp. RISC complexes and we argue that this set acts as decoy out-titrating the AGO proteins and thereby protecting the CaMV transcripts.

50 Inquiry into the variability of HCV IRES and its impact on function by developing and evaluation of a large-scale mutation database that also unfolds potential of some new nucleotides.

Anas Khawaja[1], Vaclav Vopalensky[1], Ludek Roznovsky[2], Jakub Mrazek[4], Ondrej Horvath[3], Martin Pospisek[1]

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Synthesis of the hepatitis C virus polyprotein is fully controlled by an IRES located within 5'UTR of the viral RNA. We developed a new flow-cytometry-based approach allowing us to monitor patient to patient differences in IRES activities of their whole viral populations. Simultaneously we employed more conventional methods comprising cloning of PCR fragments, DGGE, TGGE, sequencing and bicistronic reporter assay for finding new HCV IRES mutations and measuring their activities. Sequence data from patients' samples along with analysis of migration patterns by DGGE and/or TGGE allowed us to estimate the variation that may have persisted over time within one or group of patients individually and collectively. We also compiled from the literature a comprehensive database comprising ~1300 IRES mutations further categorized by frequency, structural and functional behavior, experimental parameters, clinical data etc. Some IRESs bearing multiple mutations in various domains displayed either higher or lower levels of efficacy than expected after simple counting of known effects of the individual mutations. These differences in IRES activities raise the question of how some mutations are capable of sustaining an IRES WT activity. Could it be a long-range interaction between mutated nucleotides, and if yes, then how much is the extent of structure adaptability that IRES may need to go through for conservation of its function. We also collected data from published studies dealing with induced therapeutic response of interferon and ribavirin and its possible outcome in individuals with reference to occurrence of mutations in HCV IRES. By mapping out these mutations in sustained and non-sustained responders we observed no specific distribution that may conclude a direct impact in determining the therapy response between the two populations. We also illustrate impact of ~25 novel mutations found in our patients on the HCV function.

51 Novel Insights from Structural Analysis of Lentiviral and Gammaretroviral Reverse Transcriptases in Complex with RNA/DNA Hybrids.

Stuart Le Grice[1], Mikalai Lapkouski[7], Lan Tian[6], Jennifer Miller[1], Enzbieta Nowak[4], Wojciech Potrzebowski[3], Peter Konarev[2], Jason Rausch[1], Marion Bona[1], Dmitri Svergun[2], Janusz Bujnick[3], Marcin Nowotny[4], Wei Yang[5]

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Structures of HIV-1 reverse transcriptase (RT) have been reported in several forms, but only one contains an RNA/DNA hybrid, the conformation of which has been controversial. We have been successful in obtaining three structures of HIV-1 RT complexed with a non-nucleoside RT inhibitor (NNRTI) and an RNA/DNA hybrid[1]. In the presence of an NNRTI, our RNA/DNA structure differs from all prior nucleic acid bound to RT including the previously-reported RNA/DNA hybrid derived from the polypurine tract. The enzyme structure observed in our cocrystals also differs from all previous RT-DNA complexes. As a result, the hybrid has ready access to the ribonuclease H (RNase H) active site. These observations collectively reinforce previous proposals that an RT-nucleic acid complex may be required to adopt independent structural states competent for DNA synthesis and the other for RNA degradation. RT mutations that confer drug resistance but are distant from the inhibitor-binding sites map to the unique RT-hybrid interface that undergoes conformational changes between two catalytic states. Structural features of the nucleoprotein complex, including drug resistance mutations, have been verified by site-directed mutagenesis, and will be presented.

Although the single-subunit RT of Moloney murine leukemia virus (Mo-MLV) has been extensively characterized biochemically, structural information is lacking that describes the substrate binding mechanism for this RT species. We also present data on the first crystal structure of a complex between an RNA/DNA hybrid and the 72 kDa single-subunit RT from the related xenotropic murine leukemia virus-related virus (XMRV)[2]. A comparison of this structure with its HIV-1 counterpart shows that substrate binding around the DNA polymerase active site is conserved but differs between the two enzymes in their thumb and connection subdomains. Small-angle X-ray scattering (SAXS) was used to model full-length XMRV RT, demonstrating its flexible RNase H domain becomes ordered in the presence of substrate, a key difference between monomeric and dimeric RTs.

1. Lapkouski, M., Tian, L., Miller, J.T., Le Grice, S.F.J., and Yang, W. (2013). Complexes of HIV-1 RT, an NNRTI and an RNA/DNA hybrid reveal a structure compatible with RNA degradation. Nat. Struct. Mol. Biol. 20: 230-236.

2. Nowak, E., Potrzebowski, W., Konarev, P.V., Rausch, J.W., Bona, M.K., Svergun, D.I., Bujnicki, J.M., Le Grice, S.F.J., and Nowotny, M. (2013). Structural analysis of monomeric retroviral reverse transcriptase in complex with an RNA/DNA hybrid. Nucleic Acids Res., in press.

Workshop 1: Function of Non coding Transcriptome

Time: Wednesday, June 12, 17:00 - 18.30
Location: Davos Ballroom
Abstracts: 52 - 58
Chair(s): Constance Ciaudo, *ETHZ-IHMS*
Claus Azzalin, *ETHZ-IHMS*

52	**RNAi dependent and independent control of LINE1 mobility and accumulation in mouse ES cells**
53	**Role of Telomeric Repeat-containing RNA in Alternative Lengthening of Telomeres**
54	**RNA and DNA Targeting CRISPR-Cas Immune Systems of Pyrococcus furiosus**
55	**Involvement of TERT-RdRP in heterochromatin maintenance**
56	**The role of the Arabidopsis exosome complex in siRNA-independent silencing of heterochromatic loci**
57	**Involvement of the novel complex consisting of the splicing factor Prp14p/DHX38 RNA helicase and centromeric non-coding RNAs in the regulation of chromosome segregation**
58	**A genome-wide RNAi screen draws a genetic framework for transposon control and primary piRNA biogenesis in Drosophila**

52 RNAi dependent and independent control of LINE1 mobility and accumulation in mouse ES cells

Constance Ciaudo[1], Florence Jay[3], Ikuhiro Okamoto[4], Chong-Jian Chen[2], Nicolas Servant[2], Emmanuel Barillot[2], Edith Heard[2], Olivier Voinnet[3], Alexis Sarazin[3]

[1]ETHZ-IHMS; [2]Curie Institute; [3]ETHZ; [4]Kyoto University

In most mouse tissues, long-interspersed elements-1 (L1s) are silenced *via* methylation of their 5'-untranslated regions (5'-UTR). A gradual loss-of-methylation in pre-implantation embryos coincides with L1 retrotransposition in blastocysts, generating potentially harmful mutations. Here, we show that Dicer- and Ago2-dependent RNAi restricts L1 accumulation and retrotransposition in undifferentiated embryonic stem cells (mESCs), derived from blastocysts. RNAi correlates with production of Dicer-dependent 22-nt small RNAs mapping overlapping sense/antisense transcripts produced from the L1 5'-UTR. However, RNA-surveillance pathways simultaneously degrade these transcripts, consequently confounding the anti-L1 RNAi response. In *Dicer*$^{-/-}$ mESC complementation experiments, L1 silencing was rescued in cells strongly depleted of microRNAs, which proliferated and differentiated normally unlike their non-complemented counterparts. These results shed new light on L1 biology and, possibly, on the differentiation defects of *Dicer*$^{-/-}$ mESCs.

53 Role of Telomeric Repeat-containing RNA in Alternative Lengthening of Telomeres

Rajika Arora[1], Claus Azzalin[1]

[1]Institute of Biochemistry, Eidgenössische Technische Hochschule (ETH) Zürich

The large majority of cancer cells maintain their telomeres indefinitely by re-activation of the specialized reverse transcriptase telomerase. However, certain tumors especially osteosarcomas, gastric and bladder carcinomas, and cancers of the central nervous system bypass telomerase re-activation and maintain telomere length by the so called 'alternative lengthening of telomeres' (ALT) pathway, which relies on homologous recombination between telomeric sequences. Recent studies have revealed that ALT cells can also originate from telomerase-positive cancers treated with telomerase inhibitors. Despite the relevance of ALT in cancer biology, the molecular mechanisms behind ALT activation and progression remain poorly defined. A few years ago we demonstrated that human telomeres are transcribed into long non-coding nuclear transcripts ranging in size between approximately 500 and more than 9000 bases and named 'telomeric repeat-containing RNA' (TERRA). We show here that ALT cells are characterized by extremely elevated rates of TERRA transcription as compared to telomerase positive cancer cells and primary fibroblasts. TERRA co-localize with telomeres and PML within ALT-associated PML bodies (APBs), which are thought to be the nuclear compartments where telomeric homologous recombination takes place. Strikingly, we also find that ALT cells, but not telomerase positive cancer cells and primary fibroblasts, express shorter telomeric transcripts comprised between 300 and 36 bases (small TERRRA: smTERRA). Down-regulation of Dicer leads to a decrease in smTERRA cellular levels and concomitant accumulation of longer transcripts. On the contrary, depletion of Drosha does not affect smTERRA levels, suggesting a non-canonical role for Dicer in processing TERRA into short RNA species. Our data reveal an unexpected scenario where different TERRA species might support ALT establishment and/or maintenance, and should pave the way towards the development of therapies to specifically cure or prevent ALT tumors.

54 RNA and DNA Targeting CRISPR-Cas Immune Systems of Pyrococcus furiosus

Joshua Elmore[1], Caryn Hale[1], Sonali Majumdar[1], Jason Carte[1], Hong Li[2], Sara Olson[3], Brenton Graveley[3], Lance Wells[1], Claiborne Glover[1], Rebecca Terns[1], Michael Terns[1]

[1]University of Georgia; [2]Florida State University; [3]University of Connecticut

CRISPR-Cas systems are recently discovered, small RNA-based immune systems that control invasions of viruses and plasmids in archaea and bacteria. Prokaryotes with CRISPR-Cas immune systems capture short invader sequences within the CRISPR loci in their genomes, and small RNAs produced from the CRISPR loci (CRISPR (cr)RNAs) guide Cas proteins to recognize and degrade (or otherwise silence) the invading nucleic acids. There are multiple variations of the pathway found among prokaryotes, each mediated by largely distinct components and mechanisms that we are only beginning to delineate.

Using the hyperthermophile *Pyrococcus furiosus*, we have delineated several key steps in CRISPR-Cas invader defense pathways. *P. furiosus* has 7 transcriptionally active CRISPR loci distributed throughout the genome that together encode a total of ~200 crRNAs. The 27 Cas proteins in this organism represent 3 distinct pathways and are primarily encoded in two large gene clusters. The Cas6 protein "dices" large precursor CRISPR transcripts to generate the individual invader-targeting crRNAs. The mature crRNAs include a signature sequence element ("5' tag") derived from the CRISPR repeat that is important for function. crRNAs are tailored into distinct species and integrated into three distinct crRNA-Cas protein complexes that are likely immune effector complexes. The complex formed by the Cmr (type IIIB) system proteins cleaves complementary target RNAs and can be programmed to cleave novel target RNAs in a prokaryotic RNAi-like manner. The other two CRISPR-Cas systems in *P. furiosus*, Csa (type IA) and Cst (type IB), target invaders at the DNA level. Recent work will be presented on the structure and function of CRISPR-Cas immune effector complexes from this organism. The results of our work in *P. furiosus* have revealed mechanisms of crRNA biogenesis and silencing for 3 of the diverse CRISPR-Cas pathways, and reveal that organisms like *P. furiosus* possess an arsenal of multiple RNA-guided mechanisms to resist diverse invaders.

55 Involvement of TERT-RdRP in heterochromatin maintenance

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Constitutive expression of telomerase in human cells prevents the onset of senescence and crisis by maintaining telomere homeostasis. Recent evidence suggests that the human telomerase catalytic subunit (hTERT*)* contributes to cell physiology independent of its ability to elongate telomeres. We have recently reported that hTERT form an RNA dependent RNA polymerase (RdRP) and produce double-stranded RNAs that can be processed into small interfering RNA. These observations identify a mammalian RdRP composed of hTERT (Maida et al. Nature 2009).

In fission yeast, centromeric heterochromatin is maintained by an RNA-directed RNA polymerase complex (RDRC) and the RNA-induced transcriptional silencing (RITS) complex in a manner that depends on the RNAi pathway. In association with hTERC, the telomerase reverse transcriptase (TERT) forms telomerase and counteracts telomere attrition; however, TERT has also been implicated in the regulation of heterochromatin at locations distinct from telomeres. Here, we describe a complex comprising human TERT (hTERT), Brahma-related gene 1 (BRG1), and nucleostemin (NS) that contributes to heterochromatin maintenance at centromeres and transposons. Acting as an RNA dependent RNA polymerase (RdRP), this complex produced double stranded RNAs that were processed into small interfering RNAs targeted to heterochromatin regions. These small interfering RNAs promoted heterochromatin assembly and mitotic progression in a manner dependent on the RNA interference (RNAi) pathway. These observations implicate the hTERT/BRG1/NS (TBN) complex in heterochromatin assembly at particular sites in the mammalian genome.

56 The role of the Arabidopsis exosome complex in siRNA-independent silencing of heterochromatic loci

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To maintain genomic stability and prevent expansion of invasive genomic sequences such as transposable elements (TEs), eukaryotes have evolved defensive mechanisms to control them. Here, we examine the role of the *Arabidopsis* exosome complex in such mechanisms. Depletion of the exosome allows some repetitive sequences to escape from silencing. Most of these transcripts emanate from centromeric and pericentromeric chromosomal regions and other heterochromatic loci, and many derive from repetitive and transposable elements [1]. In plants, TEs are targeted for *de novo* DNA methylation by smRNA-mediated pathways. To investigate whether exosome interacts with RdDM, we profiled Arabidopsis smRNAs by deep sequencing in exosome and RdDM mutants and also analyzed RdDM-controlled loci. We found that exosome loss had a very minor effect on global smRNA populations, suggesting that, in contrast to fission yeast, in Arabidopsis the exosome does not control the spurious entry of RNAs into smRNA pathways. Exosome defects resulted in decreased histone H3K9 dimethylation at RdDM-controlled loci, without affecting smRNAs or DNA methylation. Exosome also exhibits a strong genetic interaction with RNA Pol V, but not Pol IV, and physically associates with transcripts produced from the scaffold RNAs generating region. We also show that two *Arabidopsis rrp6* homologues act in gene silencing [2]. Our data suggest that Arabidopsis exosome may act in parallel with RdDM in gene silencing, by epigenetic effects on chromatin structure, not through siRNAs or DNA methylation. We also propose that the *Arabidopsis* exosome may coordinate the transcriptional interplay of different RNA polymerases to modulate repression of some repetitive sequences. Results of further examination of exosome involvement in gene silencing in *Arabidopsis* will be presented.

1) Chekanova et al., (2007) A hidden component of the transcriptome and specialization of function of the exosome complex in *Arabidopsis*. *Cell*, 131(7), pp.1340-1353.

2) Shin et al., (2013) "The role of the *Arabidopsis* exosome in siRNA-independent silencing of heterochromatic loci". *PLoS Genetics*, accepted.

57 Involvement of the novel complex consisting of the splicing factor Prp14p/DHX38 RNA helicase and centromeric non-coding RNAs in the regulation of chromosome segregation

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prp14, of which the responsible gene encodes RNA helicase with the DEAH box motif, is one of the pre-mRNA splicing mutants in fission yeast *Schizosaccharomyces pombe*. In fission yeast, formation of heterochromatin at the centromere is induced by the RNAi-mediated system. Interestingly, *prp14* shows defective formation of the centromeric heterochromatin, resulting in a high incidence of lagging chromosomes during anaphase. *prp14* accumulates the unprocessed centromeric noncoding RNAs and shows decreased level of H3K9 methylation and Swi6p localization at the centromere, suggesting that the Prp14p helicase is involved in not only the splicing reaction, but also the RNAi-mediated heterochromatin assembly at the centromere. We found the mRNA-type intron in the *dg* non-coding RNA transcribed from the centromere region (Chinen et al, 2010). The IP analyses demonstrated that Prp14p interacts with Cid12p, a subunit of RDRC (RNA-directed RNA polymerase complex), implying that the spliceosome or sub-spliceosome assembled on the *dg* ncRNA intron works as a platform for recruitment of RDRC. To examine a role of the *dg* intron in the formation of centromeric heterochromatin, we constructed a minichromosome containing the centromere with or without the *dg* intron. Interestingly, the minichromosome without the *dg* intron showed the decreased H3K9 methylation comparing the minichromosome with the *dg* intron, supporting that the *dg* intron plays an important role in the methylation process of H3K9.

We also tested the involvement of the human homologue of Prp14p (DHX38) in the process of the chromosome segregation. Knockdown of DHX38 in HeLa cells caused the abnormal chromosome segregation, due to the defective attachment of microtubles to the kinetochores at metaphase. No significant defects were observed in splicing of tested pre-mRNAs in the knockdown cells. Interestingly, knockdown of satellite RNAs, non-coding RNAs transcribed from the human centromere, resulted in the similar defects in the chromosome segregation. In addition, the RIP assay showed that DHX38 associates physically with the satellite ncRNAs, suggesting that the DHX38/satellite ncRNA complex plays an essential role in the control of chromosomal segregation in mammalian cells.

58 A genome-wide RNAi screen draws a genetic framework for transposon control and primary piRNA biogenesis in Drosophila

Paloma M Guzzardo[*1], Felix Muerdter*[*5], Jesse Gillis[4], Yicheng Luo[1], Yang Yu[1], Caifu Chen[2], Richard Fekete[3], Gregory J Hannon[1]*

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A large fraction of our genome consists of mobile genetic elements. Governing transposons in germ cells is critically important, and failure to do so compromises genome integrity, leading to sterility. In animals, the piRNA pathway is the key to transposon constraint, yet the precise molecular details of how piRNAs are formed and how the pathway represses mobile elements remain poorly understood. In an effort to identify general requirements for transposon control and novel components of the piRNA pathway, we carried out a genome-wide RNAi screen in Drosophila ovarian somatic sheet cells. We identified and validated 87 genes necessary for transposon silencing. Among these were several novel piRNA biogenesis factors. We also found a novel gene, which we named *asterix*, to be essential for transposon silencing, most likely by contributing to the effector step of transcriptional repression. Asterix loss leads to decreases in H3K9me3 marks on certain transposons but has no effect on piRNA levels.

*These authors contributed equally to this work

Workshop 2: RNA chemistry

Time: Wednesday, June 12, 17:00 - 18.30
Location: Sanada
Abstracts: 59 - 65
Chair(s): Ronald Micura, Leopold Franzens University
Mark Helm, University of North Carolina at Chapel hill

59	**New Approaches in RNA Chemical Biology**
60	**Resolving functional RNA dynamics by NMR**
61	**RNA-based fluorescent biosensors for live cell imaging of second messengers cyclic di-GMP and cyclic AMP-GMP.**
62	**Sequence Specific Modulation of G-Quadruplex Folding**
63	**Structural stabilization of toxic CUG repeats reverses mis-splicing associated with myotonic dystrophy**
64	**A chemo-enzymatic approach for selective modification of the RNA cap**
65	**Bromomethylcoumarins as selective reagents for RNA labeling**

59 New Approaches in RNA Chemical Biology

Jonathan Hall[1], Andreas Brunschweiger[2], Luca Gebert[1], Jochen Imig[1], Mario Rebhan[1], Ugo Pradere[1], Boris Guennewig[1]

[1]ETH Zürich; [2]TU Dortmund

Many types of oligonucleotide-based tools have advanced our knowledge of RNA biology, including microRNA (miRNA) mimics, LNAs and TaqMan probes. Other examples are functionalities employed for imaging RNAs, or identifying binding partners in cells. One of the limitations of RNA tools for chemical biology applications is their length: RNA synthesis using standard reagents is mostly limited to <80 nucleotides (nt). Beyond this, success is uncertain and characterization of the molecules by for example mass spec. is challenging. The length of pre-miRNAs typically spans 50-90 nt. MiRNA hairpin precursors are increasingly appreciated as functional RNAs with their own regulatory elements. For example, a variety of RNA binding proteins (RBPs) has been shown to bind to conserved sites in the terminal loop regions of miRNA precursors and regulate their biogenesis. We recently synthesized a library containing a subset of biotin-labeled pre-miRNAs mostly with phylogenetically-conserved terminal loop regions. We used it to screen for interactions with RBPs Lin28, hnRNP A1, KSRP and others, in their native state, i.e. using cell lysates. We showed that Lin28 as well as hnRNP A1 bind pre-let-7 family members with remarkable selectivity and that KSRP binds prominently to pre-miR-1-2 (1,2). Furthermore, we measured the affinities of these interactions in the presence of cellular factors. In a chemistry program, we used a biotin-labeled pre-miR-122 in a surface plasmon resonance assay to show that miravirsen, an LNA-based drug in clinical trials inhibits biogenesis of miR-122(submitted).

At the heart of these approaches are the chemical methods used to synthesize the RNAs. We have now developed protocols to label RNA of up to 75 nt with single and double functionality. Groups can be positioned so that they protrude into the major and minor grooves of RNA duplexes. A variety of groups with orthogonal functions have been examined. One particular combination of functions is that of biotin-psoralen, which we have used for cross-linking and deep sequencing experiments. Considerable experimentation was required to position groups so that the RNAs would be incorporated into functional RISC complexes. These RNAs were delivered into cells and under mild cross-linking conditions captured their RNA targets, including known and new targets.

(1) Loughlin et al., Nat Struct Mol Biol. 2012, **22**, 109; Towbin et al., Nucleic Acids Res 2013, **41**, e47. Funding: SNF [CRSII3_127454; 205321_124720]

60 Resolving functional RNA dynamics by NMR

Christoph Wunderlich[1], Romana Spitzer[1], Thomas Moschen[1], Martin Tollinger[1], Christoph Kreutz[1]

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The research focus in our group lies on the dynamic characterization of RNA by solution NMR spectroscopy. In the field of protein structural biology it is well established that biological function is often encoded in transiently populated conformational states, which are hard to characterize by conventional biochemical and biophysical experiments. Only recently the dynamic nature of ribonucleic acid was also recognized to be a crucial aspect in manifold functions carried out by RNA. For example, such dynamics are presumed to be of special importance in ligand recognition and/or in catalytic processes controlled by recently discovered noncoding RNAs.

For that purpose we develop sophisticated isotope labeling protocols for RNA relying on the site-specific introduction of ^{13}C-reporter spins by chemical and enzymatic methods. We then use state-of-the-art NMR experiments that exploit the particular features of the site-specific ^{13}C-modified RNAs to address functionally important dynamics occurring at the milli- to microsecond time frame. In detail, we used 6-^{13}C-modified pyrimidine labels to address the excited state structure of the HIV-1 TAR RNA further corroborating the conformational selection mechanism of this RNA to recognize the ligand argininamide. The approach is currently applied to RNAs with enzymatic function and biologically relevant RNAs involved in protein free gene regulation (i.e. riboswitches)

61 RNA-based fluorescent biosensors for live cell imaging of second messengers cyclic di-GMP and cyclic AMP-GMP.

Colleen Kellenberger[1], *Stephen Wilson*[1], *Jade Sales-Lee*[1], *Ming Hammond*[2]

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Cyclic di-nucleotides are a newly expanded class of signaling molecules in bacteria that regulate important processes including biofilm formation, motility, and virulence. Furthermore, cyclic di-nucleotides have been shown to trigger the mammalian innate immune response upon bacterial secretion of these molecules into the host cell cytosol. However, many of the components of cyclic di-nucleotide signaling remain poorly understood, so we aim to create tools for monitoring their role and regulation *in vivo*. We have designed fluorescent RNA biosensors for cyclic di-GMP and cyclic AMP-GMP through fusion of the Spinach aptamer to variants of a natural GEMM-I riboswitch. In live cell imaging experiments, these biosensors demonstrate fluorescence turn-on in response to cyclic di-nucleotides, and they were used to confirm *in vivo* production of the newly discovered natural molecule, cyclic AMP-GMP. Ongoing work is focused on applying these sensors to different cell types and on studying other phylogenetic variants of the GEMM-I riboswitch class for biosensor optimization. It is envisioned that these cyclic di-nucleotide biosensors will help elucidate how the different second messengers are integrated and transduced in bacterial and mammalian signaling.

62 Sequence Specific Modulation of G-Quadruplex Folding

Samuel Rouleau[1], *Jean-Denis Beaudoin*[1], *Jean-Pierre Perreault*[1]

[1]Université de Sherbrooke

G-quadruplexes (G4) are highly stable non canonical structures that can be adopted by guanine rich nucleic acids. Over the past years, many important biological roles have been attributed to DNA as well as RNA G4. Hence, many research studies have focused on targeting G4 with chemical compounds that specifically bind these structures and either prevent or enhance their folding. Although these compounds are able to discriminate between G4 and other nucleic acid structures, they are not able to recognize one particular G4 sequence. Since there are more than 370 000 potential G4 sequences in the human genome, off-target effects remain a major issue. The aim of this project was to target specific G4 sequences with chemically modified RNA oligonucleotides. The specificity is obtained using Watson-Crick base pairing between the oligonucleotide and the targeted G4. To ensure that there would not be any off-target effects, we focused on G4 harboring a particular topology, namely a long loop 2. Oligonucleotides with specific chemical modifications (i.e. 2'O-methylated and LNA) were used for their high affinity and their great stability in human cells. By using *in line* probing, we showed that the oligonucleotides could modulate the folding of G4 either positively or negatively, depending on where the oligonucleotide is bound. This modulation was obtained on artificial G4 sequences as well as on sequences present in the 5' UTR of human genes. By inserting them in the 5'UTR of a luciferase reporter gene, we also demonstrated that long loop 2 G4 motif can decrease translation in human cells. We showed that this translation inhibition can be either increased or decreased by co-transfecting oligonucleotides with the luciferase gene. To our knowledge this is the first report of targeting a specific G4, and it also paves the way for a new kind of therapeutic tool that could modulate the expression of specific genes.

63 Structural stabilization of toxic CUG repeats reverses mis-splicing associated with myotonic dystrophy

Elaine deLorimier[1], Jeremy Copperman[1], Alex Taber[1], Leslie Coonrod[1], Emily Reister[1], Feras Ackall[2], Kush Sharma[2], Peter Todd[2], Marina Guenza[1] and J. Andrew Berglund[1]

[1]University of Oregon, [2]University of Michigan

Myotonic dystrophy type 1 (DM1) is a dominant RNA gain of function disease caused by a trinucleotide repeat (CTG) expansion in the 3' untranslated region of the DMPK gene. When transcribed, these expanded CUG repeats sequester the muscleblind (MBNL) family of RNA binding proteins (MBNL1, MBNL2 and MBNL3). The MBNL proteins are regulators of alternative splicing, and sequestration to the CUG repeats results in the mis-splicing of MBNL target pre-mRNAs, leading to some of the disease symptoms.

Model CUG repeat RNA substrates and purified MBNL1 protein were used to determine how modifying the CUG repeats with pseudouridine and 2' O-methyl would affect the interaction between MBNL1 and the toxic RNA. A reduction or full elimination of MBNL1 binding to the CUG repeats was observed with modification. The higher number of modifications correlated with increased inhibition of protein binding to the RNA. Thermal melting studies demonstrated that pseudouridine and 2' O-methyl modifications significantly stabilized the CUG repeats in a helical conformation. Crystallographic and molecular dynamic simulations of CUG repeats containing pseudouridine suggest that helix stability is the result of a reduction in the dynamics of the U-U mismatch in the helical CUG repeats.

Cell and animal DM1 models were used to determine if modification of CUG repeats could reduce molecular and phenotypic consequences. In the DM1 cell model no mis-splicing defects were observed when CUG repeats containing 50% and 100% pseudouridylation were used. The RNA lacking modification resulted in the sequestration of MBNL proteins and mis-splicing in the cell model. Preliminary experiments in a DM1 zebrafish model showed that pseudouridylation of CUG repeats increased viability compared to unmodified RNA. Motor defects (spontaneous coiling and touch evoked escape) observed in the CUG repeat fish were reduced in the fish containing CUG repeats with pseudouridines. Our studies suggest that stabilization of toxic RNA with modifications or others means is a strategy that could lead to new therapeutic approaches for myotonic dystrophy and other diseases with toxic RNA components.

