2021
RNA Biology SYMPOSIUM

April 14-16, 2021

NIH **NATIONAL CANCER INSTITUTE**
RNA Biology Initiative

U.S. Department of Health & Human Services | National Institutes of Health

NATIONAL CANCER INSTITUTE
RNA Biology Initiative

Welcome to the 2021 National Cancer Institute Symposium in RNA Biology

RNA biology has emerged as one of the most influential areas in modern biology and biomedicine. The discovery of numerous novel classes of RNAs, coupled with the identification of the many critical processes in which they function, has revolutionized molecular biology and has profound implications for clinical science. RNA molecules are widely used as powerful tools to manipulate biological functions. Most recently, groundbreaking progress has been made that establishes the utility and immense potential of RNA-based and RNA-targeted therapies in preventing viral infections and treating previously intractable human diseases.

Organized by the NCI RNA Biology Initiative, this symposium brings together international leaders in both basic and translational areas of RNA Biology to report the latest developments in their fields and to explore the future of RNA biology and therapeutics. This symposium signals the NCI's commitment to this field and offers you an opportunity to learn more about the current status of RNA biology in development and disease, to share your research, to network with leading scientists, and to discuss the implications and use of these advances in enhancing the diagnosis of disease and the development of novel therapeutics.

Sincerely,

The NCI RNA Biology Initiative Steering Committee,

Ashish Lal, Ph.D.	Xinhua Ji, Ph.D.
Natasha Caplen, Ph.D.	Stavroula Mili, Ph.D.
Pedro Batista, Ph.D.	John 'Jay' Schneekloth, Jr., Ph.D.
Susan Gottesman, Ph.D.	Bruce Shapiro, Ph.D.
Shuo Gu, Ph.D.	John Shern, M.D.
Stephen Hughes, Ph.D.	Sandra Wolin, M.D., Ph.D.
Wei-Shau Hu, Ph.D.	Joseph Ziegelbauer, Ph.D.

CENTER FOR CANCER RESEARCH
RNA BIOLOGY SYMPOSIUM

AGENDA

Wednesday, April 14, 2021
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9:50 a.m.	**Welcome Remarks** Sandra Wolin, M.D., Ph.D., Center for Cancer Research, NCI
SESSION 1	Chair: Joana Vidigal, Ph.D., Center for Cancer Research, NCI
10:00 a.m.	**Assembly of the RNA Silencing Complex (and Beyond)** Yukihide Tomari, Ph.D., University of Tokyo
10:30 a.m.	**Mad About U: Regulating the let7 Pre-miRNA** Leemor Joshua-Tor, Ph.D., Cold Spring Harbor
11:00 a.m.	**BREAK**
SESSION 2	Chair: Astrid Haase, M.D., Ph.D., National Institute of Diabetes and Digestive and Kidney Disease, NIH
11:15 a.m.	**IsomiRs: How miRNAs