64 A chemo-enzymatic approach for selective modification of the RNA cap

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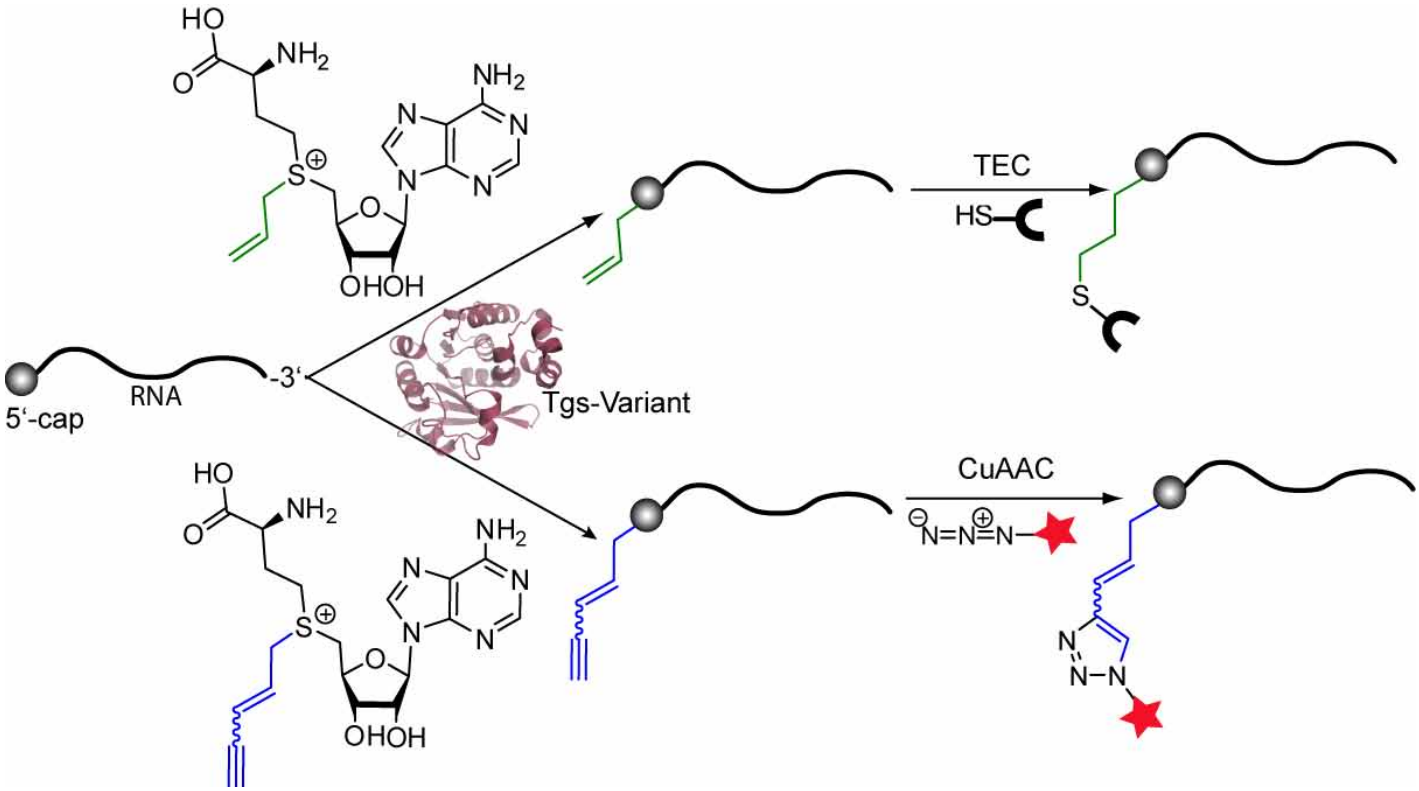
The 5'-cap is a hallmark of eukaryotic mRNAs and methods for selective modification of this particular biomolecule are highly sought in order to produce labeled capped RNA or to isolate mRNA from total RNA. Presently, there are no methods for selective covalent modification of natural cap-bearing RNAs, although these are the most important study object in terms of expression and also subcellular localization. We developed a two-step strategy, combining an enzymatic and a chemical conversion to achieve this goal. It is selective for capped RNAs and site-specific for the exocyclic N^2-position of the guanosine in the cap. We used enzymes that specifically recognize and hypermethylate the 5'-cap, so-called trimethylguanosinsynthases (Tgs), and engineered variants that successfully transfer alternative alkyl groups suitable for bioorthogonal chemistry. Although wildtype enzymes showed no or very low promiscuous activity on the AdoMet-analogs, we can now produce site-selectively modified caps with up to 91 % yield. We demonstrated further chemical modification by thiol-ene and copper-catalyzed click chemistry and introduced biotin as well as a fluorescent dye. Importantly, the chemo-enzymatic modification was also possible in cell lysate.

Potential applications of our approach include isolation of mRNAs from total RNA of eukaryotic cells for further use in RNA sequencing, which has become increasingly important to study gene expression in various cell types and states. Commonly, isolation proceeds via the 3'-end harboring a poly(A)-tail. However, the length of this poly(A)-tail itself is regulated and interesting to study. Our method might allow to isolate mRNAs via the 5'-end via a covalent or non-covalent interaction with the matrix.



65 Bromomethylcoumarins as selective reagents for RNA labeling

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Selective alkylation of RNA nucleotides is an important field of RNA biochemistry, e.g. in applications of fluorescent labeling or in structural probing experiments, yet detailed structure-function studies of the labeling agents are rare. Here, bromomethylcoumarins as reactive dyes for fluorescent labeling of RNA are developed as an attractive scaffold on which electronic properties can be modulated by variegated substituents. Using semi-quantitative LC-MS/MS analysis, the selectivity of various coumarin derivatives in labeling reaction with respect to the four major and a number of minor, i.e. modified nucleotides was assessed. This allowed the development of coumarins (i) as adaptor modules carrying an azide or alkyne function permitting further labeling by click chemistry; (ii) as photocrosslinking probes at uridine moieties, (iii) tagging the naturally occurring nucleotide modification 4-thiouridine with a selectivity of four orders of magnitude. This establishes bromomethylcoumarins as versatile reagents with a wide range of applications in RNA science.

Plenary 3A: RNA and disease

Time: Thursday, June 13, 8:30 - 10:15
Location: Davos Ballroom
Abstracts: 66 - 72
Chair(s): Tom Cooper, Baylor College of Medicine

66	**Somatic spliceosomal factor mutations in bone marrow neoplasms lead to alterations in alternative splicing patterns that relate to the splicing mechanism**
67	**Mnk2 alternative splicing inactivates its tumor suppressor activity as a modulator of the p38-MAPK stress pathway**
68	**Multiple myeloma-associated hDIS3 mutations cause perturbations in cellular RNA metabolism and suggest potential drug targets**
69	**Defective RNP Assembly in Prostate and Other Cancers**
70	**Loss of MBNL1 function impairs neuronal morphology in myotonic dystrophy type 1**
71	**Mutations in the gene encoding U11/U12-65K protein leads to pituitary hypoplasia and isolated growth hormone deficiency type I**
72	**miR-34c-5p is a novel regulator of naive T-cell activation that impacts HIV replication**

66 Somatic spliceosomal factor mutations in bone marrow neoplasms lead to alterations in alternative splicing patterns that relate to the splicing mechanism

Richard Padgett[1], Bartloniej Przychodzen[1], Amina Kozaric[1], Hideki Makishima[1], Magda Konarska[2], Jaroslaw Maciejewski[1]

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Frequent somatic mutations of spliceosomal proteins such as U2AF1, SF3B1, SRSF2 and PRPF8 have been identified in myeloid malignancies. These mutations are usually single heterozygous missense mutations at highly recurrent sites. While all of these mutations affect proteins in the common splicing pathway, their downstream consequences may be diverse and involve distinct oncogenic pathways. To identify affected genes and gain mechanistic insight into the effects of these mutations, we have analyzed the tumor transcriptomes of multiple AML patients with and without spliceosomal factor mutations. For U2AF1, we analyzed 6 tumors with U2AF1 mutations and 14 tumors that had no known splicing factor mutations. Focusing on cases of alternative exon inclusion, we identified 35 exons in 35 genes whose inclusion was altered using stringent statistical cutoffs. Of these, 8 exons were more included while 27 were more excluded in the U2AF1 mutant tumors. We examined the splice site signals flanking the alternative exons and noted a highly unusual sequence pattern adjacent to the 3' AG dinucleotide which is the recognition site of U2AF1. The sequence at this position showed a mutually exclusive pattern upon comparing the excluded to the included exons and this pattern also differed from the consensus 3' splice site sequence. We speculate that the RNA recognition activity of U2AF1 is altered by these mutations. We also found that these splicing changes are not seen in tumors expressing low levels of unmutated U2AF1 nor are the patterns the same in similar tumors with mutations in splicing factors SF3B1, SRSF2 or PRPF8.

The core splicing factor PRPF8 is also often mutated or is expressed at low levels in myeloid malignancies. We identified 9 different missense mutations in AML patients. Eight of these positions were conserved in yeast and the analogous mutations were introduced into the Prp8 gene. None of these mutations produced a growth, temperature sensitive or cold sensitive phenotype. Most showed weak suppression of a subset of splice site mutations. The mutations map to a surface of Prp8 that appears to associate with Brr2. Transcriptome analyses of tumors from mutant and low PRPF8 expressers showed characteristic alterations in exon inclusion. There was a low overlap between the genes most affected in the mutant cases compared to the low expression cases suggesting that the mutant PRPF8 has an altered function.

67 Mnk2 alternative splicing inactivates its tumor suppressor activity as a modulator of the p38-MAPK stress pathway

Avi Maimon[1], Maxim Mogilevsky[1], Asaf Shilo[1], Ben Davidson[3], Rikiro Fukunaga[2], Rotem Karni[1]

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The kinase Mnk2 is a substrate of the MAPK pathway and phosphorylates the translation initiation factor eIF4E. In humans, *MKNK2*, the gene encoding for Mnk2 is alternatively spliced yielding two splicing isoforms with differing last exons: Mnk2a, which contains a MAPK binding domain and Mnk2b which lacks this domain. We found that the Mnk2a isoform is downregulated in breast, lung and colon tumors and is tumor suppressive. Mnk2a directly interacts with, activates and translocates p38a-MAPK into the nucleus, leading to activation of its target genes, increasing cell death and suppression of Ras-induced transformation in vitro and in vivo. Alternatively, Mnk2b is pro-oncogenic and does not activate p38-MAPK, while still enhancing eIF4E phosphorylation. Using mutants of Mnk2a that localized either to the cytoplasm or the nucleus we show that Mnk2a colocalization with p38a-MAPK in the nucleus is both required and sufficient for its tumor suppressive activity; induction of p38a-MAPK target genes and apoptosis. Thus, Mnk2a downregulation by alternative splicing is a new tumor suppressor mechanism, which is lost in some breast, colon and lung tumors.

68 Multiple myeloma-associated hDIS3 mutations cause perturbations in cellular RNA metabolism and suggest potential drug targets

Rafal Tomecki[1], Karolina Drazkowska[1], Iwo Kucinski[2], Krystian Stodus[2], Roman Szczesny[2], Jakub Gruchota[2], Andrzei Dziembowski[1]

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Dis3 is a human nuclear exosome catalytic subunits which contains exonucleolytic (RNB) and endonucleolytic (PIN) active domains. Approximately 10% of multiple myeloma (MM) cases have mutations in Dis3 which locate mostly in the conserved residues of RNB domain. Moreover, Dis3 mutations were also found in other cancers like Acute Myeloid Leukemia or Medulloblastoma.

We show that mutations found in MM patents interfere with Dis3 exonucleolytic activity leading to overall decrease of enzymatic activity, loss of processivity and inability to degrade double-stranded regions. MM Dis3 mutations when introduced into yeast Dis3 ortholog causes growth alterations and changes in nuclear RNA metabolism typical for yeast exosome dysfunction. Since the role of human Dis3 has not been intensively studied we generated conditional Dis3 knockout in chicken B-cell derived DT40 cell line and found out that Dis3 is essential for cell survival suggesting that in its function cannot be taken over by other human exosome associated nucleases: Dis3l and Rrp6. Construction of human cellular model in which endogenous WT Dis3 is replaced with MM mutants reveled substantial changes in RNA metabolism as detected by northern blot and genome wide RNA-seq experiments. For example, Dis3 mutations interfered with rRNA processing and leaded to accumulation of unstable RNAs arising from pervasive transcription. Finally, we analyzed vulnerabilities caused by Dis3 dysfunction what revealed potential drug targets for cancers bearing Dis3 mutations.

All data mentioned above point to the conclusion that hDIS3 mutations found in MM influence cell physiology, disturb exosome function and may potentially cause global defects in the posttranscriptional regulation of gene expression. Such defects are a source of vulnerabilities which can be used for cancer drug development.

69 Defective RNP Assembly in Prostate and Other Cancers

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H/ACA RNPs, one of the two major families of snoRNPs, are important for many basic cellular processes including protein synthesis, pre-mRNA splicing, and genome integrity. Consisting of only one small non-coding H/ACA RNA and four core proteins, their biogenesis is surprisingly complex depending on at least two H/ACA-specific and four general assembly factors. SHQ1 is a chaperone of the central H/ACA core protein and pseudouridine synthase NAP57, aka dyskerin and Cbf5 in yeast and archaea. Through tight interaction, SHQ1 functions in the essential first step of H/ACA RNP biogenesis protecting the inherently unstable NAP57 from degradation and aggregation. It is this NAP57-SHQ1 interaction that is the target of mutations in NAP57 causing the inherited bone marrow failure syndrome dyskeratosis congenita. Surprisingly, although mutations in other components of H/ACA RNPs have been identified in this often-fatal disease, none have been uncovered in SHQ1.

Now a prostate cancer genomics project implicates SHQ1 as a tumor suppressor in cooperation with the androgen-driven serine protease-transcription factor translocation TMPRSS2-ERG, which is observed in ~50% of prostate cancers. Indeed, knockdown of SHQ1 or NAP57 (in combination with ERG expression) in a prostate tissue recombination assay leads to intraepithelial neoplastic lesions in 4-6 weeks. Additionally, SHQ1 is the only one in a cluster of genes with copy number alterations that also carries tumor-associated mutations. Importantly, prostate cancer mutations are also identified in NAP57 implicating both proteins as tumor suppressors. Additional somatic mutations in SHQ1 and NAP57 are identified in other types of tumors, i.e., colon, lung, uterine, and glioblastoma, with most amino acid substitutions adorning the interface between the two proteins. We demonstrate that these cancer mutations, unlike SNPs or mutations of conserved amino acids required for catalysis, impair the interaction of SHQ1 with NAP57 thereby impeding overall H/ACA RNP biogenesis. We conclude that, through SHQ1 and NAP57, H/ACA RNP biogenesis is a target for inherited and somatic diseases and we will speculate on the underlying molecular mechanisms.

70 Loss of MBNL1 function impairs neuronal morphology in myotonic dystrophy type 1

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Myotonic dystrophy (DM) is the most common cause of adult onset muscular dystrophy. Cognitive deficits are found in a high percentage of individuals with DM, type 1 (DM1). The cognitive and behavioral abnormalities include mental retardation, attention deficit and hyperactivity disorder, excessive daytime sleepiness and psychiatric disorders. The genetic basis of DM1 is caused by an expansion of CTG repeats in the 3' untranslated region (UTR) of the Dystrophia Myotonica Protein Kinase (*DMPK*) gene. *DMPK* mRNA containing expanded CUG repeats accumulates in nuclear foci and affect nuclear and cytoplasmic functions of RNA binding proteins such as muscleblind like 1 (MBNL1) and CELF1 (CUGBP and ETR3 Like Factor). Dysfunction of MBNL1 and CELF1 resulting in mis-regulated alternative splicing has been known to involve in the pathogenesis of DM1 skeletal muscle and heart respectively. However the molecular mechanism of how expanded CUG RNA affects central nervous system (CNS) functions in DM1 remains unknown. We have established a brain specific DM1 mouse model, EpA960/CaMKII-Cre, in which expanded CUG RNA is specifically expressed in brain. We found that mouse brains expressing expanded CUG RNA displayed several features of DM1 brain including mis-regulation of alternative splicing and atrophy of cortex and corpus callosum. Hippocampal cultured neurons expressing expanded CUG RNA or knockdown of MBNL1 exhibited similar defects in axonal outgrowth and dendrite development suggesting that loss of MBNL1 function plays an important role in DM1 CNS pathogenesis. In studying the phenotype progression in DM1 mouse brain, we found the progressive loss of MBNL1 in the cytoplasm that is reminisced of changes in alternative splicing of Mbnl1 exon 5. Using time-lapse microscopy we found that MBNL1 cytoplasmic isoform resulting from exclusion of exon 5 is involved in trafficking in cultured hippocampal neurons. We further demonstrated that overexpression of this spliced variant enhances neurite outgrowth through mediating BDNF signaling by interacting with cytoplasmic dynein light chain. More importantly neurons expressing expanded CUG RNA fail to respond to BDNF treatment and loss of BDNF responsiveness can be rescued by expression of MBNL1 cytoplasmic isoform. Thus our results provide a novel mechanism for MBNL1 in DM1 CNS pathogenesis.

71 Mutations in the gene encoding U11/U12-65K protein leads to pituitary hypoplasia and isolated growth hormone deficiency type I

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We describe here the second human disease that is caused by mutations in a specific component of the U12-dependent spliceosome. Earlier recent work by He et al. (2011) and Edery et al. (2011) have described recessive mutations in the U4atac snRNA that cause Microcephalic Osteodysplastic Primordial Dwarfism type 1 or Taybi-Linder syndrome (MOPD1/TALS). The patients with this disease suffer from severe developmental defects and death during early childhood, most likely due to splicing defects in a subset of ~800 genes carrying U12-type introns.

Here we have studied a family with three sisters affected with severe postnatal growth retardation due to isolated GH deficiency and otherwise normal development. Brain MRI scans revealed hypoplasia of the anterior pituitary. The therapeutic response to GH replacement has been excellent to date, suggesting a diagnosis of familial isolated GH deficiency with associated pituitary hypoplasia. Mutational and segregation analyses discarded involvement of all known genes of the GH axis. Exome sequencing revealed biallelic mutations in the RNPC3 gene encoding for the U11/U12-65K protein. This protein is one of the seven unique protein components in the U11/U12 di-snRNP that recognizes the 5'ss and BPS of U12-type introns. The 65K protein is known to bind to the 3' terminal stemloop of U12 snRNA and form a molecular bridge between U11 and U12 snRNPs via interactions with 59K and 48K proteins (Benecke et al., 2005; Turunen et al., 2008). The mutations are in the second RNA recognition motif (RRM2), which binds to the U12 snRNA.

Our preliminary RNAseq analyses from patient lymphocytes have identified defects in splicing of a subset of U12-type introns, which indicate a partial loss of function and is consistent with the observed mild pathological effects of the mutations. Furthermore, glycerol gradient analyses and biotinylated 2'-O-Me RNA oligo pulldown experiments indicate significantly reduced cellular levels of U11/U12 di-snRNPs, which is consistent with predicted consequences of the RRM2 mutations. Finally, we observed unexpected ~2-fold upregulation in the cellular levels of U4atac snRNA alone and as an U4atac/U6atac di-snRNA complex, which may be a possible compensatory mechanism for the defects in the 65K function.

References

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72 miR-34c-5p is a novel regulator of naive T-cell activation that impacts HIV replication

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CD4+T-cells are the main orchestrators of immune responses. They patrol the body as so-called "naïve" T-cells and become activated through their specific T-cell receptor (TCR) upon encounter with a cognate antigen, a process essential for their clonal expansion and differentiation into memory and effector T-cells. These processes involve a delicate balance of cell proliferation, differentiation and death that have been shown to highly dependent upon microRNA regulation.

Activated CD4+ T-cells are the main targets of HIV infection. Moreover, HIV critically depends upon the cellular activation-state for effective integration into the host genome and productive viral replication. We hypothesize that HIV may subvert the miR profile induced by TCR stimulation.

Based on a massive parallel sequencing study of the microRnome of primary human naive CD4+T-cells, we have identified miR-34c-5p as a novel candidate regulator of T cell activation that is targeted during HIV infection.

miR-34c-5p was found to be expressed almost below detection limits in naive CD4+ T-cells but was induced as a late response to TCR stimulation. miR-34c-5p up-regulation was absent in memory cells, providing novel insights into the differential regulation of these two cell populations. Profiling of the naive cell response to HIV infection revealed that miR-34c-5p was consistently down-regulated by the virus. By generating a T-cell line stably expressing high levels of miR-34c-5p we showed that this microRNA induced a quiescent, non-proliferative phenotype and identified several relevant target genes for the regulation of T-cell function and response to HIV infection. Additionally, the ability of HIV to infect this T cell line was significantly reduced. Our results identified miR-34c-5p as a critical regulator of HIV infection and provide data regarding the mechanisms controlling the expression of this miR during cell activation and virus infection.

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Plenary 3B: **RNA systems biology**

Time: Thursday, June 13, 10:45 - 12.30
Location: Davos Ballroom
Abstracts: 73 - 80
Chair(s): Graveley Graveley, *University of Connecticutt*

73	**Genome-wide mapping of RBM10 binding sites reveals its role in splicing regulation: Implications for cleft palate and TARP syndrome**
74	**Muscleblind-like proteins negatively regulate embryonic stem cell-specific alternative splicing and reprogramming**
75	**A pair of RNA binding proteins regulate neuron-subtype specific alternative splicing in C. elegans**
76	**CFlm25 Links Global change in APA to Cell Growth Control and Glioblastoma Survival**
77	**Subsets of introns are abundant in poly(A)+ RNA**
78	**Genome-wide analysis of pre-mRNA splicing in budding yeast from the perspective of the intron**
79	**Lariat Sequencing in a Unicellular Yeast Identifies Regulated Alternative Splicing of Exons that are Evolutionarily Conserved with Humans**
80	**Global Analysis of Phosphorylation by SR Protein Kinases and Their Effects on Genome-wide Splicing in Schizosaccharomyces pombe**

73 Genome-wide mapping of RBM10 binding sites reveals its role in splicing regulation: Implications for cleft palate and TARP syndrome

Julie Rodor[1], *David Fitzpatrick*[1], *Javier Caceres*[1]

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RBM10 is an RNA-binding protein of unknown function, which comprises several motifs that bind RNA, including two RNA recognition motifs (RRMs) and Zinc fingers. Mutations in human RBM10, which maps to the X-chromosome, results in a syndromic form of cleft palate, termed TARP syndrome. This syndrome comprises Pierre–Robin sequence (micrognathia, glossoptosis, and cleft palate), talipes equinovarus, atrial septal defect (ASD), and persistence of the left superior vena cava and results in pre- or postnatal lethality in affected males[1]. The loss of function of RBM10 in TARP syndrome shows that this gene is critical for normal mammalian development.

In order to understand the role of RBM10 and how its disruption results in human disease, we aimed to identify endogenous RMB10 RNA targets in a tissue that is relevant to the phenotype observed in TARP syndrome. For this, we used the Cross-Linking Immunoprecipitation protocol (iCLIP) that allows the mapping of protein-RNA interactions at an individual nucleotide resolution. Given the strong conservation between the human and the orthologous murine RBM10 protein and the pattern of expression of the mouse RBM10 gene being consistent with the human malformations of the TARP syndrome, we performed the iCLIP experiment in a mouse mandibular cell line.

We identified around 57,000 RBM10 binding sites at the genomic level, with significant enrichment in intronic regions, in agreement with a putative role for this protein in pre-mRNA splicing. We also uncovered a variety of cellular RNAs, including non-coding RNAs that are bound by RBM10. We used an exon-junction array to analyze changes in gene expression and alternative splicing in a male X-linked Rbm10 mouse deficient cell line. This analysis revealed significant changes in the expression level of approximately 1,400 genes as well as changes in 200 splicing events. Importantly, we observed that those pre-mRNAs that display changes in splicing also contain RBM10 iCLIP tags, suggesting a direct role of RBM10 in splicing regulation. Furthermore, transcriptome changes in the RBM10 KO ES cells revealed that RBM10-regulated genes are in cellular pathways that may help explain the phenotype observed in the TARP syndrome. These experiments will shed light on the pathways that are disturbed in the syndromic form of cleft palate associated with loss of function of RBM10.

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74 Muscleblind-like proteins negatively regulate embryonic stem cell-specific alternative splicing and reprogramming

Hong Han[1], Manuel Irimia[1], Joel Ross[6], Hoon-Ki Sung[2], Babak Alipanahi[5], Laurent David[3], Azadeh Golipour[3], Mathieu Gabut[1], Iacovos Michael[2], Emil Nachman[1], Eric Wang[4], Dan Trcka[3], Tadeo Thompson[6], Christopher Burge[4], Jason Moffat[1], Brendan Frey[5], Andras Nagy[2], James Ellis[6], Jeffrey Wrana[3], Benjamin Blencowe[1]

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Previous investigations of the core gene regulatory circuitry that controls embryonic stem cell (ESC) pluripotency have largely focused on the roles of transcription, chromatin and non-coding RNA regulators. Alternative splicing (AS) represents a widely acting mode of gene regulation, yet its role in the regulation of ESC pluripotency and differentiation is poorly understood. Here, I identify the Muscleblind-like RNA binding proteins, MBNL1 and MBNL2, as conserved and direct negative regulators of a large program of AS events that are differentially regulated between ESCs and other cell types. Knockdown of MBNL proteins in differentiated cells causes switching to an ESC-like AS pattern for approximately half of these AS events. Among the events is an ESC-specific AS switch in the forkhead family transcription factor FOXP1 that controls pluripotency. Consistent with a central and negative regulatory role for MBNL proteins in pluripotency, their knockdown significantly enhances the expression of key pluripotency genes and the formation of induced pluripotent stem cells (iPSCs) during somatic cell reprogramming.

75 A pair of RNA binding proteins regulate neuron-subtype specific alternative splicing in C. elegans

Adam Norris[1], *John Calarco*[1]

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Alternative splicing is an important and pervasive means of increasing the diversity of the transcriptome and proteome in metazoans. An increased transcriptomic repertoire has likely played a particularly important role in the cellular diversification of the nervous system, where different classes of neurons have evolved to perform distinct functions. However, it has remained a challenge to study the mechanisms and physiological impact of alternative splicing regulation at the level of individual neuronal subtypes. Utilizing the nematode *C. elegans*, with its genetic tractability and simple but well-differentiated nervous system, we created fluorescent two-color reporters to observe alternative splicing *in vivo* and at single neuron resolution. Our results reveal a remarkable diversity of alternative splicing patterns among individual neuron types. One striking example involved differential inclusion of exon 16 in UNC-16/JIP3 transcripts between GABAergic and cholinergic neurons (the two major classes of motor neurons in the animal). We conducted a genetic screen for regulators of this neuron-type specific splicing pattern and identified two broadly conserved RNA binding proteins, UNC-75/CELF and EXC-7/ELAV, both of which facilitate inclusion of the alternative exon. Analysis of splicing patterns in mutant animals and expression studies of the two factors showed that UNC-75 and EXC-7 act combinatorially to achieve neuron-type specificity through partially non-overlapping expression patterns. We next used mRNA-Seq experiments to profile the transcriptomes of wild type and mutant animals and performed CLIP-Seq to assay which transcripts were directly bound by the splicing factors. We found several hundred differentially regulated alternative splicing events when either or both factors are absent, a substantial overlap in targets between the two RNA binding proteins, and distinct modes of combinatorial regulation. Gene Ontology analysis indicated that targeted alternative splicing events were enriched in genes associated with synaptic transmission and locomotion. We are now using knockout mutant strains to explore how perturbing the network of splice isoforms impacts behavioral phenotypes. Initial results indicate that the splicing regulatory network can be utilized to implicate both known and previously uncharacterized genes in aspects of locomotory behavior and synaptic transmission, and that the impact of individual targeted isoforms on neuronal phenotypes can be teased apart *in vivo*. Taken together, our findings suggest that the combinatorial action of splicing factors help shape the regulatory networks contributing to the identity and function of distinct cell types in the nervous system. We speculate that similar regulatory strategies have been frequently utilized in the specification and diversification of the mammalian central nervous system.

76 CFlm25 Links Global change in APA to Cell Growth Control and Glioblastoma Survival

Chioniso Masamha[1], *Zheng Xia*[2], *Wei Li*[2], *Ann-Bin Shyu*[1], *Todd Albrecht*[1], *Eric Wagner*[1]

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A growing body of evidence implicates alternative cleavage and polyadenylation (APA) as an important mechanism in regulating gene expression. In rapidly proliferating cells or tumors, widespread shortening of 3'UTRs is posited to result in the increased expression of genes important for these altered states. While this observation has been known for some time, the underlying molecular events that lead to APA in these cells are not known.

Here, we systematically depleted each member of the mammalian cleavage and polyadenylation machinery and measured APA of three test genes: Cyclin D1, Timp2, and Dicer1. Depletion of only the members of the CFIm complex led to shortening of these 3'UTRs with the most dramatic shortening after reduction in CFIm25. To determine its global targets, we developed a novel algorithm capable of identifying and quantifying APA events from standard RNA-Sequencing. Using this algorithm, we identified over 1000 genes that switched from distal to proximal PAS selection after knockdown of CFIm25 in Hela cells. Among the top genes whose APA is under CFIm25 regulation is Glutaminase (GLS), which converts glutamine to glutamate in highly proliferative tumors to support the cancer metabolic phenotype. Depletion of CFIm25 leads to significant shortening of the GLS 3'UTR that causes a profound increase in GLS protein levels. In response, cells with CFIm25 depleted not only proliferate faster but also are sensitive to glutamine withdrawal demonstrating a metabolic change in response to global 3'UTR shortening.

To investigate the biological role of CFIm25 in APA we used our algorithm on data deposited in the TCGA database. This analysis uncovered a previously unsuspected link between CFIm25 levels and survival in glioblastoma patients. Moreover, using our algorithm we compared the 3'UTR usage in RNA seq data from samples of glioblastoma patients who expressed either high or low levels of CFIm25 and identified a group of genes with 3'UTR shortening in conditions of reduced expression. Top on this list is MeCP2, which has been associated with the increased proliferation of several cancers and its altered expression in neurons leads to a spectrum of human neurological disorders. These findings identify a pivotal role of the CFIm25 in governing APA, demonstrate APA identification through standard RNA-Seq, and reveal a previously unknown link between APA and metabolic pathways important for enhanced cell proliferation.

77 Subsets of introns are abundant in poly(A)+ RNA

Paul Boutz[1], *Arjun Bhutkar*[1], *Phillip Sharp*[1]

[1]David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology

Deep sequencing of poly(A)-selected RNA from mouse embryonic stem cells revealed many transcripts in which specific internal introns were significantly more abundant than the other introns within the transcript. We validated a number of these high read-coverage introns by qRT-PCR and found them to be 3-80 fold more abundant than their downstream neighbors. A computational pipeline was designed that identified thousands of high-coverage introns with a stringent statistical cutoff in both human and mouse, using our own as well as ENCODE-generated poly(A)+ RNA-seq data. These introns flank both constitutive and alternatively spliced exons, on average exhibit higher phylogenetic sequence conservation, and the genes containing them are enriched for transcription and RNA binding/ processing factors as well as cell cycle and stress response genes. After transcriptional inhibition by flavopiridol (30 minutes), several measured high-coverage introns were predominantly nuclear and their abundance was mostly unchanged; flanking downstream introns were removed by this time. During longer flavopiridol treatments the high-coverage introns ultimately decayed but with significantly slower kinetics than their rapidly spliced neighbors. These results are consistent with high-coverage introns being posttranscriptionally-spliced introns within transcripts that are detained in the nucleus until all introns have been removed. We refer to these as "detained" introns (DIs) to distinguish them from "retained" introns, which are unspliced introns present in transcripts that are transported to the cytoplasm. DIs flanking cassette exons exhibited exon-specific, cotranscriptional and posttranscriptional splicing in response to knockdown of the splicing factor Fox2. A number of DIs are associated with alternative splicing events that are predicted to function as a switch between the production of nonsense-mediated decay (NMD)-substrates and protein coding mRNAs. Among four human cell lines, evidence suggests that DIs are dynamically regulated and comprise a separate pool from previously identified mRNAs that are substrates for the NMD pathway. We propose a modified exon-definition mechanism to explain how intron detention can occur, and suggest that transcripts containing DIs may function as a nuclear detained pool for rapid mobilization of coding mRNAs in response to cellular needs.

78 Genome-wide analysis of pre-mRNA splicing in budding yeast from the perspective of the intron

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In eukaryotes, genes are interrupted by non-coding introns that are spliced out in the process of pre-mRNA splicing. Monitoring pre-mRNA splicing genome-wide is vital to annotating gene structure and understanding the role of pre-mRNA splicing in gene regulation. Pre-mRNA splicing was initially analyzed genome-wide by array-based methods. However, microarrays suffer from low dynamic range and cross hybridization and they typically require prior knowledge of splicing junctions. Recently, RNA-seq, which can reveal splice junctions, has provided another opportunity to monitor splicing with unprecedented dynamic range and resolution. However, the mRNA population ,the target of array and RNA-seq approaches, is underrepresented for splicing events that yield mRNAs subject to non-sense mediated decay. Moreover, due to slow turnover, mRNA can be sluggish in reflecting changes in splicing. Additionally, mRNA sequence does not reveal the branch point, the nucleophile for 5' splice site cleavage. Notably, the limitations of focusing on mRNA to monitor pre-mRNA splicing can be overcome by an alternative approach targeting the intron. We and others have developed approaches to target the intron. In our approach, we sequence the ends of the excised intron to reveal the 5' splice site and the branch point. We have tested the utility of our method in budding yeast and confirmed the majority of annotated intron (~240 introns) that are expressed and spliced under vegetative growth conditions. Furthermore, we detected novel introns in coding regions, 3' UTRs and antisense transcripts. Additionally, given the depth of our analysis, we observed evidence for splicing errors. Finally, although alternative splice site usage is extremely rare in budding yeast, we have uncovered and validated cases of alternative 5' splice site usage and alternative branch point usage. Intriguingly, some genes that undergo alternative splicing code for splicing factors. We hypothesize that alternative splicing of these transcripts enables auto-regulation by novel regulatory mechanisms, which we are currently testing. Our study underscores the value of viewing splicing from the perspective of the intron.

79 Lariat Sequencing in a Unicellular Yeast Identifies Regulated Alternative Splicing of Exons that are Evolutionarily Conserved with Humans

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[1]Cornell University

Alternative splicing is a potent regulator of gene expression that vastly increases proteomic diversity in multicellular eukaryotes, and is associated with organismal complexity. Although it is widespread in vertebrates, little is known about the evolutionary origins of this process owing in part to the absence of phylogenetically conserved events that cross major eukaryotic clades. Here we describe a lariat sequencing approach, which offers high sensitivity for detecting splicing events, and its application to the unicellular fungus, *Schizosaccharomyces pombe*, an organism that shares many of the hallmarks of alternative splicing in mammalian systems but for which no previous examples of exon-skipping had been demonstrated. Over 200 previously unannotated splicing events were identified, including examples of regulated alternative splicing. Remarkably, an evolutionary analysis of four of the exons identified here as subject to skipping in *S. pombe* reveals high sequence conservation and perfect length conservation with their homologs in scores of plants, animals, and fungi. Moreover, alternative splicing of two of these exons have been documented in multiple vertebrate organisms, making these the first demonstrations of identical alternative splicing patterns in species that are separated by over one billion years of evolution.

80 Global Analysis of Phosphorylation by SR Protein Kinases and Their Effects on Genome-wide Splicing in Schizosaccharomyces pombe

Michael Marvin[1], Jesse Lipp[1], Kevan Shokat[1], Christine Guthrie[1]

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Phosphorylation of both core splicing factors and other co-transcriptionally associated proteins has been shown to influence multiple steps in pre-mRNA splicing. Some phosphorylation events have been linked to specific kinases, but a global analysis of splicing kinases and their substrates, as well as how these events affect splicing genome-wide, is currently lacking. Known substrates of splicing kinases include the conserved serine/arginine (SR) proteins, which are vital in both constitutive and alternative splicing. *S. pombe* has two SR proteins (Srp1 and Srp2), two SR specific kinases (Dsk1 and Kic1/Lkh1), and almost 5,000 introns making it an attractive but manageable system to study the genome-wide effects of phosphorylation on splicing. In order to identify specific phosphorylation events, we took a chemical genetic approach by mutating the "gatekeeper" residue of specific kinases. This mutation provides access to a hydrophobic region at the back of the ATP binding pocket enabling selective use of bulky ATP analogs. We mapped the specific sites of phosphorylation in extract followed by LC-MS/MS for both the Srpk1 homolog Dsk1 and the Clk/Sty homolog Kic1/Lkh1. We found extensive phosphorylation within the RS domains of the SR proteins Srp1 and Srp2, as well as the SR-like proteins U2AF^{59} and Rsd1. In addition, we identified proteins that are also phosphorylated in humans, such as Srrm1 and U1-70K, even though their fission yeast homologs have a reduced RS domain. Perhaps most surprisingly phosphorylation was identified in core splicing factors such as Prp8, Bpb1, and Sap155. We next found that *dsk1-?* results in a defect in spliceosome complex A assembly in extracts, which further underlines the importance of these phosphorylation events in splicing. Expanding on this result, we analyzed the genome-wide effect of *dsk1-?* and *kic1-?* on splicing and found that *dsk1-?* has a broad splicing defect. To confirm the importance of phosphorylation on splicing in a titratable manner, we then integrated a gatekeeper mutated *dsk1* into yeast and observed splicing defects by specifically inhibiting its activity using a small molecule inhibitor. In the future, we will use varying amounts of inhibitor to analyze genetic interactions using an epistatic miniarray profile (EMAP) and *in vitro* assembly assays. We are currently investigating the *in vivo* importance of specific sites of phosphorylation by integrating alanine mutants of kinase substrates into yeast. Early analysis of these alanine mutants indicates that the Dsk1 phosphorylation of Bpb1 is required for normal growth. We now plan to use the microarrays to analyze splicing globally with the alanine mutants as well as genetic interactions via an EMAP and *in vitro* assembly assays. Our results from yeast support parallel results in humans and provide an evolutionary view of how phosphorylation is vital for splicing.

Keynote: Thomas R. Cech

Time:	Thursday, June 13, 20:00 - 20:45
Location:	Davos Ballroom
Abstract:	81
Chair(s):	Eric Westhof, Université de Strasbourg

81 **The Future of RiboScience**

81 The Future of RiboScience

Thomas R. Cech[1]

[1]University of Colorado, Boulder

Big Data and computational biology have accelerated the pace of discovery in RNA research. From riboswitches to lncRNAs to CRISPR to circular RNAs that act as miRNA sponges, new classes of functional noncoding RNAs are appearing with a dizzying frequency. The power of combining genome-wide and transcriptome-wide approaches with traditional biochemistry and cell biology is influencing the vision for new research institutions around the world. It also needs to influence how we educate our graduate and undergraduate students, so that they see the connections between physics, chemistry, computer science, engineering and biology and can harness multiple disciplines to address problems in RNA research and in biology more generally.

Plenary 4: **Architecture of RNPs**

Time: Thursday, June 13, 20:45 - 12.30
Location: Davos Ballroom
Abstracts: 82 - 88
Chair(s): Eric Westhof, Université de Strasbourg

82	**The architecture of Tetrahymena telomerase holoenzyme**
83	**Deciphering the assembly of box C/D snoRNP complexes**
84	**Crystal Structure of the Bacterial Pnkp1/Rnl/Hen1 Heterohexamer: A New RNA Repair Complex**
85	**Molecular basis of translation activation by the non-coding RNA RsmZ**
86	**Single-molecule analysis of L7Ae protein binding to a k-turn : induced fit or conformational selection ?**
87	**The structural basis of SRP receptor recruitment and GTPase activation by SRP RNA**
88	**Crystal structure of Prp8 and its implications for the spliceosomal active site**

82 The architecture of Tetrahymena telomerase holoenzyme

Jiansen Jiang[1], Edward J. Miracco[3], Kyungah Hong[6], Barbara Eckert[6], Henry Chan[4], Darian D. Cash[3], Bosun Min[6], Z. Hong Zhou[5], Kathleen Collins[6], Juli Feigon[2]

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Telomerase adds telomeric repeats to chromosome ends using an internal RNA template and specialized telomerase reverse transcriptase (TERT), thereby maintaining genome integrity. Little is known about the physical relationships among protein and RNA subunits within a biologically functional holoenzyme. Here we describe the architecture of *Tetrahymena thermophila* telomerase holoenzyme determined by electron microscopy. Six of the 7 proteins and the TERT-binding regions of telomerase RNA (TER) have been localized by affinity labeling. Fitting with high-resolution structures reveals the organization of TERT, TER, and p65 in the RNP catalytic core. Among the other holoenzyme proteins, p50 has an unanticipated role as a hub between the RNP catalytic core, p75-p19-p45 subcomplex, and the DNA-binding Teb1. A complete *in vitro* holoenzyme reconstitution assigns function to these interactions in processive telomeric repeat synthesis. These studies provide the first view of the extensive network of subunit associations necessary for telomerase holoenzyme assembly and physiological function.

83 Deciphering the assembly of box C/D snoRNP complexes

Jonathan Bizarro[1], Bérengère Pradet-Balade[1], Marc Quinternet[2], Xavier Manival[2], Bruno Charpentier[2], Christiane Branlant[2], Céline Verheggen[1], Edouard Bertrand[1]

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Box C/D small nucleolar Ribonucleoproteins (C/D snoRNPs) are essential complexes for gene expression as they direct post-transcriptional modifications of rRNAs in the nucleolus. Box C/D snoRNAs are assembled with a set of four core proteins. Their assembly occurs in the nucleoplasm but the mechanisms involved are not yet clearly defined although we know several assembly factors such as Nufip and the HSP90/R2TP system, which contain the key AAA+ ATPases Rvb1 and Rvb2.

Here, we described a new snoRNP assembly factor named Trip3. We showed that Trip3 dimerizes with Nufip and is important for first steps of snoRNP assembly. By quantitative SILAC proteomics, we found that Nufip/Trip3 associates with part of the R2TP complex and some snoRNP core proteins to form a complex devoid of RNA. This complex is subsequently recruited on nascent box C/D snoRNA, and this occurs concomitantly with removal of Trip3 while Nufip is removed at a later stage. By performing detailed mutagenesis coupled to NMR studies and structural modeling, we obtained a model of the interaction of a fragment of Nufip with pre-snoRNP. This model shows that Nufip keeps pre-snoRNPs in an inactive state, and that Nufip must be removed to allow formation of the active snoRNP structure. We propose that this is catalyzed by the AAA+ ATPases Rvb1/2.

84 Crystal Structure of the Bacterial Pnkp1/Rnl/Hen1 Heterohexamer: A New RNA Repair Complex

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Ribotoxins cleave essential RNAs for cell killing *in vivo*, and we have previously shown that the bacterial Pnkp/Hen1 RNA repair complex was able to repair ribotoxin-cleaved RNAs *in vitro*. Because of 2'-O-methylation by bacterial Hen1 during RNA repair, the repaired RNA resists future cleavage by the same ribotoxin that causes the original damage. Through bioinformatic analysis, we have recently found a new bacterial RNA repair complex that is related to, but also distinct from, the Pnkp/Hen1 complex. The newly discovered RNA repair complex is composed of three proteins, named Pnkp1, Rnl, and Hen1, which form a heterohexamer *in vitro*. To provide insight into the mechanism of RNA repair and shed light on potential *in vivo* RNA substrates, we solved the crystal structure of the 270 kDa Pnkp1/Rnl/Hen1 heterohexamer with cofactors (ATP, SAH, and Mg^{2+}) occupying all eight enzymatic active sites. The structure reveals the architecture of Pnkp1/Rnl/Hen1 heterohexamer as two ring-shaped Pnkp1/Rnl/Hen1 fused at Pnkp1, which forms a homodimer. Each Pnkp1/Rnl/Hen1 ring is formed through pairwise protein-protein interactions between these three proteins, but the interactions are *trans* in nature (e.g., Pnkp1a-to-Rnla-to-Hen1a-to-Pnkp1b, and vice versa). Four active sites that are required for RNA repair (kinase, phosphatase, methyltransferase, and ligase) are located on the inner rim of each ring, allowing RNA repair to be carried out efficiently. A lack of a specific RNA-binding groove/cleft and a wide opening at the center of the ring allow a variety of damaged RNAs to access the four active sites for repair, indicating that the Pnkp1/Rnl/Hen1 heterohexamer might be an all-purpose RNA repair complex.

85 Molecular basis of translation activation by the non-coding RNA RsmZ

Olivier Duss[1], *Maxim Yulikov*[1], *Erich Michel*[1], *Mario Schubert*[1], *Gunnar Jeschke*[1], *Frédéric Allain*[1]

[1]ETH Zürich

In bacteria, sRNAs (small regulatory/ non-coding RNAs) coordinate global changes in gene expression. The most important global post-transcriptional regulatory system responsible for bacterial virulence is the Csr/Rsm system, in which a sRNA (CsrB/RsmZ) activates translation initiation by sequestering a homo-dimeric protein (CsrA/RsmE) that is binding to the ribosome binding site of a subset of mRNAs [1, 2]. However, the mechanism of translation derepression is only partially understood on the molecular and atomic level.

Here we show for *Pseudomonas fluorescens* that several RsmE protein dimers are assembling sequentially, specifically and cooperatively onto the sRNA RsmZ, while binding of the third RsmE protein dimer changes RsmZ from an RNase E accessible to a protected form. Furthermore, we elucidated the 70 kDa solution structure of RsmZ bound to three RsmE proteins using a combinatorial approach consisting of nuclear magnetic resonance and electron paramagnetic resonance spectroscopy as well as multiple segmental isotope labeling of the RNA [3].

Strikingly, we discovered two similarly populated conformations in solution, which cannot directly interconvert between each other. Their interconversion requires the dissociation of all three proteins and thus, shows that the first RsmE protein binds the sRNA RsmZ by conformational selection. To our knowledge, both conformations represent a global RNA fold, which has not been described before. The structures visualize how the sRNA can bind several RsmE protein dimers with high affinity by using the helical stems as clamps which tightly grab the proteins. Furthermore, both conformations explain why the third RsmE protein dimer binding to RsmZ is protecting the RNA from RNase E mediated cleavage.

In conclusion, our findings illustrate the molecular basis of translation activation by the sRNA RsmZ and propose how the targeted proteins could ultimately be released from sRNA sequestration.

[1] Waters L. *et al*. (2009) Cell

[2] Lapouge K. *et al*. (2010) Mol Microbiol

[3] Duss O. *et al*. (2010) Nucleic Acids Res

86 Single-molecule analysis of L7Ae protein binding to a k-turn : induced fit or conformational selection

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When binding of a protein causes a conformational transition to occur in the target RNA this can in principle result from an active induced fit process, or a passive conformational selection. The k-turn is a commonly-occurring structural motif that introduces a tight kink into duplex RNA. In free solution it can exist in an extended form, or be folding into the kinked structure. Binding of proteins including the L7Ae family generate the formation of the kinked geometry, raising the question of whether this occurs by conformational selection of the kinked structure, or a more active induced fit process in which the protein manipulates the RNA structure.

We have devised a single-molecule FRET experiment whereby immobilized L7Ae protein binds Cy3-Cy5-labelled RNA from free solution. We find that all bound RNA is in the kinked geometry, with no evidence for transitions to an extended form at a millisecond timescale. Furthermore, real-time binding experiments provide no evidence for a more extended transient intermediate during the binding process.

The data support a passive model by which the protein selects a fraction of RNA that is already in the kinked conformation, thereby drawing the equilibrium into this form.

87 The structural basis of SRP receptor recruitment and GTPase activation by SRP RNA

Nikolaus Schmitz[1], *Felix Voigts-Hoffmann*[1], *Kuang Shen*[2], *Shu-ou Shan*[2], *Sandro F. Ataide*[3], *Nenad Ban*[1]

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The Signal Recognition Particle (SRP) pathway is a universally conserved membrane protein targeting system, which recognizes ribosome nascent chain complexes (RNCs) exposing a SRP signal sequence (cargo). The cargo is subsequently transferred to the translocon, which mediates the co-translational transport of the emerging polypeptide across the lipid bilayer and also guides the insertion and folding of membrane proteins.

Transfer of the cargo to the translocon involves a large scale rearrangement of the SRP:receptor complex during which the SRP RNA plays a pivotal role in both receptor recruitment and GTPase activation. We have determined the crystal structure of prokaryotic SRP:receptor GTPase domain heterodimers bound to SRP RNA at the *tetraloop* and the *distal site* at 2.6 Ångstrom resolution.

The interactions at the *tetraloop* reveal the structural basis of receptor recruitment and rationalize previously accumulated biochemical and structural data on the dynamics of this process. Strikingly, interactions between the SRP receptor and the SRP RNA at the *tetraloop* as well as at the *distal site* involve the insertion box domain (IBD), a domain that is unique to the SRP-GTPase family.

At the *distal region*, a flipped-out base of the SRP RNA inserts into the cleft between the two proteins and stimulates hydrolysis of receptor bound GTP by reordering of a side chain provided *in trans* by Ffh. Biochemical data confirm that these fully conserved residues are essential for GTP hydrolysis and efficient protein translocation.

The structural findings combined with biochemical experiments reported in this study reveal the molecular basis of the SRP receptor recruitment to the tetraloop of the SRP RNA and allow us to suggest a possible mechanism for GTPase activation at the distal site of the SRP RNA.

88 Crystal structure of Prp8 and its implications for the spliceosomal active site

Wojciech Galej[1], *Chris Oubridge*[1], *Andy Newman*[1], *Kiyoshi Nagai*[1]

[1]MRC Laboratory of Molecular Biology

The active centre of the spliceosome consists of an intricate network formed by U5, U2 and U6 small nuclear RNAs, and a pre-messenger-RNA substrate. Prp8, a component of the U5 small nuclear ribonucleoprotein particle, crosslinks extensively with this RNA catalytic core. I will present the crystal structure of yeast Prp8 (residues 885-2413) in complex with Aar2, a U5 small nuclear ribonucleoprotein particle assembly factor. The structure reveals tightly associated domains of Prp8 resembling a bacterial group II intron reverse transcriptase and a type II restriction endonuclease (Figure 1). Suppressors of splice-site mutations, and an intron branch-point crosslink, map to a large cavity formed by the reverse transcriptase thumb, the endonuclease-like and RNaseH-like domains. Our structure provides first structural insights into the architecture of the spliceosome active site, and reinforces the notion that nuclear pre-mRNA splicing and group II intron splicing have a common evolutionary origin.

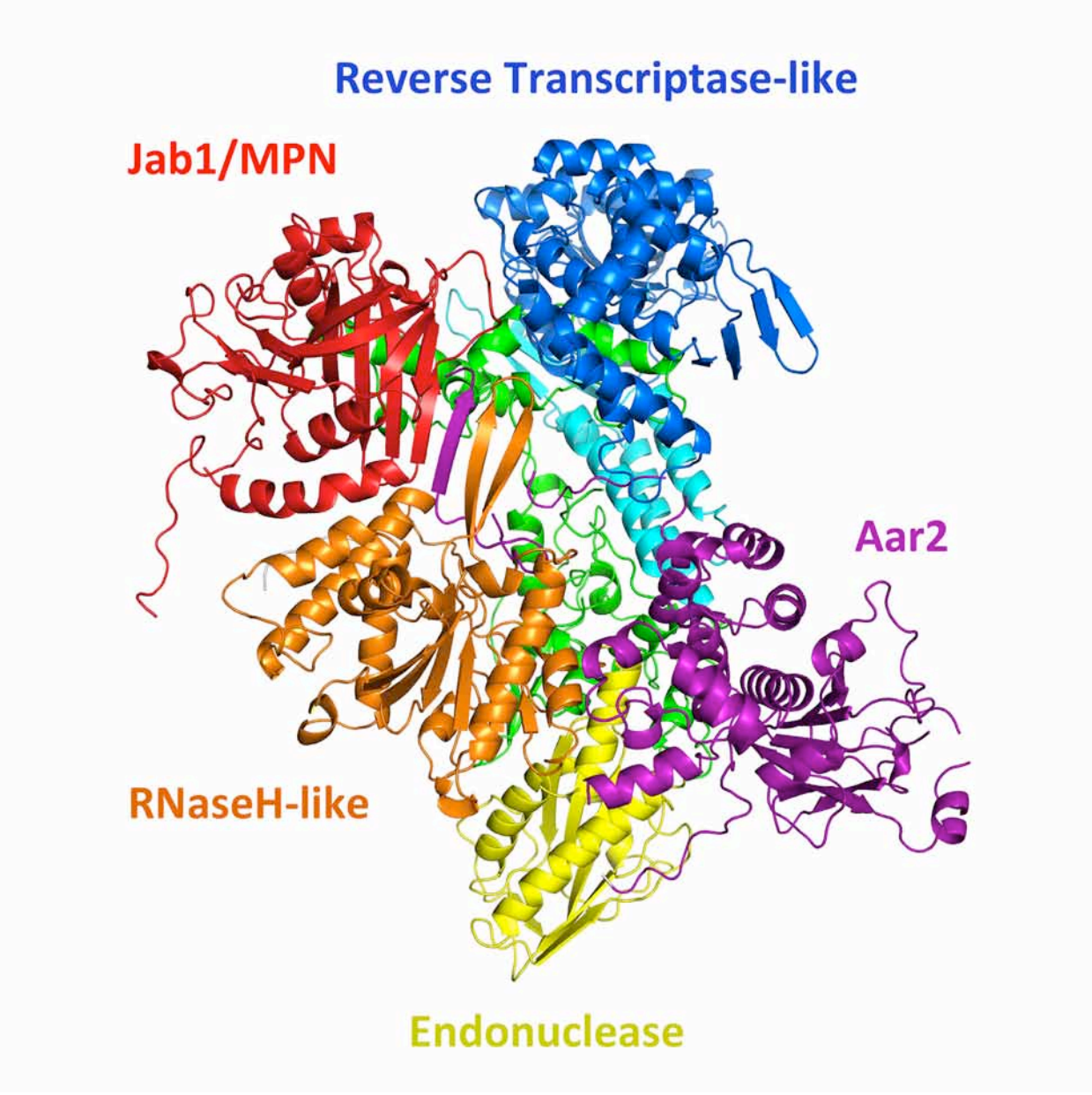

Figure 1

Plenary 5A: **RNA processing**

Time: Friday, June 14, 8:30 - 10:15

Location: Davos Ballroom

Abstracts: 89 - 95

Chair(s): Joan Steitz, *HHMI, Yale University*

89 **Structural and functional studies of pre-mRNA 5' and 3'-end processing**

90 **Analysis of eukaryotic orthologous groups reveals Archease as a crucial factor in human tRNA splicing.**

91 **Control of myogenesis by rodent SINE-containing lncRNAs**

92 **DNA Damage induces targeted, genome-wide variation of poly(A) sites in budding yeast**

93 **Polyadenylated histone mRNAs accumulate upon PARN knock-down**

94 **Non-coding Y1/3 RNAs promote the 3'-processing of canonical histone pre-mRNAs**

95 **Dicer-2 is involved in mRNA activation through cytoplasmic polyadenylation**

89 Structural and functional studies of pre-mRNA 5’ and 3’-end processing

Liang Tong[1]

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Most eukaryotic mRNA precursors (pre-mRNAs) must undergo extensive processing, which includes 5'-end capping, splicing and 3'-end cleavage and polyadenylation. We have been studying the structural basis for the organization and activity of the 3'-end processing machinery, and have reported the crystal structures of several proteins in this machinery. Histone pre-mRNAs are distinct in that they possess a stem-loop (SL) structure near the 3'-end. Stem-loop binding protein (SLBP) specifically recognizes the SL and regulates most aspects of histone mRNA metabolism. We have determined the crystal structure of a 26-nt histone mRNA SL in complex with human SLBP RNA binding domain and the human 3'-5' exonuclease 3'hExo (also known as Eri-1).

5'-end capping occurs early during transcription and it was generally believed that the capping process is always successful and no quality control mechanism was known. Our studies of yeast Rai1, a protein partner of the nuclear 5'-3' exonuclease Rat1, unexpectedly revealed that it is a novel eukaryotic enzyme with RNA 5'-end pyrophosphohydrolase (PPH) activity as well as decapping activity (but only toward unmethylated caps). These observations led us to demonstrate the presence of RNA 5'-end capping defects in yeast, and that Rai1 is a central player in a novel RNA quality surveillance pathway, promoting the degradation of such defective RNAs.

Our recent studies with Dom3Z, the mammalian homolog of Rai1, showed that it has PPH, decapping as well as 5'-3' exoribonuclease activities, and hence we have renamed it DXO (decapping exonuclease). DXO preferentially degrades defectively capped pre-mRNAs in cells, consistent with its role in 5'-end capping quality surveillance. More importantly, we found that incompletely capped pre-mRNAs are inefficiently spliced at internal introns, in contrast to current understanding where capping is linked to the splicing of only the first intron. We have also determined the crystal structures of mouse DXO in complex with substrate mimic and product RNAs at up to 1.5 Å resolution, which provide elegant insights into the catalytic mechanism of DXO and the molecular basis for its three apparently distinct catalytic activities.

Supported in part by NIH grants GM077175 and GM090059.

90 Analysis of eukaryotic orthologous groups reveals Archease as a crucial factor in human tRNA splicing.

Johannes Popow[1], Alexander Schleiffer[2], Javier Martinez[1]

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RNA ligases catalyze the ligation of RNA in eukaryotes, archaea and bacteria and play essential roles in many processes including RNA repair and stress-induced splicing of mRNA. In archaebacteria and eukaryotes, RNA ligases also join exonic sequences from transfer RNA precursors (pre-tRNAs) to generate functional tRNAs required for protein synthesis. We recently identified the RNA ligase required for tRNA splicing in humans, a multimeric protein complex having RTCB (also known as HSPC117, C22orf28, FAAP or D10Wsu52e) as the essential subunit. The human RNA ligase complex catalyzes RNA ligation only when purified from its native host indicating that additional, unknown components are required for its full activity. Here, taking advantage of clusters of eukaryotic orthologous groups (KOGs), we identified one protein of unknown function, Archease, as sharing its specific phyletic distribution with RTCB suggesting that these proteins cooperate to ligate RNA substrates. We show that Archease is required for maturation of tRNAs in human cells. Archease physically interacts with the human tRNA ligase complex, facilitating the formation of an RTCB- guanylate intermediate by the release of GMP associated after an initial round of catalysis. The widespread co-occurrence of Archease and RtcB proteins in bacterial and archaebacterial operons implies evolutionary conservation of the functional interplay between these two proteins.

91 Control of myogenesis by rodent SINE-containing lncRNAs

Jiashi Wang[1], *Chenguang Gong*[1], *Lynne Maquat*[1]

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Staufen1 (STAU1)-mediated mRNA decay (SMD), which also involves STAU2[1], degrades mRNAs that harbor a STAU-binding site (SBS) in their 3'-untranslated regions (3'UTRs)[2]. We have reported that human SBSs can be formed by intramolecular base-pairing, as exemplified by a 19-base-pair stem and 100-nucleotide apex within mRNA encoding human ADP ribosylation factor 1 (ARF1)[3]. More recently, we have found that human SBSs can also form by intermolecular base-pairing between an mRNA 3'UTR Alu element and an Alu element within a cytoplasmic and polyadenylated long noncoding RNA (lncRNA), called a ½-sbsRNA[4]. Such long (up to ~300-base-pair) duplexes undoubtedly bind more than one STAU1 and/or STAU2 molecule, which are known to homo- and hetero-dimerize[5] if not multimerize. Alu elements are a type of short interspersed element (SINE) and derive from 7SL RNA. Since Alu elements are confined to primates, it was unclear how SMD occurs in rodents. SMD is clearly important to rodents as evidenced by our finding that the efficiency of SMD increases during the differentiation of mouse C2C12 myoblasts to myotubes in a way that promotes the myogenic process[6]. SINEs in rodents include B1, B2 and B4 elements, which are evolutionarily unrelated. Here we mine the mouse transcriptome and report that 13.2% of annotated mouse mRNAs contain a single 3'UTR B SINE, and 28.4% of known mouse lncRNAs contain one or more B SINE. We show that SMD occurs in mouse cells via mRNA-lncRNA base-pairing of partially complementary SINEs (i.e., B1-B1, B2-B2 or B4-B4). A single mRNA can be regulated by multiple lncRNAs, and one lncRNA can regulate multiple mRNAs. We also provide evidence that mouse (m)½-sbsRNA-triggered SMD regulates C2C12 cell myogenesis. For example, the SMD of tumor necrosis factor receptor-associated factor 6 (mTraf6) mRNA by m½-sbsRNA2, whose abundance is upregulated during the differentiation of C2C12 myoblasts to myotubes, promotes myogenesis. Our findings define new roles for lncRNAs and B SINEs in mouse that undoubtedly influence many developmental and homeostatic pathways.

[1]Park E, Gleghorn ML, Maquat LE (2013) *Proc Natl Acad. Sci USA*, 110: 405-412.

[2]Park E, Maquat LE (2013) *Wiley Interdiscip Rev RNA*, in press.

[3]Gong C, Maquat LE (2011) *Cell Cycle* 10: 1882-1883.

[4]Gong C, Maquat LE (2011) *Nature* 470: 284-288.

[5]Gleghorn ML, Gong C, Kielkopf CL and Maquat LE (2013) *Nat Struct Mol Biol*, in press.

[6]Gong C, Kim YK, Woeller CF, Tang Y, Maquat LE (2009) *Genes Dev* 23: 54-66.

92 DNA Damage induces targeted, genome-wide variation of poly(A) sites in budding yeast

Joel Graber[1], Fathima Nazeer[4], Pei-chun Yeh[4], Jason Kuehner[2], Sneha Borikar[3], Derick Hoskinson[4], Claire Moore[5]

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Systemic response to DNA damage and other stresses is a complex process that includes changes in the regulation and activity of nearly all stages of gene expression. One gene regulatory mechanism used by eukaryotes is selection among alternative transcript isoforms that differ in polyadenylation (poly(A)) sites, resulting in either changes to the coding sequence or portions of the 3'-UTR that govern translation, stability, and localization. To determine the extent to which this means of regulation is used in response to DNA damage, we conducted a global analysis of poly(A) site usage in *Saccharomyces cerevisiae* after exposure to the UV mimetic, 4-nitroquinoline 1-oxide (4NQO). 2031 genes were found to have significant variation in poly(A) site distributions following 4NQO treatment, with a strong bias towards loss of shortened transcripts, including many with poly(A) sites located within the protein coding sequence (CDS). The change in poly(A) site profile was associated with an inhibition of cleavage and polyadenylation in cell extract and a decrease in the levels of several subunits in the mRNA 3'-end processing complex. Sequence analysis identified differences in the cis-acting elements that flank putatively suppressed and enhanced poly(A) sites, as well as a common pattern that suggests a general model for differentiating variable from constitutive poly(A) sites. Our analysis suggests that variation in transcripts that differ in their poly(A) sites is an important part of the regulatory response to DNA damage.

93 Polyadenylated histone mRNAs accumulate upon PARN knock-down

Claudia Weißbach[1], Christiane Harnisch[1], Heike Berndt[3], Lars Anders[1], Elmar Wahle[2]

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The poly(A)-specific ribonuclease (PARN) preferentially degrades poly(A). Although a 3' exonuclease, the enzyme is stimulated by a 5' cap, thus mRNAs are likely substrates. However, PARN is not involved in the degradation of bulk mRNA; instead it processes H/ACA box snoRNAs[1] and degrades a small subset of specific mRNAs[2 3]. Using PARN knock-down we identified histone mRNAs as likely substrates by microarray and qRT-PCR analyses. Whereas histone mRNAs normally do not carry poly(A) tails, cDNA sequencing, Northern blot and ribonuclease protection assays show that polyadenylated histone mRNAs accumulate upon PARN knock-down. The poly(A) tails were up to ~200 nt long, and in many cases a canonical polyadenylation signal was present just upstream of the polyadenylation site, suggesting that the regular mRNA 3' end processing machinery is responsible for poly(A) addition. In cells transfected with a histone construct carrying a mutation in the histone downstream element, read-through transcripts appear in large amounts. Using this construct, we will address whether PARN plays a role in a "fail-safe mechanism" to produce correctly processed histone mRNAs lacking a poly(A) tail or whether the enzyme participates in the degradation of misprocessed histone mRNAs.

Preliminary data suggest that a PARN-knock-down may affect the cell cycle. This is interesting as improper histone stoichiometry leads to genomic instability[4].

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2. Lee, J. E. et al. The PARN deadenylase targets a discrete set of mRNAs for decay and regulates cell motility in mouse myoblasts. PLoS Genet. 8, e1002901 (2012).

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94 Non-coding Y1/3 RNAs promote the 3'-processing of canonical histone pre-mRNAs

Marcel Köhn[1], *Stefan Hüttelmaier*[2]

[1]Martin-Luther-University Halle-Wittenberg, Institute of Molecular Medicine, Sect. of Molecular Cell Biology; [2]Martin-Luther-University Halle-Wittenberg

The human Y RNAs constitute a family of four small (85-115nt) non-coding (nc) RNAs (Y1, Y3, Y4 and Y5). They are transcribed from individual genes by RNA-polymerase III and fold into characteristic stem-loop-structures. The depletion of Y RNAs in *Xenopus* and *Zebrafish* leads to developmental defects, resulting in embryonic lethality at the mid-blastula-stage. Despite their essential role during development the cellular role of Y RNAs remains largely elusive.

To characterize the function of Y RNAs in mammalian cells, we aimed at identifying proteins associated with these ncRNAs using Streptavidin-pulldown-assays. MS-analyses identified various Y RNA-associated proteins involved in the 3'-processing of pre-mRNAs, for instance subunits of CPSF (Cleavage and polyadenylation specificity factor). To test a potential role of Y RNAs in the 3'-processing of mRNAs, we used *chimeric antisense-Oligonucleotide* (ASO)-mediated knockdown in HEK293 cells. The depletion of Y1 and more prominently Y3 but not Y4 impaired the processing of replication-dependent histone mRNAs. Notably, the depletion of Y3 also induced an up-regulation of histone mRNA polyadenylation supporting a role of this RNA in the processing of histone mRNAs. This was essentially mediated via the U-rich region within the Y3 loop that promotes association with the histone pre-mRNA cleavage complex (HCC). However, binding of the HCC to the Y3 RNA was facilitated via FIP1L1/CPSF30 that directly associates with this U-rich region. Both factors are essential components of the HCC and facilitate the RNA dependent anchorage of the HCC on Y3 as well as histone pre-mRNAs. Pre-formation of this complex on Y3 enhances the association of the HCC with Histone-reporter RNAs *in vitro* and promotes the processing of histone pre-mRNAs in cells. We propose that Y3 RNA serves as a binding scaffold required to activate the HCC in order to facilitate efficient histone pre-mRNA processing in *trans*. This provides a new concept suggesting a pivotal role of ncRNAs in the assembly of mRNA processing complexes.

95 Dicer-2 is involved in mRNA activation through cytoplasmic polyadenylation

Ana Villalba[1], Olga Coll[1], Tanit Guitart[1], Catherine Papin[2], Martine Simonelig[2], Fátima Gebauer[1]

[1]Centre for Genomic Regulation (CRG) and UPF, Barcelona (Spain); [2]Institute of Human Genetics, CNRS, Montpellier (France)

Cytoplasmic poly(A) tail elongation is a widespread mechanism to regulate mRNA translation. The biochemistry of cytoplasmic polyadenylation has been elucidated in vertebrates, where it requires two sequence elements in the 3' UTR of substrate mRNAs: the U-rich cytoplasmic polyadenylation element (CPE) and the AAUAAA hexanucleotide (Hex). We have recently found CPE- and Hex- independent cytoplasmic polyadenylation in *Drosophila* (1). This "non-canonical" polyadenylation mechanism operates on maternal *Toll* mRNA and requires a region distal to the 3' end of the mRNA which we term PR (Polyadenylation Region).

To identify the components of the non-canonical cytoplasmic polyadenylation machinery we have performed RNA affinity chromatography using PR as bait. We have found that the cytoplasmic poly(A) polymerase Wispy and the siRNA processing factor Dicer-2 specifically associate to PR. Depletion and co-immunoprecipitation studies indicate that Dicer-2 interacts with Wispy and is necessary for polyadenylation of *Toll* reporters *in vitro*. Furthermore, embryos derived from Wispy or Dicer-2 mutant mothers show defects in polyadenylation of endogenous *Toll* mRNA. These results uncover a novel function of Dicer-2 in activation of mRNA translation through cytoplasmic polyadenylation.

(1) Coll et al. A novel, non-canonical mechanism of cytoplasmic polyadenylation operates in *Drosophila* embryogenesis. Genes Dev. 2010, 24: 129-134.

Plenary 5B: **Session P5B: RNA decay**

Time: Friday, June 14, 10:45 - 12:30
Location: Davos Ballroom
Abstracts: 96 - 102
Chair(s): Elena Conti, *MPI Biochemistry*

96	**Assembly and function of the NOT module of the CCR4-NOT complex**
97	**Structural insights into the Not module of the Ccr4-Not complex**
98	**Roquin Promotes Constitutive mRNA Decay via a Conserved Class of Stem-Loop Recognition Motifs**
99	**The crystal structure of the nucleolar exosome engaged with RNA**
100	**Nonsense mediated mRNA decay is inefficient on long ORF transcripts**
101	**eIF4E-bound mRNPs are substrates for nonsense-mediated mRNA decay in mammalian cells**
102	**PAXT-1 binds XRN-2 and promotes its activity**

96 Assembly and function of the NOT module of the CCR4-NOT complex

Ying Chen[1], Andreas Boland[1], Tobias Raisch[1], Stefanie Jonas[1], Duygu Kuzuoglu-Öztürk[1], Lara Wohlbold[1], Oliver Weichenrieder[1], Elisa Izaurralde[1]

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The CCR4-NOT complex plays a crucial role in post-transcriptional mRNA regulation in eukaryotic cells. It catalyzes the removal of mRNA poly(A) tails, thereby repressing translation and committing mRNAs to degradation. The complex consists of a catalytic module comprising two deadenylases (POP2/CAF1 and CCR4) and the NOT module minimally containing the NOT1, NOT2 and NOT3 subunits. It is known that NOT1 acts as a scaffold protein for the assembly of the CCR4-NOT complex. However, the mechanism by which the NOT2 and NOT3 proteins interact with each other and dock onto the NOT1 scaffold remains unknown. NOT2 and NOT3 are related proteins that both contain a highly conserved C-terminal domain referred to as "NOT-box". Here we show that the NOT-box is a heterodimerization domain mediating the assembly of the NOT2-NOT3 subcomplex. We have solved the crystal structures of the human NOT2 and NOT3 NOT-boxes at 2.4Å and 2.5Å resolution, respectively. The NOT-box consists of a four-stranded C-terminal open b-barrel as well as N-terminally located a-helices, which are required for heterodimerization. We also defined the domains of NOT1, NOT2 and NOT3 required for the assembly of the NOT1-NOT2-NOT3 module. Functional studies in *Drosophila melanogaster* cells revealed that depletion of NOT1, NOT2 or NOT3 inhibits mRNA deadenylation with a stronger effect for the NOT1 depletion, followed by NOT3. Importantly, NOT3 depletion destabilizes both NOT1 and NOT2 indicating that one important function of NOT3 is the stabilization of the NOT1 scaffold. We used mutagenesis and functional studies to identify key residues in the NOT module required for mRNA deadenylation. These studies revealed that the interaction of NOT1 with NOT2-NOT3 heterodimers is required for deadenylation in *D. melanogaster* cells. Similarly, NOT3 mutants that do not interact with NOT1 cannot restore deadenylation in cells depleted of endogenous NOT3. Collectively, our data shed light on the assembly of the CCR4-NOT complex and provide the basis for dissecting the role of this complex in mRNA deadenylation.

97 Structural insights into the Not module of the Ccr4-Not complex

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Deadenylation is the first and rate-limiting step in mRNA turnover. Shortening of the poly-A tail is catalyzed by the processive deadenylation activity of the Ccr4-Not complex and the distributive deadenylation activity of the Pan2-Pan3 complex. Both complexes are conserved in all eukaryotes examined to date and have been recently shown to be involved in microRNA-mediated silencing in human cells. The Ccr4/Not complex is a multisubunit complex organized around a large scaffold protein, Not1. The complex has a modular architecture with at least two distinct modules: a "deadenylase module" and a "Not module". Previous studies have revealed the structure and have dissected the activities of the deadenylase module. However, very limited information on the structure and function of the Not module is currently available. We have mapped the interacting domains of yeast Not1, Not2 and Not5 proteins and have determined the 2.8 Å structure of this ternary complex that forms the core of the Not module. The structure shows how the Sm-like domains of Not2 and Not5 dock on to an extensive HEAT-repeat region in Not1. Based on the structural information, we are testing possible functions of the Not module using biochemical *in vitro* assays and *in vivo* experiments in yeast.

98 Roquin Promotes Constitutive mRNA Decay via a Conserved Class of Stem-Loop Recognition Motifs

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Tumor necrosis factor alpha (TNFa) is the most potent pro-inflammatory cytokine in mammals. Numerous posttranscriptional mechanisms control the expression of this potentially harmful cytokine, including an AU-rich element (ARE) and a constitutive decay element (CDE) in the 3'UTR. Whereas ARE-mediated mRNA decay is transiently blocked during macrophage activation, the CDE causes constitutive mRNA decay, thereby limiting the expression of this cytokine under pro-inflammatory conditions. We now demonstrate that the CDE is a 17 nt long sequence that does not serve as a microRNA binding site. Rather, structural probing and mutagenesis provide evidence that it forms a short RNA stem-loop in its active conformation.

Using an improved streptavidin-binding aptamer-tag for RNA affinity purification, we identified CDE-associated proteins by mass spectrometry. Thereby, we found that the CCCH-type zinc and RING finger proteins Roquin (Rc3h1) and Roquin2 (Rc3h2) are CDE-binding proteins. RNA-IP, crosslinking and EMSA experiments confirmed that the ROQ domain of Roquin specifically and directly binds to the CDE stem-loop in cells and *in vitro*. Overexpression and knockdown analyses further showed that Roquin suppresses TNFa mRNA and protein expression in macrophages. TNFa mRNA expression was also upregulated by introduction of a morpholino that interferes with the Roquin-CDE interaction. By affinity purification followed by mass spectrometry, we found that Roquin and Roquin2 recruit the Ccr4-Caf1-Not deadenylase complex and thereby promote mRNA degradation.

To identify CDEs on a genome-wide scale, we developed a bioinformatics approach based on experimentally determined sequence and structural requirements for an active CDE. Thereby, we identified more than 50 highly conserved CDEs in the mouse transcriptome, many of which encode regulators of development and inflammation. RNA-Seq of Roquin-associated mRNAs in macrophages confirmed that CDE-containing mRNAs are the primary targets of Roquin. Taken together, we demonstrate that Roquin proteins act as major mediators of mRNA deadenylation by recognizing CDEs, a novel class of conserved stem-loop RNA degradation motifs.

99 The crystal structure of the nucleolar exosome engaged with RNA

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The RNA exosome is an essential, multisubunit and multipurpose ribonuclease required for processing a large variety of coding and noncoding RNAs. The exosome core includes nine distinct subunits (Exo9) with a six-membered RNase PH-like ring on the bottom, and three S1/KH binding proteins on the top (Liu *et al*., 2006). The exosome core is catalytically inert but includes a prominent central channel that is lined with RNA binding surfaces. RNA exosome ribonuclease activity is catalyzed by two enzymes that associate with the exosome core, Rrp6 and Rrp44. Rrp6, a member of the RNase D family, is a distributive 3' to 5' exoribonuclease that associates with Exo9 in the nucleolus (Tomecki *et al*., 2010) to form a 10 subunit complex, Exo10^{6}. In the nucleus, Rrp6 associates with Exo9 and the processive 3' to 5' exoribonuclease and endoribonuclease, Rrp44 (Allmang et al., 1999). A longstanding question in the field of exosome biology is how RNA is directed to either the Rrp6 or Rrp44 active sites for processing or decay. A recent crystal structure has illustrated how an Rrp44-associated exosome uses the entire length of the Exo9 central channel to funnel RNA from the top of the S1/KH ring, through the PH-like ring, and into the Rrp44 active site (Makino et al., 2013). However, how Rrp6 engages RNA, and its position on the Exo9 core, has remained unclear. Using x-ray crystallography, we have obtained a preliminary atomic resolution structure of reconstituted Exo10^{6} bound to an RNA substrate, the results of which will be discussed.

100 Nonsense mediated mRNA decay is inefficient on long ORF transcripts

Laurence Decourty[1], Antonia Doyen[1], Christophe Malabat[1], Emmanuel Frachon[3], Delphine Rispal[2], Bertrand Séraphin[2], Alain Jacquier[1], Cosmin Saveanu[1], Alain Jacquier[1]

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Nonsense mediated decay (NMD) is the most studied translation dependent mechanism for "quality control" of mRNAs, proposed to serve in removing aberrant mRNAs with premature termination codons from the cell. Conserved in eukaryotes and essential for mammals development, NMD was found to require the presence of a long 3' UTR region (Amrani et al., *Nature* 2004). Our results suggest that a long 3'UTR is required but not sufficient for efficient NMD.

We performed a large scale study of the importance of the region upstream the stop codon in triggering NMD. We have built *Saccharomyces cerevisiae* strains that allowed the testing of hundreds of natural transcripts expressed from their chromosomal location and modified to have the same long 3'UTR region, known to induce NMD. We have used our previously developed Genetic Interactions Mapping (GIM) method (Decourty et al., *PNAS* 2008) to test the effect of NMD inactivation on the steady state levels of 650 barcoded reporter mRNAs. We measured the barcoded transcript level changes in cells lacking Upf1 (Nam7) or Upf2 (Nmd2) in comparison with the corresponding wild-type strains. In contrast with short ORF containing RNAs, transcripts with long ORFs were only marginally affected by the addition of a long, 1.4 kb 3'UTR, a surprising result that was not predicted and could not be explained by current NMD models. The large scale results were confirmed on individual strains and mRNAs. Long ORF transcripts were only slightly destabilized by a long 3'UTR, with steady-state levels higher than 50% of those of the corresponding unmodified transcripts. Both long ORF and short ORF 3' extended mRNAs were highly enriched in a Upf1 (Nam7)-TAP purification to the same extent. Increasing the ORF length of a PGK1 NMD reporter gene, with an mRNA half-life of 3 minutes led to the stabilization of the corresponding mRNA despite the fact that the 3'UTR length, promoter, initiation and stop codon were identical in the two reporter mRNAs.

These data provide an essential piece of evidence for an "early termination" model in NMD, which proposes that translation termination efficiency is intrinsically dependent on the length of the translated ORF. According to this model, early termination is inefficient and, in this context only, NMD competes with "normal" termination to avoid ribosome stalling at stop codons. Thus, while a long 3' UTR is required for NMD, presumably through an increase in the local concentration of Upf1 (Hogg & Goff, *Cell* 2010; Kurosaki & Maquat *PNAS* 2013), our results show that only transcripts with short ORFs will be efficiently recognized and degraded through NMD. Our conclusions correlate with the fact that short ORFs are present in the vast majority of natural NMD substrates in yeast (He et al., *Mol. Cell*, 2003), are compatible with previous publications and give a new perspective on how a premature termination codon could be detected by the NMD process in eukaryotes.

101 eIF4E-bound mRNPs are substrates for nonsense-mediated mRNA decay in mammalian cells

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Eukaryotic mRNAs with premature translation-termination codons (PTCs) are recognized and degraded by a process referred to as nonsense-mediated mRNA decay (NMD). The evolutionary conservation of the core NMD factors UPF1, UPF2 and UPF3 would imply a similar basic mechanism of PTC recognition in all eukaryotes. However, unlike NMD in yeast, which targets PTC-containing mRNAs irrespectively of whether their 5' cap is bound by the cap-binding complex (CBC) or by the eukaryotic initiation factor 4E (eIF4E), mammalian NMD has been claimed to be restricted to CBC-bound mRNAs during the pioneer round of translation. In our recent study we compared decay kinetics of two NMD reporter systems in mRNA fractions bound to either CBC or eIF4E in human cells. Our findings reveal that NMD destabilizes eIF4E-bound transcripts as efficiently as those associated with CBC. These results corroborate an emerging unified model for NMD substrate recognition, according to which NMD can ensue at every aberrant translation termination event. Additionally, our results indicate that the closed loop structure of mRNA forms only after the replacement of CBC with eIF4E at the 5' cap.

102 PAXT-1 binds XRN-2 and promotes its activity

Takashi Miki[1], *Stefan Rueegger*[1], *Hannes Richter*[1], *Helge Grosshans*[1]

[1]Friedrich Miescher Institute for Biomedical Research

XRN-2 is a conserved 5'-to-3' exoribonuclease in eukaryotes, which is predominantly localized in the nucleus. Although XRN-2 functions in turnover and processing of various classes of RNA such as rRNA, snoRNA, and microRNA, little is known about how XRN-2 is regulated in these processes. In order to address this issue, we screened for XRN-2 interacting partners in *C. elegans*. Using two independent approaches, immunoprecipitation of XRN-2 and screening for synthetic lethality of a newly engineered *xrn-2* temperature-sensitive allele, we have identified PAXT-1 (PArtner of Xrn-Two) as a cofactor of metazoan XRN-2. Endogenous XRN-2 and PAXT-1 co-immunoprecipitate in an RNase-insensitive manner, and depletion of one reduces levels of the other. Although wild-type animals are unaffected by RNAi-mediated depletion of PAXT-1, *paxt-1* null animals, generated by genome engineering using TALENs (Transcription Activator-Like Effector Nucleases), are inviable at elevated temperature. Consistent with XRN-2 stabilization being a major function of PAXT-1, overexpression of XRN-2 can suppress this lethality. Thus we identified PAXT-1 as a novel cofactor of XRN-2, which stabilizes XRN-2 to promote its activity. We are currently investigating whether PAXT-1 enhances exoribonuclease activity of XRN-2 beyond stabilization of the protein. We will also examine functional conservation for a mammalian counterpart of PAXT-1.

Concurrent 4: Regulation by small non-coding RNAs

Time: Friday, June 14, 14:00 - 16:30
Location: Davos Ballroom
Abstracts: 103 - 111
Chair(s): Jennifer Doudna, HHMI, University of California - Berkeley

103	**Molecular Clues to Tissue-Specific Control of MiRNA Biogenesis**
104	**Mouse Tudor domain containing 12 (Tdrd12) is essential for biogenesis of piRNAs associating with the nuclear Piwi protein Miwi2**
105	**Regulation of miRNAs and endo-siRNAs during oocyte-to-zygote transition in the mouse**
106	**Kinetic and biophysical models improve identification of miRNA targets**
107	**Insights into the recruitment of the PAN2-PAN3 deadenylase complex to miRNA targets by the GW182/TNRC6 proteins**
108	**Translational repression and eIF4A2 activity are critical for microRNA-mediated gene regulation.**
109	**The conserved concave surface of the MIF4G domain of CNOT1 is involved in miRNA-mediated translational repression**
110	**Single-molecule observation of DNA targeting and cleavage by the RNA-guided Cas9 endonuclease**
111	**Processing-Independent CRISPR RNAs Limit Natural Transformation in Neisseria meningitidis**

103 Molecular Clues to Tissue-Specific Control of MiRNA Biogenesis

Nila Roy Choudhury[1], Flavia de Lima Alves[1], Luisa de Andrés-Aguayo[2], Thomas Graf[2], Javier F. Cáceres[4], Juri Rappsilber[3], Gracjan Michlewski[1]

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At present, around 2000 miRNAs are annotated in the human genome. In spite of the great effort to understand the various biological roles of individual miRNAs there is a huge void of knowledge about the regulation of their own biogenesis. Until now only a handful of miRNAs have been investigated individually for their features and trans-acting factors that play significant roles in their production.

We show the first report of tissue-specific control of brain-enriched miRNA biogenesis by defined tissue-specific factors. We demonstrate that the level of miR-7, which is processed from the ubiquitous hnRNP K pre-mRNA transcript, is shaped by the inhibition of its biogenesis in non-neural cells. Using SILAC combined with RNA pull down we reveal MSI2 and HuR to be factors that cooperatively inhibit miR-7 biogenesis by binding to its conserved terminal loop. We provide evidence that the role of HuR is to assist MSI2 binding, which is the actual factor regulating miR-7 maturation. Importantly, we present evidence that the MSI2/HuR complex increases the rigidity of the pri-miR-7 structure. Due to the fact that Microprocessor cleavage is dependent on DEAD-box RNA helicase activity, such stabilization provides the first mechanistic clue to the inhibitory effect of tissue-specific factors on the miRNA processing. Finally, we confirm the physiological relevance of MSI2/HuR-mediated inhibition of miR-7 biogenesis in human neuronal differentiation system and in MSI2 KO mouse.

Currently we are working on the mechanisms controlling biogenesis of other tissue-specific miRNAs. Importantly, our methodological approach provides a platform for future studies on miRNA biogenesis and on all other processes involving RNA-protein interactions.

104 Mouse Tudor domain containing 12 (Tdrd12) is essential for biogenesis of piRNAs associating with the nuclear Piwi protein Miwi2

Radha Raman Pandey[1], Zhaolin Yang[2], Ramesh S Pillai

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Transposons are selfish genetic elements that spread in the population by mobilizing themselves in the germline. A gonad-specific class of small RNAs (25-30 nucleotides long), called Piwi-interacting RNAs (piRNAs), provide protection against these elements, ensuring genome integrity and fertility. The initial pool of primary piRNAs is derived by processing single-stranded transcripts originating from discrete genomic loci called piRNA clusters, which then guide endonulceolytic cleavage of transposon transcripts. An epigenetic memory of this encounter is recorded in germ cells by generating a secondary piRNA from one of the transposon cleavage fragments. While flies use the newly generated secondary piRNA to initiate a piRNA amplification loop, mice integrate this information to effect DNA methylation of the loci containing active transposon elements. Here we identify a Tudor and helicase domain-containing protein, Tdrd12, as a novel component of the secondary piRNA biogenesis machinery in mice. Tdrd12 is detected in complexes containing the Piwi protein Mili and other factors already implicated in this process. While primary piRNAs are present in mice lacking Tdrd12, the nuclear Piwi protein Miwi2 is devoid of piRNAs. Consequently, unloaded Miwi2 remains in the cytoplasm, contributing to the reduced promoter DNA methylation and derepression of transposons observed in the mutant. We used the Bombyx mori (Silkworm) BmN4 cell culture model to molecularly dissect the formation of a complex between BmTdrd12 and Piwi. Additionally, our studies point to the importance of an intact ATPase motif in BmTdrd12 for its proper sub-cellular localization in the Nuage. We propose that Tdrd12 function as a molecular scaffold that dynamically bridges the association between Piwi proteins engaged in piRNA amplification.

105 Regulation of miRNAs and endo-siRNAs during oocyte-to-zygote transition in the mouse

Matyas Flemr[1], Radek Malik[1], Vedran Franke[2], Jana Nejepinska[1], Kristian Vlahovicek[2], Petr Svoboda[1]

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In the mouse, transcription ceases prior to ovulation and it is renewed during the major phase of the zygotic genome activation, which takes place at the 2-cell stage. Thus, mechanisms of post-transcriptional control of maternal transcripts play key roles during the oocyte-to-zygote transition. RNA silencing is a common name for a group of pathways utilizing small RNAs as sequence-specific guides regulating gene expression, usually post-transcriptionally. Previous research on small RNAs revealed that three different classes of small RNAs are present in the mouse oocyte: piRNAs, miRNAs, and endo-siRNAs. Mutation studies however, suggest that only the endogenous RNAi is essential for the oocyte. Furthermore, while miRNA biogenesis appears intact in the oocyte, biological activity of miRNAs is suppressed. This is apparently because the effector complex (miRISC) does not efficiently form in the oocyte. Our new data suggest that GW182 is the limiting factor for GW182-AGO2 interaction.

Our earlier analysis of dsRNA processing in transgenic animals revealed preferential production of siRNAs in the oocyte while siRNA production in somatic tissues was negligible. Therefore, we investigated control of small RNA biogenesis in the female germline. We have found that growing mouse oocytes express a unique isoform of Dicer, which is truncated at its N-terminus. Several lines of evidence suggest that this Dicer isoform is the factor responsible for increased production of endogenous siRNAs in the oocyte. The oocyte-specific Dicer isoform is the first evidence for molecular divergence of mammalian miRNA and RNAi mechanisms. Notably, poduction of oocyte-specific Dicer is controlled by a rodent-specific retrotransposon. This fact has interesting implications regarding evolution and conservation of RNA silencing pathways in animals.

106 Kinetic and biophysical models improve identification of miRNA targets

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MicroRNAs (miRNAs) are small RNA regulators that are involved in the establishment of gene expression patterns, frequently at transitions between cellular states or developmental stages. Many types of methods have been developed to uncover the mRNA targets of miRNAs. Experimental methods frequently employ transfection or knockdown of individual miRNAs followed by the profiling (with microarrays or deep sequencing) of mRNAs, 1-2 days after the miRNA expression was manipulated. Based on previously published data as well as new experimental measurements we inferred the parameters that determine the kinetics of miRNA-dependent responses, including the rate of loading and dissociation of miRNAs to/from Argonaute proteins, their decay rate and the relative magnitude of the miRNA effect on protein translation and mRNA decay rates. We show that the inferred computational models predict with good accuracy the mRNA, protein and ribosome footprinting response of mIRNA targets following miRNA transfection. The model further predicts that protein decay rates introduce substantial bottlenecks in miRNA-dependent regulation of protein levels and that active miRNA turnover and increased protein decay are necessary to achieve appreciable protein changes over the time scale of a cell cycle. We further developed a biophysical model of miRNA-target interaction that enables accurate identification of non only canonical but also non-canonical targets. We found that the latter are particularly frequent when the level of miRNA expression is high. These results provide new insights into the dynamics of miRNA-dependent regulation of mRNA decay and translation rates.

107 Insights into the recruitment of the PAN2-PAN3 deadenylase complex to miRNA targets by the GW182/TNRC6 proteins

Mary Christie[1], *Andreas Boland*[1], *Eric Huntzinger*[1], *Oliver Weichenrieder*[1], *Elisa Izaurralde*[1]

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The PAN2-PAN3 deadenylase complex functions in general and miRNA-mediated mRNA degradation and is specifically recruited to miRNA targets by the TNRC6 proteins. PAN3 is an adaptor protein that recruits the PAN2 deadenylase to mRNA targets. PAN3 contains three prominent regions: an N-terminal region that is predicted to be unstructured, a central pseudokinase (PK) domain and a highly conserved C-terminal domain (C-term), which is unique to the PAN3 protein family. While the N-terminal region of PAN3 contains a well-characterized PAM2 motif, studies on the roles of the PK and C-term domains have been restricted to the yeast protein. In particular, yeast two-hybrid assays have demonstrated that a C-term domain of yeast PAN3 is required for PAN2 binding, while both the PK+C-term domains mediated self-interaction. However, the stoichiometry and significance of PAN3 oligomerization has remained unclear. Here we show that PAN3 forms dimers *in vitro* and *in vivo*. Surprisingly, despite containing nonconservative substitutions in all the sequence motifs required for kinase activity, the PAN3 pseudokinase domain is capable of binding ATP and the integrity of the nucleotide binding pocket is required for deadenylation *in vivo*. Through a combined mutational and functional analysis, we have identified critical residues that mediate PAN3 interaction with PAN2 and TNRC6 proteins, as well as additional residues required for deadenylation *in vivo*. Collectively, our data provides a framework for understanding the role of PAN3 in recruiting PAN2 to mRNA targets to initiate their deadenylation and reveals a role for ATP-binding in mRNA deadenylation.

108 Translational repression and eIF4A2 activity are critical for microRNA-mediated gene regulation.

Anna Wilczynska[1], Hedda Meijer[1], Wei-Ting Lu[1], Yi-Wen Kong[1], Ruth Spriggs[1], Jack Godfrey[1], Sue Robinson[1], Anne Willis[1], Martin Bushell[1]

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In the cytoplasm, miRNAs play a major role in the control of gene expression by down-regulating the protein product of their target mRNAs. The binding of miRISC has been shown to exert both translational repression and degradation of target mRNA. However, the interplay between these processes and the precise molecular mechanisms involved remain unclear. We show that translational inhibition is the primary event required for mRNA degradation. Moreover, we demonstrate that miRNA-mediated repression is only enhanced by the poly(A) tail nor does it require deadenylation. The use of IRES-containing mRNAs allowed us to determine that translational inhibition depends on miRNAs impairing the function of the eIF4F initiation complex. We further define the RNA helicase eIF4A2 as the critical factor involved in this process. We uncover a correlation between the presence of miRNA target sites in the 3'UTR of mRNAs and secondary structure in the 5'UTR, and show that mRNAs with unstructured 5'UTRs are refractory to miRNA repression. Finally, immunoprecipitation studies reveal that *in vivo* eIF4A2 associates with the Ccr4-NOT complex while eIF4A1 is predominantly found to bind eIF4G. eIF4A2 may interact with CNOT1 through its MIF4G domain and impede the helicase activity of the eIF4F complex by clamping onto the structure in the 5'UTR of target mRNAs. These data support a linear model for miRNA-mediated gene regulation in which translational repression via eIF4A2 is required first, followed by mRNA destabilization, and suggest a mechanism by which the repression machinery at the 3' end of the mRNA inhibits translational initiation on the 5' end.

109 The conserved concave surface of the MIF4G domain of CNOT1 is involved in miRNA-mediated translational repression

Hansruedi Mathys[1], *Witold Filipowicz*[1]

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MiRNAs are endogenous ~21-nt non-coding RNAs that act as post-transcriptional regulators of gene expression by base pairing to target mRNAs. Mature miRNAs form part of ribonucleoprotein complexes, miRISCs, that contain Argonaute (AGO) and GW182 as core proteins. Recently, we have shown that GW182 proteins recruit the CCR4-NOT deadenylase complex via tryptophan-containing motifs to repress both poly(A)-containing and poly(A)-free mRNAs, indicating that recruitment of the CCR4-NOT complex, in addition to catalyzing deadenylation, also mediates translational repression.

We have now investigated the functional role of the CCR4-NOT complex in miRNA-mediated translational repression. Pull-down experiments revealed that the C-terminal effector domain (CED) of the GW182 protein TNRC6C interacts, in a W-motifs-dependent manner, with two different non-overlapping regions of CNOT1, the large scaffold subunit of the CCR4-NOT complex. Deletion analysis of CNOT1 also identified a central part of CNOT1 (named CNOT1-R) as a region mediating translational repression of mRNA. A highly conserved region of CNOT1-R adopts a MIF4G-like fold and its convex surface interacts with the deadenylase CAF1 (Basquin et al., Mol Cell 2012, Petit et al., NAR 2012). Mutations in the convex surface of MIF4G abrogating the interaction of CNOT1 with CAF1 alleviated the CNOT1R-mediated repression of a poly(A)$^+$ mRNA reporter but had no effect on repression of a poly(A)$^-$ mRNA. Furthermore, the repression of a poly(A)$^-$ reporter mRNA by tethered CAF1 was largely dependent on the ability of CAF1 to interact with CNOT1, strengthening the conclusion that it is CNOT1 that acts, via its CNOT1-R region, as an inhibitor of translation.

The concave surface of the MIF4G domain of the initiation factor eIF4G serves as a binding platform for the initiation factor eIF4A (Schutz et al., PNAS 2008). To test whether the CNOT1 MIF4G competes with eIF4G for binding to eIF4A and thereby interferes with translation initiation, we introduced mutations to the CNOT1 MIF4G expected to interfere with the eIF4A binding. Indeed, the mutations alleviated the CNOT1R-mediated repression of poly(A)$^+$ and poly(A)$^-$ mRNAs. However, this effect appears not to be due to the compromised interaction with eIF4A since we were unable to detect interaction with either its eIF4AI or eIF4AII isoform. We will present our progress towards identification of the factor acting downstream of the CNOT1 MIF4G in mediating translational repression.

110 Single-molecule observation of DNA targeting and cleavage by the RNA-guided Cas9 endonuclease

Samuel Sternberg[1], *Eric Greene*[2], *Sy Redding*[2], *Martin Jinek*[3], *Jennifer Doudna*[1]

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Bacteria and archaea maintain a history of viral infections by integrating small fragments of foreign DNA into specialized genomic loci called clustered regularly interspaced short palindromic repeats (CRISPRs). Subsequent infections trigger an adaptive immune response that relies on CRISPR-derived RNAs (crRNAs) functioning together with CRISPR-associated (Cas) proteins to identify and destroy complementary viral DNA sequences known as protospacers. In type II CRISPR/Cas immune systems, the Cas:crRNA ribonucleoprotein surveillance complex comprises two RNA molecules and the Cas9 endonuclease, which cleaves both strands of the protospacer following initial recognition. Prior studies have used oligonucleotide substrates to show that DNA targeting proceeds via RNA–DNA base-pairing interactions and requires melting of the double-stranded substrate, as well as protein-mediated recognition of a protospacer adjacent motif (PAM) to discriminate self from non-self. Nevertheless, the mechanism by which Cas9:RNA locates and recognizes its target sequence within the larger context of genomic DNA is unknown.

To gain deeper insights into this critical step of CRISPR/Cas-based immunity, we used single-molecule fluorescence microscopy to visualize Cas9:RNA as it searches for DNA targets in real-time. We demonstrate that Cas9:RNA exhibits specific DNA binding in a highly programmable fashion. Both bulk experiments and single-molecule observation of off-target interactions reveal that the search process relies in part on facilitated diffusion, and that PAM sites recruit Cas9:RNA to potential targets. Surprisingly, the Cas9:RNA complex stays tightly bound to DNA protospacers after the cleavage reaction, suggesting the possibility that product association is an important component of CRISPR interference *in vivo*. Collectively, these experiments have elucidated mechanistic details of DNA targeting and cleavage by Cas9:RNA and will facilitate future efforts to develop this system for genome engineering.

111 Processing-Independent CRISPR RNAs Limit Natural Transformation in Neisseria meningitidis

Yan Zhang[1], Nadja Heidrich[5], Biju Joseph Ampattu[4], Carl Gunderson[3], Hank Seifert[3], Christoph Schoen[4], Jörg Vogel[5], Erik Sontheimer[2]

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CRISPR interference confers adaptive, sequence-based immunity against viruses and plasmids in bacteria and archaea. Interference is specified by CRISPR RNAs (crRNAs) that are transcribed and processed from spacer-repeat units. Most forms of CRISPR interference involve RNA-directed DNA targeting, and this mechanism is being exploited for facile, RNA-guided genome engineering applications. Processing of monomeric crRNAs (i.e., each containing only a single spacer sequence) from a multimeric precursor is essential for CRISPR interference in all natural systems studied thus far.

Several bacterial strains from the genus *Neisseria* have "Type II" CRISPR/Cas systems in which the Cas9 protein provides the catalytic activities required for crRNA-directed DNA targeting. Type II systems are those that have proven most effective for eukaryotic genome engineering. Our analyses of crRNA biogenesis and CRISPR interference in *Neisseria* have revealed a unique crRNA biogenesis pathway in which crRNAs are transcribed from promoters that are embedded within each CRISPR repeat, yielding crRNA 5' ends that are formed by transcription and not by processing. This is in contrast with previously studied systems, where pre-crRNA transcription is driven by promoter(s) within a "leader" sequence outside of the CRISPR array. Although *Neisseria* crRNA 3' end formation involves RNase III-catalyzed cleavage of duplexes formed by pre-crRNA and a *trans*-encoded tracrRNA (as in other Type II CRISPR systems), this processing is dispensable for interference, and unprocessed, multimeric crRNAs are functional. Even though pre-crRNA processing is dispensable for interference, the tracrRNA is still required, indicating that the tracrRNA likely contributes to DNA binding and cleavage steps in bacterial cells, as previously established by others *in vitro*. The pathway from the deadly human pathogen *Neisseria meningitidis* exemplifies a newly defined CRISPR/Cas subtype (Type II-C) and is the most streamlined CRISPR/*cas* system characterized to date, as indicated by the number of required components and steps. Its simplicity, as well as its ability to function via unprocessed, multimeric crRNAs, suggests the possibility of unique practical applications for engineered systems in eukaryotes. Endogenous CRISPR spacers frequently target genomic sequences of other *Neisseria* strains and so limit natural transformation, which is the primary source of genetic variation that contributes to immune evasion, antibiotic resistance, and virulence in *N. meningitidis*. We conclude that native CRISPR/Cas systems can limit all three primary routes of horizontal gene transfer (phage transduction, conjugation, and natural transformation) in bacteria and archaea.

Concurrent 5: Structure, dynamics, and catalysis

Time: Friday, June 14, 14:00 - 16:30
Location: Aspen
Abstracts: 112 - 120
Chair(s): David Rueda, Wayne State University

112	**T box riboswitch decodes both the information content and geometry of tRNA to affect gene expression**
113	**Structural basis of specific tRNA recognition by the T-box riboswitch**
114	**Crystal Structure and Biophysical Analysis of a Class 2 PreQ1 Riboswitch**
115	**A novel class of self-cleaving ribozymes is prevalent in many species of bacteria and eukarya**
116	**Spliceosomal Prp24 unwinds a minimal U2/U6 complex from yeast**
117	**CONFORMATIONAL HETEROGENEITY OF THE PROTEIN-FREE HUMAN SPLICEOSOMAL U2-U6 snRNA COMPLEX**
118	**A new class of minimal Hammerhead ribozymes conserved in the eukaryotic family of Penelope-like retroelements**
119	**Probing EF-G Power Stoke During Ribosome Translocation**
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112 T box riboswitch decodes both the information content and geometry of tRNA to affect gene expression

Ailong Ke[1], Jason Grigg[1], Yujie Chen[1], Frank Grundy[2], Tina Henkin[2], Lois Pollack[1], Ailong Ke[1]

[1]Cornell University; [2]The Ohio State University

The T box riboswitch is an RNA element that controls gene expression by directly binding to a specific tRNA and sensing its aminoacylation state. This interaction controls expression of amino acid-related genes in a negative feedback loop. The T box RNA structure is highly conserved, but its tRNA binding mechanism is only partially understood. Known sequence elements are the Specifier Sequence, which base-pairs with the tRNA anticodon, and the antiterminator loop, which base-pairs with the tRNA acceptor end. Here, we reveal the crucial function of the highly conserved Stem I distal region in tRNA recognition and report its 2.65 Å crystal structure. The apex of this region contains an intricately woven loop-loop interaction between two conserved motifs, the AG Bulge and the Distal Loop. This loop-loop structure presents a base triple on its surface that is optimally positioned for base-stacking interactions. Mutagenesis, cross-linking and small-angle X-ray scattering data demonstrate that the apical base triple serves as a binding platform to dock against the tRNA D- and T-loops. Strikingly, the binding platform strongly resembles the D- and T-loop binding elements from RNase P and the ribosome P/E sites, suggesting that this loop-loop structure may represent a widespread tRNA recognition platform. We propose a new double checkpoint model for decoding tRNA, in which the information content of tRNA is first examined through the Specifier Sequence-anticodon interaction, the length of the tRNA anticodon arm is then measured by the distal loop-loop platform. When both conditions are met, tRNA is secured and its aminoacylation state is sensed.

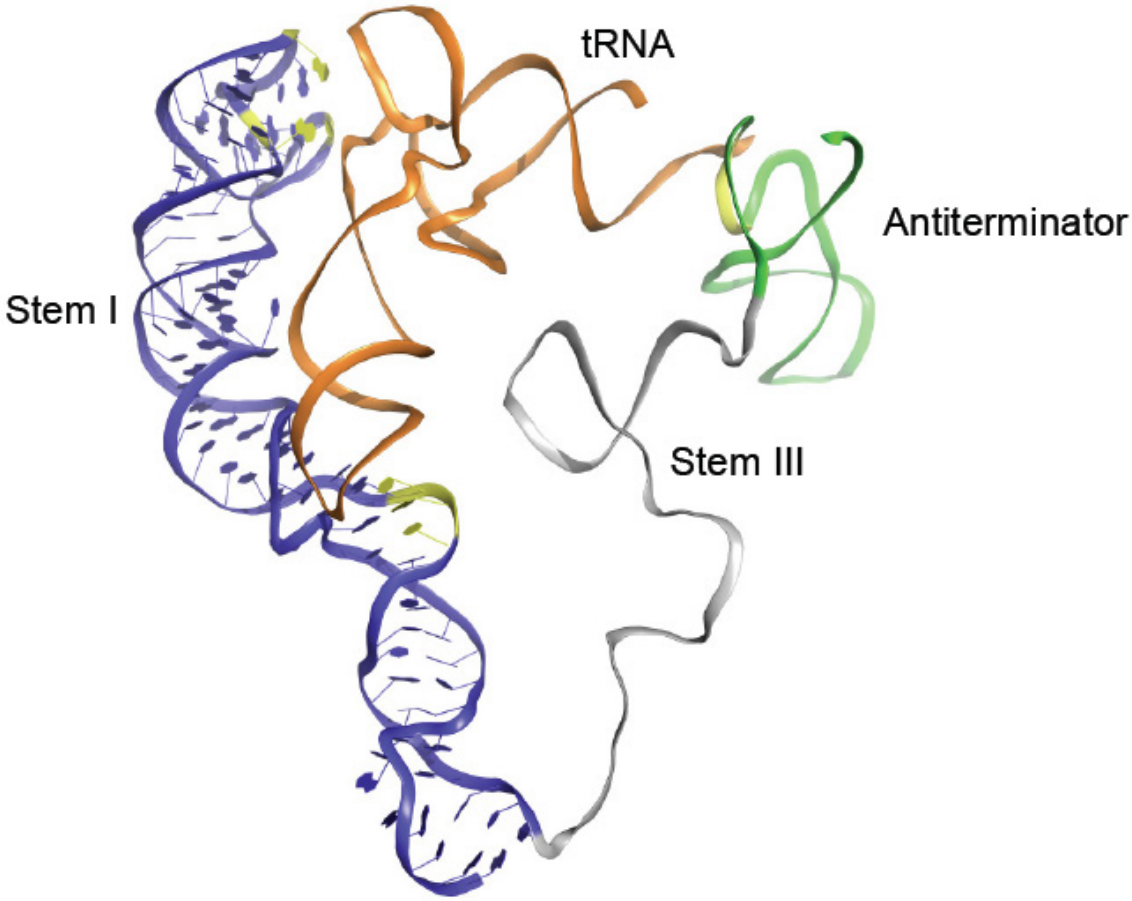

Figure 1

113 Structural basis of specific tRNA recognition by the T-box riboswitch

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The T-box riboswitches regulate tRNA aminoacylation, amino acid synthesis and transport in Gram-positive bacteria in response to cellular tRNA aminoacylation levels. They couple direct binding of non-aminoacylated tRNAs with formation of a transcription antiterminator allowing expression of downstream genes. The T-box consists of two phylogenetically conserved domains connected by a flexible, highly variable linker. These domains are an elongated Stem I that has extensive sequence conservation beyond the "specifier" loop that base pairs with the tRNA anticodon, and an antiterminator that is stabilized by base pairing with the non-aminoacylated tRNA 3' end [1]. We employed isothermal titration calorimetry to monitor tRNA binding to a T-box truncation series and found that the Stem I is necessary and sufficient for robust and specific tRNA binding (K_D~120 nM). Indeed, Stem I-tRNA association accounts for the majority of the binding free energy of full-length T-boxes. To elucidate the molecular basis of tRNA recognition by T-boxes, we have now determined the crystal structure of a representative T-box Stem I (102 nt) in complex with its cognate tRNA (75 nt) and a bacterial protein that binds the conserved Stem I K-turn. Stem I adopts an extended "C" shape that cradles the tRNA. As predicted genetically [2], the anticodon is recognized by the specifier element through base pairing. The specifier is in the middle of the C, and is flanked by loop E and K-turn motifs. The distal end of Stem I comprises two T-loop motifs that interdigitate to form a compact module with a flat molecular surface that recognizes the elbow of tRNA (where its D- and T-loops come together) through shape complementarity. Sequence conservation indicates that this bipartite mechanism of tRNA recognition, in which the specifier binds the anticodon, and the interdigitated T-loops reach out to recognize the tRNA elbow, is near-universal in T-box riboswitches. Remarkably, recognition of the tRNA elbow by two interdigitated T-loops has been also demonstrated for RNase P [3], even though the T-box and RNase P recognize the opposite arms of tRNA. Thus, it appears that these two RNAs evolved independently to converge on using the same interdigitated T-loop module for recognizing tRNA.

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114 Crystal Structure and Biophysical Analysis of a Class 2 PreQ1 Riboswitch

Joseph Liberman[1], *Mohammad Salim*[1], *Jolanta Krucinska*[1], *Joseph Wedekind*[1]

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Riboswitches are a class of *cis*-acting functional RNAs that regulate gene expression by directly binding ligands. Typically found in the 5′-leader sequences of bacterial genes, riboswitches have great potential as novel antimicrobial targets. Of special interest are riboswitches that bind to ligands that are unique to the bacterial metabolome. One such molecule is $preQ_1$, a hypermodified guanine base that interacts with a cognate riboswitch in over 29 bacterial species including pathogenic organisms. Two classes of $preQ_1$ riboswitches, termed $preQ_1$-I and $preQ_1$-II, have been discovered, but thus far structural information has been described only for class 1.

Here we present the crystallographic analysis and structure determination of a class 2 $preQ_1$ riboswitch in the ligand bound state. The overall structure comprises 77 nucleotides, and reveals the mode of ribosome-binding site sequestration by an H-type pseudoknot whose formation is fortified by the adjacent $preQ_1$-binding pocket. The ligand binds at the confluence of a three-way helical junction (figure 1) and is recognized by a readout mechanism that does not entail canonical Watson-Crick base pairing (figure 1). Thermodynamic parameters of ligand binding have been determined by isothermal titration calorimetry to both wild type and binding site mutants, which confirm the mode of interaction between the riboswitch and ligand observed in the crystal structure.

In-line probing analysis has been conducted and confirms key structural changes that occur upon ligand binding. Combined with the crystal structure, thermodynamic analysis, and comparison with similar structures such as human telomerase RNA, we propose a mechanism for gene regulation whereby binding of $preQ_1$ stabilizes formation of three base triples at the base of the pseudoknot that sequesters the RBS. The model for translational regulation will be presented as well as a comparison to other riboswitches that bind purine-like effectors.

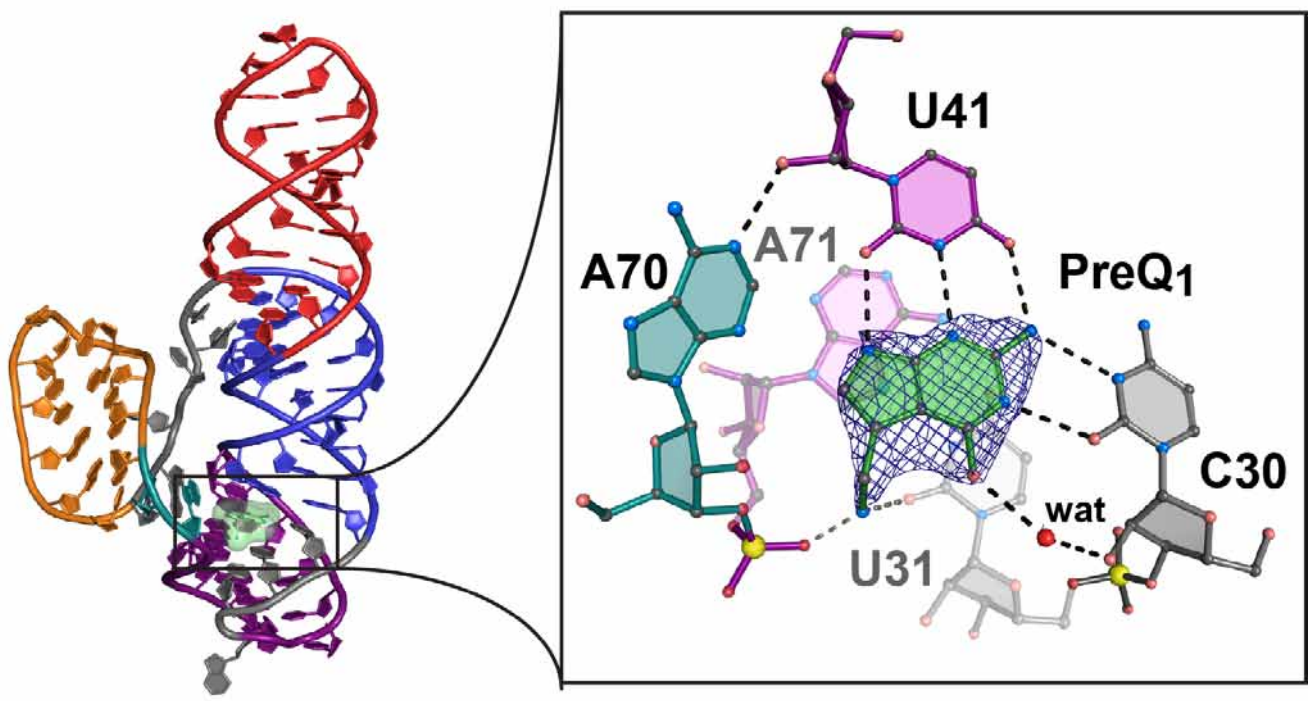

Figure 1

115 A novel class of self-cleaving ribozymes is prevalent in many species of bacteria and eukarya

Adam Roth[1], Zasha Weinberg[1], Andy Chen[2], Peter Kim[2], Tyler Ames[2], Ronald Breaker[2]

[1]Yale University; Howard Hughes Medical Institute; [2]Yale University

Ribozymes are noncoding RNAs that promote chemical transformations with rate enhancements approaching those of protein enzymes[1]. Although ancient RNA World organisms[2] likely used many types of ribozymes[3], only ten classes have been previously verified in nature. We report the discovery and analysis of an additional self-cleaving ribozyme class, called twister, which is present in many species of bacteria and eukarya. More than 2690 twister ribozymes were identified that conform to a secondary structure consensus that is small yet complex, with three stems conjoined by internal and terminal loops **(Fig 1a)**. Two pseudoknots provide tertiary structure contacts that are critical for catalytic activity. Many examples of these ribozymes are circularly permuted, bearing striking similarities to hammerhead ribozymes **(Fig 1b,c)**. Twister ribozymes cleave RNA via an internal phosphoester transfer reaction, which is identical to the general mechanism used by other five classes of self-cleaving RNAs discovered previously[4]. The discovery of twister ribozymes deepens the mystery regarding the biological roles of widespread classes of self-cleaving RNAs.

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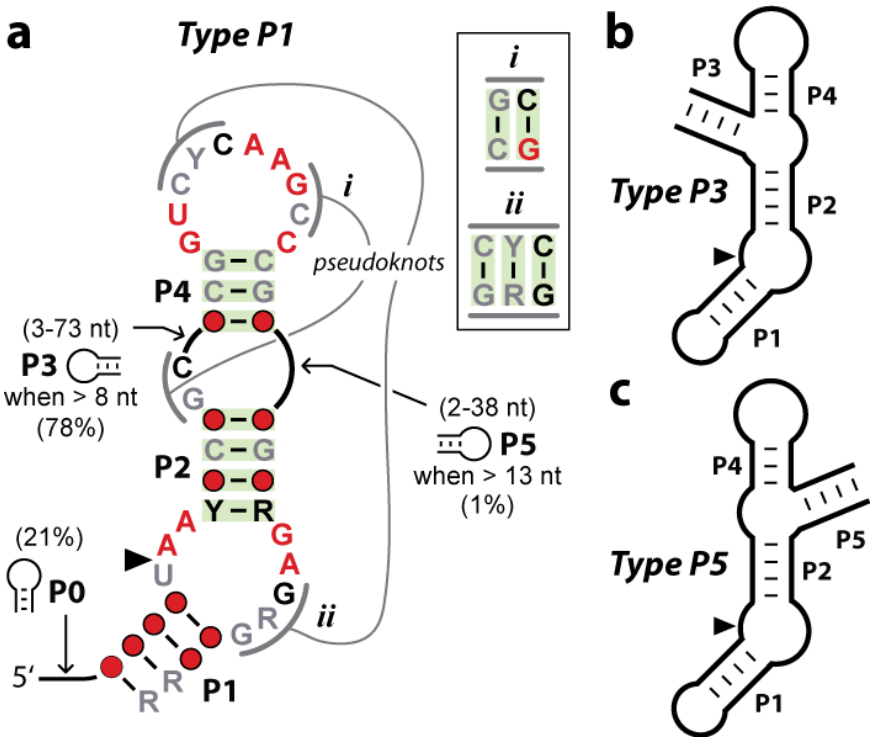

Figure 1 | Consensus sequence and secondary-structure model for twister self-cleaving ribozymes. (**a**) Consensus model based on 2690 twister ribozymes in its type P1 configuration, wherein the RNA chain begins and ends at the base of the P1 stem. The arrowhead identifies the cleavage site. Gray, black and red nucleotides designate conservation of at least 75, 90, and 97%, respectively; positions in which nucleotide identity is less conserved are represented by circles. Green shading denotes predicted base pairs supported by natural covariation. Notations *i* and *ii* identify predicted pseudoknots. Numbers in parentheses are the variable lengths of linker sequences that sometimes form stem structures as indicated. (**b**,**c**) The RNA chains of naturally occurring type P3 and type P5 configurations enter and depart the motif at the optional P3 or P5 stems, respectively.

116 Spliceosomal Prp24 unwinds a minimal U2/U6 complex from yeast

Chandani Warnasooriya[1], Zhuojun Guo[4], Samuel Butcher[3], David Brow[3], David Rueda[2]

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Splicing plays a major role in eukaryotic gene expression by processing pre-mRNA to form mature mRNA. Pre-mRNAs undergo splicing to remove introns, non-protein coding regions, and religate exons, protein coding regions. This process is catalyzed by the spliceosome, which consists of five small nuclear ribonucleoprotein particles (snRNPs: U1, U2, U4, U5 and U6) and numerous protein factors. Proper assembly of spliceosomal components is critical for function, and thus, defects in assembly can be lethal. Several spliceosomal proteins facilitate structural rearrangements important for spliceosomal assembly and function. Prp24 is an essential factor in U6 snRNP assembly, and it has been proposed to assist in U4/U6 formation and unwinding. Here, we address the question whether Prp24 affects the U2/U6 complex dynamics. Using single-molecule Fluorescence Resonance Energy Transfer (smFRET), we have previously shown that a minimal U2/U6 complex from yeast can adopt at least three distinct conformations in dynamic equilibrium. Our new single molecule data show that Prp24 unwinds U2 from U2/U6 complex and stabilizes U6 in a low FRET conformation. We also show that the RNA Recognition Motifs of Prp24 affect the binding affinity of Prp24 for U6 and unwinding activity. We propose that Prp24 plays an important role in U2 and U6 snRNP recycling by dissociating the U2/U6 complex.

117 CONFORMATIONAL HETEROGENEITY OF THE PROTEIN-FREE HUMAN SPLICEOSOMAL U2-U6 snRNA COMPLEX

Caijie Zhao[1], Ravichandra Bachu[1], Nancy Greenbaum[1]

[1]Hunter College of The City University of New York

The complex formed between the U2 and U6 small nuclear (sn)RNA molecules of the eukaryotic spliceosome plays a critical role in the catalysis of precursor mRNA splicing. We used ^{19}F NMR and enzymatic structure probing to characterize the fold of a protein-free paired construct representing the human U2-U6 snRNA complex. For the NMR studies, we included a single 5-^{19}F-cytidine residue in a region of U2 that would be part of Stem I (*i.e*. double-stranded) in a four-helix model or single-stranded in the three-helix model. Results for both enzymatic probing and NMR of the complex in the absence of Mg^{2+} are consistent with formation of a four-helix structure as a predominant conformation. However, ^{19}F NMR data also identify a lesser fraction (up to 14% at 25°C) of an alternative conformation presumed to be the three-helix conformer. In the presence of 5 mM Mg^{2+}, the fraction of the three-helix conformation increased to ~17%, suggesting a slight shift to the three-helix conformation. A mutant complex designed to favor the four-helix conformer displayed almost complete formation of Stem I, and a complex designed to favor a three-helix formation showed enhanced formation of Helix 1b, a hallmark of that conformer (Zhao C, Bachu R, Popovic M, Devany M, Brenowitz M, Schlatterer JC, Greenbaum NL. *RNA,* advance publication February 20, 2013, doi:10.1261/ rna.038265.1130). Based upon this distribution, the calculated DG for interconversion to the four-helix structure from the three-helix structure is approximately -4.6 kJ/mol. To evaluate the possibility of interconversion between the two conformations and the time scale, we acquired two-dimensional ^{19}F-^{19}F NOESY spectra of the complex labeled with ^{19}F-cytidine in the Stem I site of the U2 snRNA strand. We observed a NOE between the two peaks in a spectrum with a mixing time of 400 ms. This finding provides compelling evidence of exchange between the alternative conformations on a slow exchange time scale. These observations indicate conformational heterogeneity in the protein-free human U2-U6 snRNA complex consistent with a model in which the RNA has sufficient conformational flexibility to facilitate interconversion between steps of splicing *in situ*.

118 A new class of minimal Hammerhead ribozymes conserved in the eukaryotic family of Penelope-like retroelements

Amelia Cervera[1], *Marcos de la Peña*[1]

[1]IBMCP (CSIC-UPV)

Small self-cleaving RNAs like the paradigmatic Hammerhead ribozyme (HHR) have been recently found widespread in DNA genomes from bacteria to humans[1,2,3]. Their patchy distribution and common connection with retrotranscriptases and repetitive DNA suggest that most of these ribozymes would have a role in retrotransposition. Following bioinformatic and phylogenetic analysis, we have now discovered a new class of HHR motif conserved in the superfamily of Penelope-Like retrotransposable Elements (PLEs), a peculiar group of intron-containing retroelements different from the LTR and non-LTR retrotransposons of eukaryotes[4]. These new PLE-HHRs are minimalist variants of the canonical HHR that lack one of the three helixes and show some characteristic variations within their catalytic core. The PLE-HHR motifs consistently map in the 5' and 3' flanking repeats of PLEs from plants, invertebrates and lower vertebrates, although for some organisms, conserved PLE-HHRs can be also found in their genomes without any connection to a recognizable retrotransposon. *In vitro* analysis confirmed that these ribozymes may only work as dimeric forms during transcription of the retrotransposon. Our observations, together with those reported for HDV-like ribozymes in non-LTR retrotransposons[5,6], expand the role of RNA self-cleavage to the biology of diverse eukaryotic retroelements and leave open the question of how many more forms of RNA self-cleaving motifs can be possible.

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119 Probing EF-G Power Stoke During Ribosome Translocation

Yuhong Wang[1], Li Yao[2], Yue Li[1], Shoujun Xu[2]

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The mechanism of error control for the ribosome to move exactly three nucleotides during the translocation process and the role of EF-G remain elusive. The two possible mechanisms are the "Brownian motor" model and the "power stroke" hypothesis. Many recent studies support the former mechanism, implying that the ribosome moves by intrinsic Brownian motions and EF-G biases the random motion into the forward direction. However, the spontaneous translocation rate is several orders slower than the translocation with EF-G catalysis. This is contradictory to the "Brownian motor" hypothesis in which the maximum rate is limited by the intrinsic fluctuation. Because the EF-G can dramatically accelerate translocation comparing to the intrinsic rate, one intriguing question is whether EF-G exerts a mechanical force, i.e., a power stroke, to push the mRNA movement. At present, there has been no direct experimental evidence supporting such power stroke.

We have obtained direct force measurements in ribosome translocation using a combination of single-molecule fluorescence resonance energy transfer (FRET) and a new force-induced remnant magnetization spectroscopy (FIRMS) technique. The latter probes different noncovalent interactions and resolves them based on the binding forces. Single-basepair force resolution has been achieved. The ribosome pre-translocation complex and post-translocation complex are well resolved, and the translocation yield can be obtained. The EF-G power stroke is directly obtained by force-induced dissociation of a series of mRNA-DNA duplexes. A clear transition in magnetic signal by FIRMS indicates a substantial mechanical force produced by the EF-G. Our new results provide direct evidence, for the first time, that EF-G is the mechanical motor that generates power stroke to promote mRNA movement during its GTP hydrolysis cycle. The amplitude of the force may play an important role in determining the translocation fidelity.

120 Understanding RNA Interference One Molecule at a Time

William Salomon[1], *Victor Serebrov*[1], *Mellissa Moore*[1], *Phillip Zamore*[1]

[1]Department of Biochemistry & Molecular Pharmacology, RNA Therapeutics Institute, and Howard Hughes Medical Institute, University of Massachusetts Medical School

In mammals, small RNAs direct Argonaute2 (Ago2) to cleave complementary target mRNAs. For *Drosophila*, the development of efficient, cell-free systems that recapitulated RNAi led to a detailed mechanistic understanding of the RNAi pathway. Until recently, no such system existed for mammals because cell extracts that recapitulate RNAi are surprisingly inefficient. We find that cytoplasmic S100 extracts from *Ago2*$^{-/-}$ immortalized mouse embryonic fibroblast (MEF) cells that stably over express wild-type mouse AGO2 support highly efficient RNAi in vitro. Unlike extracts from HeLa or wild-type MEFs, extracts from the AGO2 over-expressing cells allow efficient assembly of a synthetic small RNA guide into AGO2 to form RISC, the RNA-induced silencing complex. Using a novel affinity purification strategy, we can readily purify AGO2-RISC assembled in these extracts with a specific guide sequence (1). We will present our recent studies analyzing the biochemical properties of this purified AGO2-RISC using the "CoSMoS" (co-localization single molecule spectroscopy) single-molecule technique.

CoSMoS can measure the kinetic properties of Ago2-RISC, including k_{on}, k_{off}, and k_{cat}. Our single-molecule data agrees well with recently published biochemical measurements of AGO2-RISC using population-based (ensemble) methods (2). Consistent with ensemble measurements, we find that AGO2-RISC binds to an mRNA target at the speed of macromolecular diffusion. This stands in contrast to the annealing of nucleic acids in the absence of protein, a process whose speed is limited by its on-rate. Intriguingly we find that target cleavage by AGO2-RISC is highly temperature-dependent, suggesting that a conformational rearrangement is required for RISC activity in mammals. Using both ensemble and single molecule experiments, we are now measuring the departure from RISC of the cleaved target fragments, a step proposed to limit the turnover rate of AGO2-RISC. In the future, we plan to use the CoSMoS system to determine the evolutionarily conserved and the specialized properties of Argonaute family proteins from diverse species.

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Concurrent 6: High-throughput approaches to RNA biology

Time: Friday, June 14, 14:00 - 16:30
Location: Sanada
Abstracts: 121 - 128
Chair(s): Jernej Ule, *MRC-LMB, Cambridge*

121	**Promoter directionality is controlled by U1 splicing and polyadenylation signals**
122	**Suppression of promoter upstream transcripts (PROMPTs) by polyadenylation site-induced RNA decay provides directionality to transcription of human promoters**
123	**Pathway of histone mRNA decay determined by high-throughput sequencing (HTS)**
124	**Global poly(A)-tail length measurements reveal that the relationship between tail length and translational efficiency varies between biological contexts**
125	**Capture of a microRNA targetome using chemically modified miRNA mimics (“miR-CLIP”)**
126	**Identification of multiple regulatory microRNAs by miTRAP**
127	**Neurodegenerative diseases: Quantitative predictions of protein-RNA interactions**
128	**New in vivo RNA-binding architectures discovered by RBDmap**

121 Promoter directionality is controlled by U1 splicing and polyadenylation signals

Albert Almada[1], Xuebing Wu[2], Andrea Kriz[1], Christopher Burge[1], Phillip Sharp[1]

[1]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139 USA; [2]Computational and Systems Biology Graduate Program, Massachusetts Institute of Technology, Cambridge, MA 02139 USA

Transcription of the mammalian genome is pervasive but productive transcription outside protein-coding genes is limited by unknown mechanisms. In particular, we and others recently discovered that although RNAPII initiates divergently from most active gene promoters, productive elongation occurs primarily in the sense coding direction. The data presented here reveal that asymmetric sequence determinants flanking gene transcription start sites (TSS) control promoter directionality by regulating promoter-proximal cleavage and polyadenylation. We demonstrate in various tissues of mouse and human that upstream antisense RNAs (uaRNAs) are cleaved and polyadenylated at a poly (A) site (PAS) shortly after their initiation. De novo motif analysis reveal PAS signals and U1 snRNP binding sites (U1) as the most depleted and enriched sequences, respectively, in the sense direction relative to the upstream antisense direction. In agreement with the recent finding that U1 snRNP suppresses cleavage and polyadenylation, our data suggests that a U1-PAS axis characterized by low U1 recognition and high density of PAS in the upstream antisense region results in early termination of uaRNAs whereas proximal sense PAS signals are primarily suppressed by U1. Experiments to modulate U1 snRNP levels at the upstream antisense region of divergent promoters will be discussed. We propose that the U1-PAS axis may limit pervasive transcription throughout the genome.

122 Suppression of promoter upstream transcripts (PROMPTs) by polyadenylation site-induced RNA decay provides directionality to transcription of human promoters

Evgenia Ntini[1], Aino Järvelin[5], Jette Bornholdt-Lange[3], Yun Chen[3], Mette Jørgensen[4], Robin Andersson[4], Aleks Schein[1], Peter Refsing Andersen[1], Pia Kjølhede Andersen[1], Vicente Pelechano[5], Lars Steinmetz[5], Albin Sandelin[3], Torben Heick Jensen[2]

[1]Department of Molecular Biology and Genetics, Aarhus University, Denmark; [2]Centre for mRNP Biogenesis and Metabolism, Department of Molecular Biology and Genetics, Aarhus University; [3]Department of Biology & BRIC, University of Copenhagen, Denmark; [4]Department of Biology and BRIC, University of Copenhagen, Denmark; [5]Genome Biology Unit, EMBL, Heidelberg, Germany

Active eukaryotic promoters are associated with unorthodox transcription. In human cells, this leads to the production of PROMoter uPstream Transcripts (PROMPTs), which are rapidly cleared by the ribonucleolytic RNA exosome. How PROMPT transcription is terminated and coupled to RNA decay, while its neighboring promoter-downstream mRNA production is not, is unknown. Here, we use exosome depletion to create genome-wide maps characterizing PROMPT RNAs and their termini in unprecedented detail. The clear majority of PROMPTs initiate in the antisense direction from 150-200bp upstream of the transcription start site (TSS) of the associated gene. PROMPT TSSs share features with mRNA-producing TSSs, including stalled RNA polymerase II (RNAPII) and the production of small TSS-associated (TSSa) RNAs. Thus, PROMPTs are unstable, capped full-length products of the previously reported bi-directional antisense transcription. Importantly, motif analyses around PROMPT 3'ends reveal polyadenylation (pA)-like signals, well-positioned to elicit PROMPT 3'end formation. However, contrary to their promoter-distal counterparts at mRNA 3'ends, RNA derived from TSS-proximal pA signals is highly exosome-sensitive. Interestingly, promoter-downstream regions contain significantly less pA signal information, but are instead enriched, relative to their promoter upstream counterparts, for U1 snRNP binding sites, which are known to suppress pA site utility. We conclude that asymmetric sequence distribution around human gene promoters serves to provide an overall directionality to the transcription process by rapidly terminating antisense transcription and degrading its RNA product.

123 Pathway of histone mRNA decay determined by high-throughput sequencing (HTS)

Mike Slevin[1], *Rebecca Bigler*[1], *Staci Meaux*[1], *William Marzluff*[1]

[1]University of North Carolina

Histone mRNAs are the only mRNAs in animal cells that are not polyadenylated ending instead in a conserved stemloop. Inhibition of DNA replication results in rapid histone mRNA decay directed by the stemloop. Decay of histone mRNA is initiated by addition of a non-templated oligouridine tail of ~10 nts, which binds Lsm 1-7. Previous studies used low throughput approaches for detecting non-templated additions. The low number of sequences generated limits the detailed analysis of mRNA decay. We have developed a high throughput sequencing strategy that allows detection of all intermediates in histone mRNA decay, and can specifically target mRNAs with non-templated 3' tails. This approach provides the depth of coverage necessary to detect patterns of decay intermediates for individual histone mRNAs. We find multiple oligouridylated intermediates resulting from partial decay located in the 3' side of the stem and in the coding region near the stop codon. These intermediates contain oligo(U) tails of 4-10 nts, and many are capped indicating they result from 3'-5' decay. Analysis of polyribosome gradients using HTS, show oligouridylated mRNAs are polyribosome associated, including decay intermediates degraded into the coding region. Blocking translation with pactamycin results in run-off of ribosomes and accumulation of monosomes with histone mRNAs, including capped oligouridylated decay intermediates. Thus a major pathway for histone mRNA decay after HU treatment is 3' to 5', with repeated exosome stalling, possibly when it contacts translating ribosomes. The degradation intermediate is then uridylated to re-prime 3'-5' decay. Exosome knockdown results in accumulation of the initial intermediates in the stemloop and reduction in intermediates stalled in the coding region. This is consistent with initial decay stalling in the stemloop catalyzed by 3'hExo (eri-1) and subsequent decay carried out by the exosome. To assess the simultaneous 5' and 3' pattern of decay on individual transcripts we used an anti-biotin oligonucleotide designed to isolate both capped and uncapped intermediates from the H2a mRNAs. A capped luciferase mRNA ending in a stemloop transfected into cells is rapidly degraded after treatment of cells with HU with a similar set of decay intermediates. This technique should be generally applicable to determining the degradation pathway of any set of mRNAs.

124 Global poly(A)-tail length measurements reveal that the relationship between tail length and translational efficiency varies between biological contexts

Alexander Subtelny[1], Stephen Eichhorn[1], Grace Chen[1], Hazel Sive[1], David Bartel[1]

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Poly(A) tails are found at the 3' ends of nearly all eukaryotic messenger RNAs (mRNAs) and long non-coding RNAs. The presence of a poly(A) tail promotes efficient mRNA translation, an effect mediated through poly(A)-binding protein. However, an understanding of the relationship between the length of a poly(A)-tail and translational efficiency, as well as other aspects of mRNA metabolism, has been limited, primarily because of the lack of a technology that provides high-resolution poly(A)-tail length measurements in a global manner. Here, we describe a high-throughput-sequencing-based method that measures the tails of individual mRNA molecules by coupling a fluorescence-based readout of poly(A)-tail length with sequencing of the poly(A)-proximal region. We have applied this method to diverse eukaryotic organisms and cellular contexts, including budding and fission yeasts, insect and mammalian cell lines, mouse liver, and zebrafish embryos. Poly(A)-tail lengths vary across mRNAs and show some conservation between orthologous genes. In particular, transcripts corresponding to ribosomal protein genes and certain other classes of "housekeeping" genes tend to have short tails. Poly(A)-tail lengths exhibit a notably poor correlation with translational efficiency (as measured by ribosome profiling) across genes in nearly all systems we have examined. This lack of correlation also applies to different translational states of mRNAs from the same gene, as indicated by poly(A)-tail lengths across a polysome gradient. Unlike all other systems we have studied, the early zebrafish embryo exhibits a striking correlation between poly(A)-tail length and translational efficiency (Spearman $R > 0.6$). Remarkably, this correlation disappears later, indicating a dramatic and rapid developmental switch in the nature of translational control. Our results clarify the relationship between poly(A)-tail length and translational efficiency, and enable further investigations of the determinants and consequences of poly(A)-tail length.

125 Capture of a microRNA targetome using chemically modified miRNA mimics ("miR-CLIP")

Jochen Imig[1], Andreas Brunschweiger[1], Anneke Brümmer[2], Nitish Mittal[2], Boris Guennewig[1], Mihaela Zavolan[2], André P. Gerber[3], Jonathan Hall[1]

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Identification of the targets of microRNAs (miRNAs) remains a significant barrier to understanding their function. Recently, several methods including PAR-CLIP and affinity purification by biotinylated miRNAs have been developed to achieve this goal, either in a global fashion, or for selected miRNAs. All of these methods have their strengths and weaknesses. We have developed a two-step approach to screen in straightforward fashion for targets using miRNAs chemically modified with psoralen and biotin. Prior to Ago-2 immunoprecipitation as the first step *in vivo* photo-crosslinking is performed and followed by a second step by stringent affinity purification of bound target RNAs. We have termed this method "miR-CLIP".

We applied miR-CLIP to isolate targets of miR-106a in HeLa cells by using functionally modified mimics of pre- and mature miR-106a. Deep sequencing of isolated RNAs yielded a large number of significantly enriched mRNAs compared to "mock"-treated controls. Among these were several hundred potential new targets. No enrichment was observed for predicted targets of unrelated miR-let-7a. Mostly, target recognition followed a canonical seed-binding mode however shorter non-canonical seed matches were also detected in enriched RNAs. In follow-up assays, overexpression of miR-106a significantly repressed 8 from 10 of the top-ranked potential mRNA targets detected in the analysis validating the approach. Surprisingly, the sequencing also showed enrichment of the long non-coding RNA (lncRNA) H19 as an interacting partner of miR-106a. H19 is expressed from a paternally imprinted gene which is strongly induced during muscle development. Indeed, H19 was strongly and unexpectedly elevated by overexpression of miR-106a in HeLa and in myoblasts, and conversely, its inhibition by an anti-miR showed the opposite effect. RNAi-mediated suppression of H19 led to reduced levels of known miR-106a targets in myocytes leading us to speculate a mechanism in which H19 buffers the activity of miR-106a during muscle cell differentiation.

We are currently testing the functional consequences of this new interaction. In summary, the miR-CLIP method appears to be a valuable technique to uncover the "targetome" of specific miRNAs.

*equally

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126 Identification of multiple regulatory microRNAs by miTRAP

Juliane Braun[1], *Knut Krohn*[2], *Stefan Hüttelmaier*[1]

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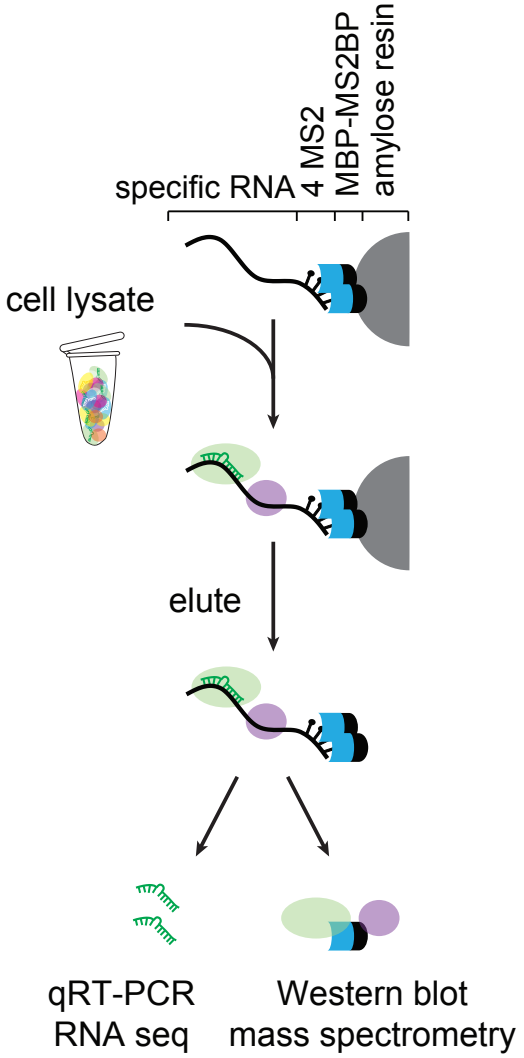
MicroRNAs (miRNAs) are endogenous, small non-coding RNAs which serve essential functions in post-transcriptional control of gene expression. Animal miRNA target sites (MTS) are typically characterized by imperfect complementarity and are mainly located in the 3'UTR of mRNAs. The length of such 3'UTRs and the number of putative MTSs could be correlated with the morphological complexity of organisms. However, for only a few human targets multiple miRNA regulation has been verified as experimental identification and *in silico* prediction remains challenging and limited by various means. Aiming to provide a procedure allowing the comprehensive identification of regulatory miRNAs, we established miTRAP (**mi**croRNA **T**rapping by **R**NA **A**ffinity **P**urification). MiTRAP co-purifies miRNAs and RNA-binding proteins (RBPs) with *in vitro* transcribed bait RNAs from cell lysates by adapting MS2-tethering (Figure 1). Therefore, miTRAP is independent of genetic manipulation of cellular systems and *in silico* predictions.

Applying MiTRAP allowed the validation of regulatory miRNAs and RBPs controlling the expression of the transcriptional regulators MYC and ZEB2. Moreover, miTRAP and subsequent deep sequencing identified 13 novel MYC-regulatory miRNAs all of which could be validated by gain- as well as loss-of-function studies. Four of these novel miRNAs target 'non-canonical' sites and thus could not have been identified by *in silico* prediction. We consider miTRAP a reliable and easy to handle technology allowing the rapid and comprehensive identification of miRNAs targeting RNAs of interest in a given cellular context. MiTRAP thus provides a powerful tool, which will significantly expedite analyses aiming the characterization of miRNA-facilitated regulation of gene expression in development, metabolic control and diseases.



127 Neurodegenerative diseases: Quantitative predictions of protein-RNA interactions

Davide Cirillo[1], *Gian Tartaglia*[1]

[1]Centre for Genomic Regulation

Increasing evidence indicates that RNA plays an active role in a number of neurodegenerative diseases. We recently introduced a theoretical framework, to predict the binding ability of protein and RNA molecules. We use our approach to investigate ribonucleoprotein interactions linked to inherited intellectual disability, amyotrophic lateral sclerosis, Creutzfeuld-Jakob, Alzheimer's, and Parkinson's diseases. In my presentation, I will focus on (1) RNA interactions with fragile X mental retardation protein FMRP; (2) protein sequestration caused by CGG repeats; (3) noncoding transcripts regulated by TAR DNA-binding protein 43 TDP-43; (4) autogenous regulation of TDP-43 and FMRP; (5) iron-mediated expression of amyloid precursor protein APP and a-synuclein; (6) interactions between prions and RNA aptamers. Our results are in striking agreement with experimental evidence and provide new insights in processes associated with neuronal function and misfunction.

128 New in vivo RNA-binding architectures discovered by RBDmap

Alfredo Castello[1], Bernd Fischer[1], Sophia Foehr[1], Anne-Marie Alleaume[1], Tomaz Curk[2], Jeroen Krijgsveld Krijgsveld[1], Matthias W Hentze[1]

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The RNA interactomes of HeLa and HEK293 cells jointly comprise 1106 RNA-binding proteins (RBPs) (1, 2), with almost half of these lacking well-defined RNA-binding domains (RBDs), suggesting the existence of numerous unknown RNA-binding architectures. Here, we report RBDmap, a new method built on interactome capture (3), to comprehensively identify the RBDs of native RBPs in proliferative HeLa cells. Making use of in vivo UV-crosslinking of RBPs to polyadenylated RNAs, capture on oligo(dT) magnetic beads, proteolytic mapping and mass spectrometry combined with a sophisticated scoring algorithm, RBDmap "re-discovered" the known RNA-binding sites (e.g. RRM, KH) of numerous well characterized RBPs, validating the approach. Strikingly, RBDmap identified dozens of additional RNA-binding architectures (e.g. thioredoxin, SSB, RAP, WD40) in multiple non-homologous proteins, also including disordered motifs such as basic patches. RBDmap thus instructs on the modes of RNA-binding of hundreds of proteins in their native cellular states, providing valuable structural and functional insights into RNA biology. For example, the identification of the thioredoxin domain as a high-confidence RBD reveals an intriguing link between the redox state of cells and RNA metabolism.

1. A. G. Baltz et al., Mol Cell 46, 674 (Jun 8, 2012).
2. A. Castello et al., Cell 149, 1393 (Jun 8, 2012).
3. A. Castello et al., Nat Protoc 8, 491 (Feb 14, 2013).

Workshop 4: RNA in pharmaceutical research

Time: Friday, June 14, 17:00 - 18.30
Location: Davos Ballroom
Abstracts: 129 - 134
Chair(s): Nicole Meisner, Novartis Institutes for Biomedical Research
David Morrissey, Novartis Institutes for Biomedical Research

129	**Development of Dynamic Polyconjugates for tissue-targeted delivery of siRNA**
130	**Specific Gene Activation by Disruption of PRC2-lncRNA Interactions**
131	**RNA-based immunotherapeutics against cancer**
132	**Antisense Oligonucleotide Therapies for the Treatment of Neurodegenerative and Muscular Diseases**
133	**Extracellular RNAs are markers of muscle myogenesis following splice switching oligonucleotide therapy in a mouse model of Duchenne muscular dystrophy**
134	**Silencing gene expression by recruiting RISC**

129 Development of Dynamic Polyconjugates for tissue-targeted delivery of siRNA

Christine Wooddell[1], So Wong[1], Jason Klein[1], Andrei Blokhin[1], Magdolna Sebestyén[1], Weijun Cheng[1], Julia Hegge[1], Qili Chu[1], Vladimir Trubetskoy[1], Collin Hagen[1], Anthony Perillo-Nicholas[1], Jacob Griffin[1], Jonathan Benson[1], Jeffrey Carlson[1], Darren Wakefield[1], Holly Hamilton[1], Stephanie Bertin[1], Jessica Montez[1], Alan McLachlan[2], David Rozema[1], David Lewis[1]

[1]Arrowhead Research Corporation; [2]University of Illinois at Chicago

Dynamic Polyconjugates (DPCs) are siRNA delivery vehicles composed of reversibly-masked, membrane-active polymers whose activity is revealed only within endosomes. A new generation of hepatocyte-targeted DPC consists of a fully biodegradable peptide with membrane lytic properties (MLP) as the membrane-active polymer that is conjugated to N-acetylgalactosamine (NAG) for targeting. This NAG-MLP is co-injected with a liver-tropic, cholesterol-conjugated siRNA (chol-siRNA). Effective target gene knockdown is accomplished without complex formation or interaction of the NAG-MLP and the chol-siRNA prior to reaching the target cell.

We are utilizing this separately-targeted siRNA delivery approach for the treatment of chronic hepatitis B virus (HBV) infection. The use of RNAi therapeutics for the treatment of HBV infection enables knockdown of viral RNAs, including the pre-genomic RNA from which the replicative intermediates are derived, thus reducing both viral load and viral proteins. A single i.v. co-injection of NAG-MLP with chol-siRNAs targeting conserved human HBV sequences resulted in profound reduction of viral RNA and multi-log knockdown of viral proteins and DNA with long duration of effect in mouse models of chronic HBV infection. Using a hybridization/HPLC-based method of detection, we are able to correlate the degree of viral repression and duration of effect with the amount of the active form of the siRNA guide strand in the liver, the 5' phosphorylated guide strand, which can be detected up to one month after a single administration.

In addition to our existing pH-labile masking reagents, we are developing DPC formulations with masking chemistry that is reversed by the action of proteases. These delivery vehicles are stable in the absence of lysosomal proteases, allowing for functional delivery of subcutaneously-administered DPC (scDPC). The protease-sensitive DPCs display long circulation times, indicating that the masking chemistry is stable in serum. Attachment of the NAG ligand to scDPCs results in rapid liver uptake and >90% knockdown at sub-mg/kg siRNA doses in mice and non-human primates. The remarkable stability and efficacy of protease-sensitive DPCs make them amenable for use in liver indications for which subcutaneous dosing is desirable as well as in targeting tumor and other extra-hepatic tissues.

130 Specific Gene Activation by Disruption of PRC2-lncRNA Interactions

James McSwiggen[1]

[1]RaNA Therapeutics

A wide variety of nucleic acid compounds are used in both the laboratory and the clinic to inhibit specific RNA expression and subsequent protein production. We are developing nucleic-acid-based therapeutics that activate genes—rather than inhibiting them—by blocking the interaction of Polycomb repressive complex 2 (PRC2) with its target long noncoding RNA (lncRNA).

Histone H3K27 trimethylation is generated by PRC2 and results in inhibition of transcription. Our platform is based on the discovery that PRC2 is recruited by non-coding RNAs to methylate histones local to the site of the lncRNA. The EZH2-containing PRC2 complex represses gene expression at thousands of sites across the genome. During its transcription, an lncRNA that contains a PRC2-recognition sequence is tethered to the site of transcription. This tethering leads to very localized repression of neighboring genes. Since each PRC2-associated lncRNA interacts with PRC2 through distinct sequences, we are able to design single-stranded oligonucleotides that specifically block the binding of PRC2 to an individual lncRNA, thus de-repressing the expression of a single mRNA to elevate protein levels. Starting with a genome-wide pool of RNA sequences that interact with PRC2, we are now developing nucleic acid compounds that selectively upregulate gene expression for therapeutic benefit. We are applying this technology to develop new treatments in the therapeutic areas of rare genetic disorders, oncology, metabolic diseases and neurodegenerative diseases.

131 RNA-based immunotherapeutics against cancer

Andreas Kuhn[1], Janina Buck[2], Florian Eberle[2], Britta Vallazza[2], Joanna Kowalska[3], Jacek Jemiliety[3], Edward Darzynkiewicz[3], Ugur Sahin[1]

[1]Ribological GmbH, Mainz, Germany & TRON, University Medicine Mainz, Germany; [2]Ribological GmbH, Mainz, Germany; [3]University of Warsaw, Poland

RNAs with optimized properties are increasingly investigated as a tool to deliver the genetic information of complete antigens into professional antigen-presenting dendritic cells for HLA haplotype-independent immunotherapies against cancer. Upon entering dendritic cells, the encoded antigen is translated from the RNA, and then processed into epitopes that are presented on MHC class I and II complexes triggering an antigen-specific T cell response (1).

The dose of the antigen and the duration of its presentation are critical factors for generating strong and sustained immune responses. Substantial increase of the amount of antigen produced from RNA can be achieved by optimizing RNA stability and translational efficiency. Both features are determined by *cis*-acting elements in the RNA, i.e. the 5' cap, the poly(A)-tail, and the sequence of the non-coding and coding regions. Continuous improvement of RNA by modifying the different elements for increased expression of foreign proteins in dendritic cells has been a main goal of our studies (2).

We have developed plasmid templates for *in vitro* transcription of RNAs with modified 3' structures (3' UTR and poly(A)-tail) stabilizing the RNA and optimizing its translational performance in dendritic cells. Moreover, we observed that RNAs capped with the D1 diastereoisomer of $m_2^{7,2'\text{-O}}\text{Gpp}_\text{S}\text{pG}$ (beta-S-ARCA) have increased stability and translational efficiency in dendritic cells. Recently, we have analyzed the effect of the codon composition on the level of antigen expression by testing up to ten different constructs for several antigens. Significant differences can be observed, and the expression of the antigen can be enhanced up to ten-fold compared to the wild-type codon composition. By combining these measures we were able to profoundly improve properties of RNA-encoded immunotherapeutics.

To initiate human clinical testing of our RNA technology, a suitable manufacturing process to produce gram amounts of RNA using the modified cap analog and optimized sequence elements as given above has been established under "Good Manufacturing Practice". The first patients have been treated, demonstrating safety of the treatment. Immunological assessment of the efficacy to induce antigen-specific immune responses in humans is in progress.

In principle, our optimized RNA technology can be generally used for exogenous protein expression in target cells (3). Importantly, cell-type dependent differences have been observed for the effects of the *cis*-acting RNA elements. Therefore we have started a screening program to identify the optimal combination of cap structures and UTR sequences for multiple cell lines and primary cells.

(1) Kreiter et al. Curr. Opin. Immunol. (2011) 23: 399-406

(2) Kuhn et al. Curr. Gene Ther. (2012) 12: 347-361.

(3) Grudzien-Nogalska et al. Methods Mol. Biol. (2013) 969: 55-72

132 Antisense Oligonucleotide Therapies for the Treatment of Neurodegenerative and Muscular Diseases

Frank Rigo[1], *C. Frank Bennett*[1]

[1]ISIS Pharmaceuticals, Carlsbad, CA

Antisense oligonucleotides (ASOs) are chemically modified oligonucleotides that are designed to specifically bind to a targeted RNA through Watson-Crick base pairing. There are a variety of mechanisms by which ASOs modulate RNA function after hybridization, including promoting the degradation of the RNA and modulating RNA processing. Neurodegenerative and muscular diseases are an attractive area for antisense technology in that there a number of diseases in which the genetic basis is known. Because ASOs target RNA, rather than protein, they can be used to directly modulate a broad range of genetic defects that cannot be targeted easily by conventional small molecule or protein based drugs. Isis Pharmaceuticals is developing antisense drugs for several inherited neurodegenerative and muscular diseases including amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), Huntington's disease (HD), tauopathies and myotonic dystrophy (DM1). Preclinical studies in rodents and non-human primates demonstrate broad distribution of single stranded ASOs in CNS tissues following central administration, directly into the CSF, with commensurate modulation of gene expression in spinal cord and different brain regions. In addition, we have demonstrated that ASOs can also modulate gene expression in skeletal muscle when administered peripherally. The tissue half lives of the drugs in the CNS and muscle of mice and non-human primates is in the order of months. We have also used ASOs in mouse models of disease and will present out work in mouse models of SMA and DM1. We recently completed an early stage clinical study in SMA demonstrating safety and tolerability of antisense drugs administered by intrathecal injection. This early clinical experience supports the continued development of antisense drugs for severe neurodegenerative and muscular diseases.

133 Extracellular RNAs are markers of muscle myogenesis following splice switching oligonucleotide therapy in a mouse model of Duchenne muscular dystrophy

Matthew Wood[1]

[1]University of Oxford

Extracellular RNAs are promising disease biomarkers for a number of disease conditions. Here we report that in the mdx dystrophin-deficient mouse model of Duchenne muscular dystrophy (DMD), extracellular dystromiRs (miR-1, miR-133a and miR-206) show dynamic patterns of expression in serum throughout disease progression and in response to exon skipping-mediated dystrophin restoration utilizing potent peptide-morpholino oligonucleotide conjugates. This dynamicDystromir expression pattern is found to be distinct from the expression of the corrected dystrophin protein, while the extracellular RNAs show a positive correlation with the expression of myogenic transcription factors suggesting that these miRNAs are indices of muscle myogenic i.e. regenerative / degenerative status and probably reflect activity of the myogenic stem cell niche within the diseased muscles. Further data obtained following experimentally-induced skeletal muscle regeneration in non-dystrophic mice support this hypothesis. Moreover the release of these extracellular miRNAs is likely to be a regulated, specific process (i.e. not simply a result of passive release from degenerating muscle) given that the extracellular RNA expression pattern is significantly different from that found within the muscle tissue. Further, the majority of the extracellular dystromiRs are bound in protein/lipoprotein complexes and only a minority are found in extracellular vesicles. These data therefore have important implications for understanding the biological and clinical significance of extracellular RNAs in DMD and other neuromuscular diseases, and in particular following splice switching oligonucleotide therapy.

134 Silencing gene expression by recruiting RISC

Jennifer Broderick[1], *Neil Aronin*[1], *Phillip Zamore*[2]

[1]RNA Therapeutics Institute; [2]RNA Therapeutics Institute, Investigator HHMI, Gretchen Stone Cook Professor of Biomedical Sciences

The capacity for small RNAs to repress gene expression depends on nucleic acid base-pairing between the target mRNA and a small RNA guide bound to an Argonaute protein. A challenge to the successful use of small interfering RNA (siRNA) in mammals has been effectively triggering RNAi in vivo while simultaneously protecting the siRNA from nucleolytic degradation and delivering it to the correct tissue and cell type. 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 microRNAs (miRNA). Our strategy employs a stablized, synthetic oligonucleotide tether that recruits endogenous microRNAs 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. A tether designed to recruit *let-7* directed a >90% reduction in luciferase reporter expression, compared to a control tether directed towards a miRNA not present in animal cells. Tethers designed to recruit miR-21 or *let*-7a to the 3´ UTR of an endogenous mRNA reduced mRNA abundance by ~70%. 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.

Workshop 6: Tutorial on prediction of RNA secondary structure

Time: Friday, June 14, 17:00 - 18.30
Location: Sanada
Abstracts: 135 - 141
Chair(s): Alain Laderach, University of North Carolina at Chapel hill

135	**What can you know about noncoding RNAs without doing any experiments?**
136	**Methods of Predicting RNA Structure Change Due to Mutation**
137	**RNAsnp: Predicting SNP Effects on Local RNA Secondary Structure**
138	**Navigating through the MC-Flashfold 2D suboptimal solution maze using simple structural transformation rules**
139	**How to determine binding affinities and binding motifs for RNA-bindingproteins from CLIP-seq data**
140	**The HIV-2 Rev-Response Element: Determining Secondary Structure and Defining Folding Intermediates**
141	**Structural spectrum of long non-coding RNAs revealed by experiment**

135 What can you know about noncoding RNAs without doing any experiments

Zasha Weinberg[1], *Ronald Breaker*[1]

[1]HHMI / Yale University

This tutorial will address computational and other non-experimental means to improve the understanding of the structure and function of non-coding RNAs whose secondary structure is conserved. The session is aimed primarily at experimental biologists, and will not assume any computational background. Concepts to be addressed include: analysis of covariation to detect and understand a family of RNAs sharing a common structure, and using flanking genetic elements as clues to RNA function. These approaches are often useful both for finding novel noncoding RNAs and for developing knowledge and hypotheses about poorly understood RNAs.

136 Methods of Predicting RNA Structure Change Due to Mutation

Matt Halvorsen[1], *Joshua Martin*[1], *Justin Ritz*[1], *Alain Laederach*[1]

[1]University of North Carolina at Chapel Hill

In determining the functional effect of a mutation in RNA, it is important to consider potential effects of the mutation on RNA structure. Alteration of the structural ensemble of an RNA due to mutation can influence the very network of regulatory interactions an RNA is involved with in its lifetime by altering the binding affinity of the transcript for various interacting RNAs and proteins, and in some cases where an RNA is enzymatically active, influence its very catalytic activity. We present various means of predicting the degree and nature of RNA secondary structure change in a given transcript from mutation. We first compare the traditional minimum free energy model of RNA structure to a probabilistic model that we utilize where a full ensemble of possible structures for a given RNA is represented. We then review the SNPfold algorithm as a means of quantifying the global RNA structure change brought about by given mutations using commonly available RNA structure software. Various flavors of the SNPfold algorithm, as well as a method of non-parametrically calculating the significance of global structure change brought about by a point mutation when compared to all other possible mutations in a transcript, are discussed. We review the use of Principal Component Analysis (PCA) as a method of visualizing structure ensembles and their disruption upon introduction of a mutation. We take measurements of structure change from Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) experiments, and compare the experimental data to predictions made using the described methodology in order to determine the sensitivity and specificity of our predictions.

137 RNAsnp: Predicting SNP Effects on Local RNA Secondary Structure

Radhakrishnan Sabarinathan[1], Hakim Tafer[5], Stefan E Seemann[3], Ivo L Hofacker[5], Peter F Stadler[4], Jan Gorodkin[2]

[1]Center for non-coding RNA in Technology and Health, IKVH, University of Copenhagen; [2]Center for non-codign RNA in Technology and Health, IKVH, University of Copenhagen; [3]Center for non-coding RNA in Technology and Health, IKVH, University of Copenhagen; [4]University of Leipzig; [5]University of Vienna

The function of many noncoding RNAs and cis-regulatory elements of mRNAs largely depends on the structure, which is in turn determined by their sequence. Single Nucleotide Polymorphisms (SNPs) and other mutations may disrupt the RNA structure, interfere with the molecular function, and hence cause a phenotypic effect. Thus it is of relevance to search for structural changes induced by SNPs. Existing methods have addressed this problem by considering global folding of the wild-type and mutant sequences respectively, and then compared them in various ways. However, if the structural change is only local, it can be hard to detect on global scale. Therefore, we have developed RNAsnp (based on RNAfold from the Vienna package), which is an efficient method for prediction of SNPs effects on local RNA secondary structure. RNAsnp searches efficiently all subsequences (local regions) for structural effects and selects the one where structural difference is largest based on some distance measure (of which a number is benchmarked). To determine the significance of the SNP effects empirical p-values are computed from the background distribution generated using random sequences, for which computational efficiency is obtained by using extensive precomputed tables describing the score distributions as a function of sequence length, G+C content, and SNP position.

Only some 30 examples are reported in the literature where the RNA structure could be the cause of dysfunction in the presence of the SNP. In four of these cases probing analysis was carried out and confirmed an actual structural change (on top of in silico predicted methods). Additionally, on a data set comprising 501 SNPs associated with human inherited diseases, we predicted 54 to have significant local structural effect in the UTR region of mRNAs. RNAsnp is available both as standalone software and as a web server. The RNAsnp Web server provides a convenient interface to provide input data to RNAsnp and to select different modes of operation. It helps to visualize the output using informative graphical representation, such as dot plot matrices comparing pair probabilities for wild-type and mutant. In addition, the web server is connected to a local mirror of the UCSC genome browser database that enables the users to select the genomic sequences of interest for analysis and to visualize the results in the UCSC genome browser. Both software and web server are available at http://rth.dk/resources/rnasnp.

138 Navigating through the MC-Flashfold 2D suboptimal solution maze using simple structural transformation rules

Paul Dallaire[1], Stefanie Schirmer[2], Francois Major[1]

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RNA 2D structure prediction from a single sequence often requires the inspection of large numbers of suboptimal solutions proposed by the software. This exercise is unproductive and the modeler is left with little insights about the native structure. However, many of the proposed structures are very similar and networks can be build, where each connected pair of structures in a network differs from each others by a simple transformation. Structures that cannot be linked by a series of simple transformations are found in other networks. We consider simple a transformation between two structures when they differ by a bulge loop migration, or the formation of a base pair. Even with only these two simple transformations, we can transform long lists of suboptimal predictions into a tractable number of independent networks that can help to: 1) Interpret the RNA 2D proposed structures; and, 2) Compare the networks of two different sequences, for instance to evaluate the structural impact of mutations. Besides, these networks are particularly informative when non-canonical base pairs are considered. We generated such networks under the statistical framework of nucleotide cyclic motifs (NCM) using a faster and leaner version of the MC-Fold program, MC-Flashfold. For instance, MC-Flashfold with a threshold of 8 kcal/mol produces a quarter million suboptimal structures, within which the native cloverleaf is found in the few first hundreds. This is a problem for the end-user who has no clue about selecting the right one. Among more than 7,000 networks build from the suboptimal structures, the native structure was found in the second one, where the networks are ranked by the free energy of its most stable member. Other concrete examples, benchmarks, as well as MC-Flashfold and network applications will be discussed.

139 How to determine binding affinities and binding motifs for RNA-bindingproteins from CLIP-seq data

Rolf Backofen[1], *Daniel Maticzka*[1], *Fabrizio Costa*[1]

[1]University of Freiburg

The analysis of binding sites of RNA-binding proteins is becoming an increasingly important topic. The standard experimental approach for determining binding sites is CLIP-seq. Since not all possible binding sites are covered due to differential expression in tissues and developmental states, the main problem is to come up with good motif descriptions to find missing binding sites and to evaluate the binding strength.

It is well know that binding motifs for RNA-binding proteins have to contain both sequential and structural information [1,2]. Previous approaches used only structural profiles. Our new approach GraphProt uses an advanced machine learning approach based on a graph-kernel [3], and is able to use both structural profiles as well as detailed 2D-structures, without the need to decide a priori about the weight of the different structural components. In addition, we are able to integrate also context information. Furthermore, we provide support for mutagenesis analysis, which is a non-trivial task, provided that structural information is used.

We have compared our approach with the current state of the art, RNAcontext, on the in-vitro data that where used for evaluation in the RNAcontext publication. We outperform RNAcompete for all 9 proteins used in this evaluation (see Figure1 below). Furthermore, we will report on our results of applying GraphProt on CLIP-seq data for PTB. Our predictions highly correlate with measured affinities. We have used the discovered motifs to search for new binding sites within a mini-gene construct, which could then be verified experimentally by our cooperation partner.

1. Michael Hiller, Zhaiyi Zhang, Rolf Backofen, and Stefan Stamm. *Pre-mRNA Secondary Structures Influence Exon Recognition*. **PLoS Genet**, 3 no. 11 pp. e204, 2007.
2. Michael Hiller, Rainer Pudimat, Anke Busch, and Rolf Backofen. *Using RNA secondary structures to guide sequence motif finding towards single-stranded regions.* **Nucleic Acids Res,** 34 no. 17 pp. e117, 2006.
3. Steffen Heyne, Fabrizio Costa, Dominic Rose, and Rolf Backofen. *GraphClust: alignment-free structural clustering of local RNA secondary structures*. **Bioinformatics,** 28 no. 12 pp. i224-i232, 2012

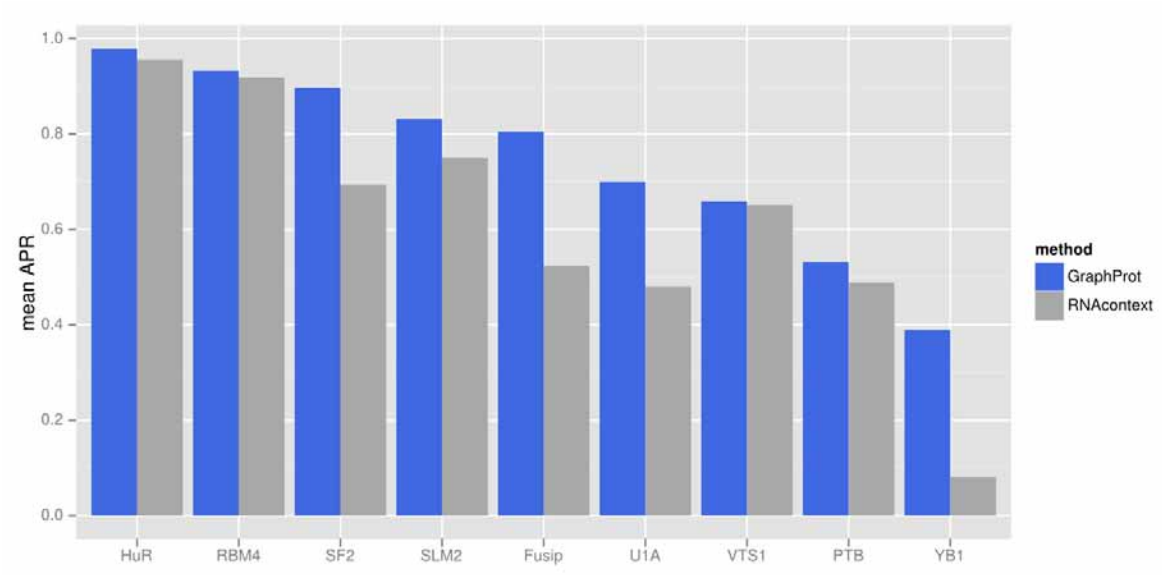

Figure 1

140 The HIV-2 Rev-Response Element: Determining Secondary Structure and Defining Folding Intermediates

Joanna Sztuba-Solinska[1], Sabrina Lusvarghi[1], Katarzyna Purzycka[1], Gary Pauly[2], Jason Rausch[1], Stuart Le Grice[1]

[1]Reverse Transcriptase Biochemistry Section, HIV Drug Resistance Program; [2]Chemical Biology Laboratory, Frederick National Laboratory for Cancer Research, Frederick, MD21702, USA

Similar to its' HIV-1 counterpart, the Rev response element of HIV-2 (RRE2) is responsible for transporting unspliced and partially spliced viral RNA from the nucleus to the cytoplasm. We applied complementary chemical and biochemical probing techniques to determine the structure of the RRE2 RNA. SHAPE analysis indicates that the low-energy form of RRE2 contains five peripheral stem loops linked by adjacent 4-way and 3-way junctions. Interestingly, electrophoretic fractionation of RRE2 RNA under non-denaturing conditions revealed additional conformers (open and intermediate) that undergo conversion to the more stable, low energy (closed) form in a time-dependent manner. This transition was verified by kinetic analysis, and the structures of the three forms were determined using a novel method for mathematically dissecting an ensemble of SHAPE profiles. Site-directed hydroxyl radical footprinting was used to examine the relative positioning of select motifs within these structures in three-dimensional space. Overall, our study provided detailed insight into the RRE2 conformation, and as such will aid in the development and application of nanoparticle-based antiviral drugs targeting *cis*-acting RNA regulatory signals.

For the workshop "Tutorial on prediction of RNA secondary structure"

141 Structural spectrum of long non-coding RNAs revealed by experiment

Irina Novikova[1], Scott Hennelly[1], Bin Zhang[3], David Spector[2], Karissa Sanbonmatsu[1]

[1]Los Alamos National Laboratory; [2]Cold Spring Harbor Laboratory; [3]Washington University in St. Louis

Thousands of long non-coding RNAs (lncRNAs) have been identified in the past few years. These RNAs have emerged as key players in development, cancer and brain function in mammalian systems. As many have been discovered only recently, very few structural studies have been performed. Fundamental questions regarding the structure of lncRNAs have yet to be addressed, including, (1) do lncRNAs have structure? and (2) are lncRNAs organized into modular sub-domains or linear chains of stem loops [1,2]? We have previously produced the first experimentally-derived secondary structure of an intact lncRNA by performing extensive biochemical studies in our wet lab using SHAPE probing, in-line probing, DMS probing and RNase V1 digestion [3]. Recently we have developed a new divide-and-conquer experimental strategy, called Shotgun Secondary Structure (3S) determination. The 3S experimental strategy allows us to produce the secondary fold with little need for computational predictions. First, we perform SHAPE probing on the entire lncRNA transcript. We repeat the probing on several overlapping fragments of the transcript. If the SHAPE profile for the fragment matches the profile for the same region in the context of the full transcript, this strongly suggests the fragment folds into a modular sub-domain or secondary motif, eliminating a large number of possible folds. We have applied this technique to the steroid receptor RNA activator (SRA1), the growth arrest-specific 5 transcript (Gas5), portions of MALAT-1 and other lncRNA systems. We find a variety of architectures, which may each be commensurate to the needs of the lncRNA system. The secondary structure of SRA1 resembles the 16S rRNA in terms of the modular organization into sub-domains and the relative number of helices, stem loops, internal loops and junctions. The Gas5 RNA consists of a roughly linear chain of stem loops separated by unstructured regions of RNA. Using SHAPE probing, we validated the triplex formation of the 3'-end of human MALAT-1, demonstrating that this motif is also structurally conserved in other species. Other systems show either a compact fold or a linear chain-like architecture. Numerous studies over the past few years have displayed a tremendous range of functional roles and disease applications for lncRNAs. Our results demonstrate that lncRNAs also have a diverse range of structural architectures.

[1] Sizing up long non-coding RNAs: do lncRNAs have secondary and tertiary structure? Novikova IV, Hennelly SP, Sanbonmatsu KY. Bioarchitecture. 2012, 2(6):189-99.

[2] Rise of the RNA machines: exploring the structure of long non-coding RNAs, Novikova IV, Hennelly SP, Tung, CS, Sanbonmatsu KY, Journal of Molecular Biology, 2013 (in press).

[3] Structural architecture of the human long non-coding RNA, steroid receptor RNA activator. Novikova IV, Hennelly SP, Sanbonmatsu KY. Nucleic Acids Res. 2012, 40(11):5034-51.

Plenary 6A: Interconnections in RNA regulation

Time: Saturday, June 15, 8:30 - 10:15
Location: Davos Ballroom
Abstracts: 142 - 148
Chair(s): Ulrike Kutay, *ETH Zürich*

142 **The transcription factors ILF2 and ILF3 are trans-acting factors for 60S ribosomal biogenesis**
143 **Novel function for human Argonaute 2 in gene regulation at the tRNA genes**
144 **The human cap-binding complex is functionally connected to the nuclear RNA exosome**
145 **A splicing-dependent transcriptional checkpoint**
146 **The Exon Junction Complex core component MLN51 interacts with eIF3 and activates translation**
147 **SRSF1-mediated translational regulation and its role in cellular transformation**
148 **The RNA kinase CLP1 is required for efficient tRNA splicing and regulates p53 activation in response to oxidative stress**

142 The transcription factors ILF2 and ILF3 are trans-acting factors for 60S ribosomal biogenesis

Franziska Wandrey[1], Christian Montellese[1], Lukas Badertscher[1], Lukas Bammert[1], Ulrike Kutay[1]

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The interleukin enhancer binding factors ILF2 (NF45) and ILF3 (NF90/NF110) were originally identified as a heterodimeric transcription factor complex required for Interleukin 2 expression in T-cells. Since then, the nuclear proteins ILF2 and ILF3 have been shown to be involved in various cellular pathways such as miRNA processing, DNA double-strand break repair and translation. Interestingly, ILF3 possesses two double-stranded-RNA binding domains (dsRBDs), allowing it to interact with different RNA species.

Using tandem affinity purification followed by mass-spectrometry analysis, we identified human ILF2 and ILF3 as novel components of premature particles of the large ribosomal subunit (pre-60S). This was supported by sucrose gradient analysis of HeLa cell extract where a fraction of NF45 and NF90, but not NF110, co-sedimented with pre-60S particles. Moreover, depletion of ILF2 or ILF3 by RNAi leads to a defect in 60S biogenesis as observed in a tetracycline-inducible RPL29-GFP cell line. Further, ILF2 or ILF3 downregulation results in a change in nucleolar size and number. These defects are not due to impaired transcription or processing of pre-28S or pre-18S ribosomal RNA (rRNA) as shown by fluorescence in situ hybridization and Northern blotting of different pre-rRNA species.

Notably, whereas most eukaryotic ribosomal trans-acting factors are conserved from yeast to higher eukaryotes, ILF2 is metazoan- and ILF3 vertebrate-specific. This suggests that the added layer of complexity in higher eukaryotes calls for additional regulatory factors in ribosomal biogenesis. Taken together, this indicates that ILF2 and ILF3 are novel, higher eukaryote-specific biogenesis factors required for the maturation of the 60S ribosomal subunit.

143 Novel function for human Argonaute 2 in gene regulation at the tRNA genes

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Enhancer sequences are critical for controlling cell-type specific gene expression. These can function at great distance from their target genes, and in some cases can be closer to non-target genes. The maintenance of specificity in enhancer-promoter interactions remains poorly understood. However, insulator sequences are critical in ensuring that only proper enhancer-promoter interactions are formed. Insulators block productive transcription when placed in between an enhancer and its promoter. In humans, the zinc-finger protein, CTCF is largely responsible for insulator function. It has recently been shown in *Drosophila* that Ago2 is essential for CTCF/CP190-dependent *Fab-8* insulator activity. Additionally, recent work in *S.pombe*, *D. melanogaster* and *H. sapiens* has shown that TFIIIC bound tRNA genes can function as insulator sequences. The mechanism of how these tDNAs can function as insulators remains unknown. An identification of the genome-wide binding sites for hAgo2 via ChIP-seq indicates an interaction with ~60% of tDNAs. We assessed TFIIIC-occupancy, which is a requirement for tDNA insulator activity in humans, and found that greater than 90% of Ago2-bound tDNAs are TFIIIC-occupied. This binding is cell cycle-dependent; binding is abolished when cells are halted in M-phase. We have further characterized the role of hAgo2 at human tDNAs by measuring changes in gene expression, histone modifications patterns, and long-range chromatin interactions in the absence of hAgo2. Furthermore, we have utilized a well-studied heterologous assay for measuring insulator activity to accurately quantify the impact the RNAi machinery has on insulator function in human cells. Our data suggests a novel function for the human RNAi machinery in regulating gene expression and large scale genome architecture.

144 The human cap-binding complex is functionally connected to the nuclear RNA exosome

Peter Refsing Andersen[1], Michal Domanski[1], Maiken S. Kristiansen[1], Evgenia Ntini[1], Celine Verheggen[3], Jakob Bunkenborg[2], Ina Poser[5], Marie Hallais[3], Anthony Hyman[5], John LaCava[4], Michael P. Rout[4], Jens S. Andersen[2], Edouard Bertrand[3], Torben Heick Jensen[1]

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Nuclear processing and quality control of eukaryotic RNA is mediated by the multi-subunit RNA exosome, which utilizes accessory factors to regulate its enzymatic activity. However, the mechanism of exosome recruitment to its ribonucleoprotein (RNP) targets remains poorly understood. We now disclose a physical link between the human nuclear RNA exosome and the cap-binding complex (CBC). The CBC associates with the ARS2 protein to form CBC-ARS2 (CBCA), and then further connects together with the uncharacterized ZC3H18/NHN1 protein to the nuclear exosome targeting (NEXT) complex, forming CBC-NEXT (CBCN). RNA immunoprecipitation analysis using CBCN factors as baits as well as the combinatorial depletion of CBCN and exosome components underscore the functional relevance of CBC-exosome bridging at the level of target RNA. Furthermore, we reveal that CBCN and the RNA Exosome together enforce a dual-level suppression of spurious transcripts in human cells. Specifically, CBCA suppresses U2 snRNA read-through- and promoter upstream-transcription by promoting transcriptional termination in these regions. We suggest that the RNP 5'cap links transcription termination to exosomal RNA degradation via CBCN.

145 A splicing-dependent transcriptional checkpoint

Keerthi Chathoth[1], *Shaun Webb*[1], *David Barrass*[1], *Jean Beggs*[1]

[1]University of Edinburgh

It is well established that pre-mRNA splicing and other RNA processing events often occur co-transcriptionally. Furthermore, high resolution kinetic studies performed in our lab showed splicing-dependent RNAPII pausing near the 3' splice site of a reporter gene[1]. Pausing requires splicing, as mutations that block splicing lead to loss of pausing, and restoring splicing restores pausing. We have proposed that RNAPII pausing may occur at splicing-dependent transcriptional checkpoints[1].

In a search for factors that are involved in coupling these processes, we identified several splicing factor mutations that cause transcription elongation defects. Prp5p is an RNA helicase that has been reported to act as a fidelity factor during pre-spliceosome formation[2]. We will present *in vivo* RNA labelling and RT-qPCR data demonstrating reduced transcription in *PRP5* mutant at the non-permissive temperature. ChIP analysis shows polymerase accumulating over intron-containing genes in the mutant strain, supporting a transcription elongation defect. We also analysed the phosphorylation status of the CTD of the large subunit of RNAPII, revealing that the apparently stalled polymerase is hyper-phosphorylated at Ser5. Furthermore, ChIP-Seq analysis in the mutant strain revealed that polymerase accumulated on 40% of the intron-containing genes genome-wide. We then identified that this transcription elongation defect in *prp5-1* is abolished upon removal of Cus2p, a U2 snRNP-associated protein. Our observations suggest that Cus2p could be a checkpoint factor in transcription prior to pre-spliceosome formation. We speculate that splicing fidelity factors may impose transcriptional checkpoints at different stages of splicing.

[1] RD Alexander et al, *Mol Cell* (2010) 40: 582-93

[2] YZ Xu and CC Query, *Mol Cell* (2007) 28: 838-49

146 The Exon Junction Complex core component MLN51 interacts with eIF3 and activates translation

Pierre-Etienne Chazal[1], Elisabeth Daguenet[2], Catherine Tomasetto[2], Bruno Sargueil[3], Herve Le Hir[1]

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The multiprotein Exon Junction Complex (EJC) that is deposited onto mRNAs by the splicing machinery nicely exemplifies the impact of nuclear history on mRNP lifecycle. This processing mark influences numerous post-transcriptional steps including splicing, transport, translation and surveillance via the nonsense-mediated mRNA decay pathway (NMD). The EJC notably offers a selective advantage to newly synthesized mRNAs by stimulating their translation efficiency. However, the exact mechanism by which the EJC functionally communicates with the translation machinery remains poorly understood.

The EJC is organized around a tetrameric core complex that accompanies spliced mRNAs to the cytoplasm. The core is composed of the DEAD-box RNA helicase eIF4AIII, MAGOH, Y14 and MLN51 (also known as Barentsz). Here, we show that among the EJC core components, the RNA-binding protein MLN51 is a translation enhancer. Overexpression of MLN51 in human cells preferentially increased the protein expression of intron-containing reporters through its incorporation within EJC cores. In contrast, its silencing decreased the translation of both spliced and unspliced reporters. In addition, modulation of MLN51 level in cell-free translational extracts confirmed its direct role in protein synthesis while metabolic labeling experiments demonstrated that it is a general translation activator. Polysome fractionation and immunoprecipitations indicated that MLN51 associates with translation initiation factors and ribosomal subunits in an RNase-insensitive manner. Finally, *in vitro* binding assays with purified factors revealed that MLN51 directly interacts with the central initiation factor eIF3 and that this interaction is compatible with EJC core assembly.

Altogether, our data define MLN51 as a new translation activator that constitutes a positive link between nuclear splicing and cytoplasmic translation machineries via the EJC.

147 SRSF1-mediated translational regulation and its role in cellular transformation

Magdalena Maslon[1], Sara Heras[2], Nicolas Bellora[3], Eneritz Aguirre[3], Eduardo Eyras[3], Javier Caceres[1]

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The serine and arginine-rich protein family (SR proteins) are highly conserved regulators of pre-mRNA splicing. The SR protein prototype, SRSF1 (also known as SF2/ASF), has been initially characterized as a splicing factor but also shown to mediate post-splicing activities such as mRNA export and translation. SRSF1 has also been proposed to act as an oncogene (1). We have previously shown that SRSF1 promotes translation initiation of bound mRNAs by suppressing the activity of 4E-BP, a competitive inhibitor of cap-dependent translation. This activity is mediated by interactions of SRSF1 with components of the mTOR signaling pathway. These findings suggest the model whereby SRSF1 functions as an adaptor protein to recruit the signaling molecules responsible for regulation of cap-dependent translation of specific mRNAs (2).

In order to dissect the importance of SRSF1 in translational control, we have performed a high-throughput deep sequencing analysis of polysomal fractions in cells overexpressing SRSF1. A group of more than one thousand mRNAs shifts from the subpolysomal fraction to the heavier polysomal fractions upon SRSF1 overexpression. Interestingly, one third of these mRNAs were previously identified as bona fide RNA targets of this SR protein by CLIP-seq. Bioinformatics analyses showed that these mRNAs encode proteins involved in cell cycle regulation, such as spindle, kinetochore and M phase proteins, which are essential for accurate chromosome segregation. In parallel, protein quantification using SILAC (Stable isotope labelling by amino acids in cell culture) confirmed that this subset of proteins was upregulated upon SRSF1 overexpression. Notably, SILAC analysis also revealed an increased expression of key glycolytic enzymes upon SRSF1 overexpression and several experiments confirmed a role for SRSF1 in enhancing a glycolytic switch (Warburg effect), a hallmark of proliferating cancer cells. Finally, we have analyzed changes in alternative splicing in response to different levels of SRSF1 protein. Interestingly, a significant proportion of those mRNAs that display alternative splicing changes are also translationally regulated by SRSF1. This suggests that SRSF1 influences several steps of an mRNA life.

Altogether, the finding that SRSF1 promotes the increased translation of genes associated with cell division and also regulates metabolic reprogramming in cancer cells could partially explain the oncogenic role of SRSF1. In summary, these data provide insights on the complex role of SRSF1 in the control of gene expression and its implications in cancer.

1. Karni et al. (2007) NatStruct Mol Biol., 14, 185-93.

2. Michlewski et al.(2008)Mol Cell, 30, 179-189

148 The RNA kinase CLP1 is required for efficient tRNA splicing and regulates p53 activation in response to oxidative stress

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Both in mammals and archaeabacteria CLP1 proteins have been identified as kinases that phosphorylate 5'-hydroxyl ends of RNA molecules. Human CLP1 is a component of the mRNA 3'-end cleavage and polyadenylation machinery and associates in mammals with the tRNA splicing endonuclease (TSEN) complex. TSEN proteins remove an intronic region present within the anticodon loop of numerous pre-tRNAs generating 5' and 3' tRNA exon halves. Within the TSEN complex, CLP1 has been shown to phosphorylate the 5'-hydroxyl group of 3' tRNA exons *in vitro*, potentially contributing to tRNA splicing in mammals. Here we characterize tRNA metabolism in kinase-dead *Clp1* (*Clp1*$^{K/K}$) mutant mice that contain a point mutation within the ATP binding motif of the genomic *Clp1* locus. We show that extracts prepared from *Clp1*$^{K/K}$ embryonic fibroblasts display reduced TSEN cleavage activity, revealing an unexpected role for CLP1 during tRNA exon generation. Affinity purified kinase-dead CLP1 containing TSEN complexes were deficient in pre-tRNA cleavage, most likely due to reduced levels of TSEN2, TSEN34 and TSEN54 subunits. Thus CLP1 is an integral component of the TSEN complex and ATP binding and/or hydrolysis is crucial for complex assembly. Interestingly, loss of CLP1 activity causes increased cell death upon H_2O_2 challenge and leads to accumulation of an entirely novel set of small tRNA fragments, comprising 5' leader and 5' exon sequences derived from aberrant processing of mainly tyrosine pre-tRNA. Overexpression of such tRNA fragments results in enhanced p53 activation in response to H_2O_2 challenge. We thus hypothesize that inactivation of CLP1's kinase activity sensitizes cells to oxidative stress-induced p53 activation and p53-dependent cell death.

Plenary 6B: **Alternative splicing**

Time: Saturday, June 15,

Location: Davos Ballroom

Abstracts: 149 - 155

Chair(s): Javier Caceres, *MRC Edinburgh*

149	**GENOME-WIDE IDENTIFICATION OF RECURSIVE SPLICING IN DROSOPHILA**
150	**Isolated pseudo-RRMs of SR proteins can regulate splicing using a non-canonical mode of RNA recognition**
151	**RBM5 OCRE domain modulates alternative splicing regulation by recognition of proline-rich motifs in spliceosomal SmN/B/B'**
152	**GSK3-Induced Regulation of the Protein- and RNA-Binding Activity of PSF Modulates Signal-Induced Alternative Splicing.**
153	**Rhythmic U2AF26 alternative splicing regulates the circadian clock in mice**
154	**RNA Binding Protein Sfpq is required for the expression of neuron-specific long pre-mRNAs essential for brain development.**
155	**The SWI/SNF subunit Brahma modulates the choice of alternative terminal exons by recruiting the BRCA1/BARD1 ubiquitin ligase**

149 GENOME-WIDE IDENTIFICATION OF RECURSIVE SPLICING IN DROSOPHILA

Sara Olson[1], Xintao Wei[1], Ahmad Osman[1], Michael Duff[1], Susan Celniker[2], Brenton Graveley[1]

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In *Drosophila*, the vast majority of introns are very short. As a result, the exons that are to be spliced together are in close proximity and can find one another easily. There are, however, many large introns (>50 kb) in *Drosophila* where it may be more difficult to juxtapose the exons that are to be spliced together. The 74 kb intron in the *Ultrabithorax* gene has been shown to be removed by a process called recursive splicing in which an upstream exon is spliced to a location within the intron, and the sequence of this splice junction re-creates a 5' splice site that can be subsequently respliced to another 3' splice site further downstream. The sites at which recursive splicing occurs are referred to as ratchet points. Though recursive splicing was discovered in 1998, only three additional genes (*kuzbanian, outspread,* and *frizzled*) have been experimentally shown to utilize recursive splicing to remove long introns. We have identified over 30 genes that undergo recursive splicing (and 70 ratchet points) by analyzing total RNA-Seq data generated from developmental time points, dissected tissues, and cultured cells. The recursive splicing events were identified by the presence of three specific features: 1) a novel splice junction that mapped to an annotated 5' splice site and an unannotated intronic 3' splice site, 2) the presence of the sequence AG/GT at the unannotated 3' splice site, and 3) a 5' to 3' gradient of RNA-Seq read intensity indicative of co-transcriptional splicing. We have validated many of these recursive splicing events by directed RT-PCR and sequencing. The average size of the intronic segments removed by recursive splicing is ~10 kb, and as many as 5 ratchet points have been identified in a single intron. Recursive splicing is detectable in all samples in which the host gene is abundantly expressed, indicating that recursive splicing is a constitutive process – we observe no evidence that recursive splicing occurs in some tissues or cell types, but not others. The ratchet points have a strong consensus sequence and are highly conserved among *Drosophila* species. We are currently performing RNA-Seq experiments in distantly related insects to investigate the functional conservation of recursive splicing. Together these results indicate that recursive splicing is commonly used in *Drosophila* and provides insight into the general mechanisms by which long introns are removed.

150 Isolated pseudo-RRMs of SR proteins can regulate splicing using a non-canonical mode of RNA recognition

Antoine Cléry[1], Rahul Sinha2, Olga Anczukow2, Anna Corrionero3, Ahmed Moursy1, Gerrit Daubner1, Juan Valcarcel[3], Frédéric H.-T. Allain[1], Adrian Krainer[2]

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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 and a pseudo-RRM. Although pseudo-RRMs are crucial for activity of SR proteins, their mode of action was unknown. By solving the structure of the human SRSF1 pseudo-RRM bound to RNA, we discovered a very unusual and sequence-specific RNA binding mode that is centered on one alpha-helix and does not involve the beta-sheet surface of the RRM. Remarkably, this mode of binding is conserved in all pseudo-RRMs tested. Furthermore, the isolated pseudo-RRM is sufficient to regulate splicing of about half of the SRSF1 target genes tested, and the bound alpha-helix is a pivotal element for this function. Our results strongly suggest that SR proteins with a pseudo-RRM frequently regulate splicing by competing with, rather than recruiting, spliceosome components, using solely this unusual RRM.

151 RBM5 OCRE domain modulates alternative splicing regulation by recognition of proline-rich motifs in spliceosomal SmN/B/B'

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RBM5 is a putative tumor suppressor gene frequently inactivated in cancers of the lung and other tissues and is down-regulated upon oncogenic Ras activation. Its main protein product is a 92 kDa, multi-domain protein that has been detected in pre-spliceosomal complexes and modulates cell proliferation and Fas mediated apoptosis. RBM5 was identified as a component of complexes involved in 3' splice site recognition, but contrary to classical mechanisms of splicing regulation, RBM5 does not affect early events of splice site recognition that lead to exon definition. Instead, RBM5 inhibits the transition between pre-spliceosomal complexes assembled around exons to mature spliceosome assembly on the flanking introns. Protein-RNA and protein-protein interactions are important for RBM5 regulation of alternative splicing of different target genes. Here, we examine the interaction of individual RBM5 domains with protein components of the spliceosome that are important to regulation of Fas exon 6 splicing.

The OCRE (OCtamer REpeat of aromatic residues) domain is important for RBM5 function in vivo and interacts with components of the U4/5/6 tri-snRNP. Using NMR spectroscopy, we show that the RBM5 OCRE domain adopts a novel ß-sheet fold with a hydrophobic surface rich in tyrosine residues that are key determinants both for the interaction with protein partners and for splicing regulation in vivo. The structure of a complex of RBM5 OCRE with a proline-rich motif (PRM) from the core spliceosomal SmN proteins and mutational analysis reveal that tyrosine residues on the hydrophobic surface of OCRE recognize a poly-proline helix, while flanking arginines in the PRM define the orientation. Interestingly, OCRE binds to individual proline-rich motifs with mM affinity, but with μMaffinity for the C-terminal tail of SmN, which contains multiple PRM motifs, suggesting avidity effects. Our data reveal how protein-protein interactions of the OCRE domain docks RBM5 to spliceosomal core protein for alternative splicing regulation.

152 GSK3-Induced Regulation of the Protein- and RNA-Binding Activity of PSF Modulates Signal-Induced Alternative Splicing.

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To maintain viability, virtually all cells in the human body must regulate their function in response to environmental stimuli. Such functional regulation requires control of gene expression in a signal dependent manner, including signal-regulated control of alternative splicing. However the molecular mechanisms by which signaling pathways impinge on splicing decisions remain largely unexplored.

We have recently identified hundreds of exons that display differential inclusion in response to antigen-stimulation of T cells. The best characterized of these T cell signal-regulated splicing events is the induced skipping of three variable exons of the CD45 gene. We have previously shown that signal-responsive repression of the CD45 exons is mediated in large part through the activity of the multifunctional RNA-processing protein PSF. PSF binds the exonic silencer elements of the CD45 pre-mRNA following T cell activation and favors variable exon exclusion. In resting cells, however, PSF is prevented from binding the ESS1 through a protein-protein interaction with TRAP150, which is dependent on phosphorylation of PSF at position T687 by the kinase GSK3.

Here we show that the two RNA recognition motifs (RRMs) of PSF are necessary and sufficient for binding to TRAP150. This interaction is direct and can be recapitulated with purified bacterial proteins. We further show that the RRMs of PSF interact in a mutually exclusive manner with TRAP150 and RNA, providing a mechanism for how TRAP150 blocks the function of PSF in the regulation of CD45 splicing. Surprisingly, the RRMs of PSF do not encompass the regulatory T687 residue, suggesting that phosphorylation of PSF-T687 regulates interaction with TRAP150 indirectly. Interestingly, limited proteolysis of PSF reveals a phosphorylation-dependent difference in digestion pattern, suggesting that phosphorylation of T687 induces a conformational change in PSF which controls accessibility to TRAP150. Together these data provide a unique example of signal-dependent control of an RNA binding protein through allosteric regulation of protein and RNA binding. Finally, we show that inhibition of GSK3, which occurs in response to T cell stimulation, is sufficient to account for approximately 40% of the alternative splicing events we observe during an antigen response in T cells. Thus we conclude that regulation of PSF by GSK3 plays a widespread role in shaping gene expression in T cells during an immune response.

153 Rhythmic U2AF26 alternative splicing regulates the circadian clock in mice

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[1]Philipps-University Marburg, Institute of Molecular Biology and Tumor Research (IMT)

The circadian clock is a cell-autonomous regulator driving daily rhythms in metabolism, behavior and physiology. At the molecular level, clock components of interlocking transcriptional feedback loops have been thoroughly investigated, whereas the impact of post-transcriptional regulation, especially alternative splicing, remains largely unexplored.

Here we describe a splice variant of mouse U2AF26 lacking exons 6 and 7 (U2AF26DE67) whose expression is strongly regulated with the circadian clock. Skipping of exons 6 and 7 induces a frameshift, thereby generating 144 novel C-terminal amino acids with interesting properties: the new C-terminus displays homology to the *D. melanogaster* core clock component Timeless and in addition substantially reduces the half-life of U2AF26DE67 protein, enabling diurnal expression of this specific isoform. Consistent with a function in regulating the circadian clock, we show that U2AF26DE67 interacts with and destabilizes Per1 protein in a cell culture model. To confirm this activity in vivo, we have generated U2AF26 deficient mice and analyzed the expression of core clock components at different circadian times. In line with our cell culture data, U2AF26 knockout mice display nearly acyclic expression of Per1 protein in the liver as well as a reduced and shifted peak of Per1 mRNA both in liver and cerebellum. mRNA expression of several other clock components such as Dbp, Npas2, and RevErba also showed a shifted expression profile, suggesting a broad circadian phenotype in U2AF26 deficient animals. We are now investigating whether these defects in the molecular clock of U2AF26 deficient mice lead to disturbed circadian locomotor activity.

In summary, we introduce U2AF26 as a novel component of the mammalian clock. We provide evidence for a circadian alternative splicing switch that directly regulates the expression of a central clock component in mammals. In addition, U2AF26 exon 6 and 7 exclusion is an interesting case for a splicing induced frameshift, resulting in translation into the 3'UTR to generate a protein with a new C-terminus with novel functions.

154 RNA Binding Protein Sfpq is required for the expression of neuron-specific long pre-mRNAs essential for brain development.

Akihide Takeuchi[1], Kei Iida[1], Kensuke Ninomiya[1], Mikako Ito[2], Kinji Ohno[2], Masatoshi Hagiwara[1]

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Recent methodological advances using microarray, deep-sequencing and biochemical analysis combining bioinformatics provide growing evidence for the essential roles of mRNA processing on the neural development. The mRNA processing is mediated by the RNA-binding proteins which regulate the expression of many genes co-transcriptionally or post-transcriptionally through direct interaction with pre-mRNAs. Here we found that RNA-binding protein, Sfpq (splicing factor, proline/glutaminerich) plays an essential function in mammalian brain. In embryonic mouse brains, *Sfpq* is specifically expressed in nascent cortical plate neurons after they differentiate and migrate from neuronal progenitor cells, suggesting its crucial functions for the development of cerebrocortical neurons. To address the *in vivo* function of Sfpq, we disrupted the *Sfpq* gene in mice brain. Neuronal tissue specific knockout of *Sfpq* caused apoptosis in neurons and massive loss of brain tissues in the developing brains including neocortex, indicating that Sfpq is essential for differentiation or maturation of neurons. Next, we produced a specific antibody against Sfpq, and conducted the iCLIP analysis to identify the target of Sfpq in the mouse embryonic neocortex. Distribution of the iCLIP tags showed saw-tooth patterns on entire pre-mRNAs of more than 7400 genes with low sequence specificity. The density of the tags was highest in 5' end of introns, and gradually declined to 3' end. In these large populations of Sfpq-binding genes, only specific gene subsets which express long pre-mRNAs were significantly down-regulated in the *Sfpq*-disrupted mice brain. According to the Gene Ontology, these genes have essential functions for the brain development, such as cell-adhesion, cell motion, axon guidance, ion channel activity or receptor related molecules, and synaptic vesicle transport and related molecules. Our comprehensive transcriptome analysis showed that 17.4% of specifically expressed pre-mRNAs exceed 262k in differentiated neurons . These data indicate that the RNA binding protein Sfpq is required for the expression of long pre-mRNAs which play essential roles for the survival of neurons, especially in the cortical plate at the developmental stages of brain.

155 The SWI/SNF subunit Brahma modulates the choice of alternative terminal exons by recruiting the BRCA1/BARD1 ubiquitin ligase

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The choice of polyadenylation sites located in alternative terminal exons is a widespread phenomenon that increases transcript and protein diversity. The choice of alternative poly(A) sites generates different 3'UTRs that can affect translation, stability and localization of the mRNA. When coupled to the inclusion of an alternative last exon (ALE) alternative polyadenylation leads to the generation of mRNA variants that not only differ in their 3'UTR but may also encode proteins with different C-terminal regions. Whereas the molecular details of mRNA polyadenylation are rather well known, little is known about the choice between alternative polyadenylation sites.

Brahma (BRM), one of the two alternative ATPase subunits of the human SWI/SNF chromatin-remodeling complex, was previously shown to control alternative splicing by slowing down RNA polymerase II. Here we show that the effect of BRM on the selection of ALEs does not involve the modulation of the elongation rate of RNAPII but rather implicates a different molecular mechanism. We report that BRM favors the selection of the distal ALE by inhibiting 3' end processing at the proximal poly(A) site. BRM accumulates on the proximal ALE promoting an interaction between the 50 kDa subunit of the 3' end processing factor CstF and the BARD1/BRCA1 E3 ubiquitin ligase. Ubiquitination of CstF50 leads to dissociation of the CstF complex and consequently to the inhibition of 3' end processing. We propose a model in which commitment of the proximal ALE to splicing and 3' end processing would require a reduction of the RNAPII elongation rate. In contrast, selection of the distal ALE would necessarily require both a highly elongating polymerase and inhibition of 3' end processing at the proximal poly(A) site to allow transcription to continue past the proximal ALE. This would result in the recruitment of splicing factors on the downstream 3' splice site, resulting in the 3' end processing complex at the distal poly(A) site and the splicing out of the proximal ALE.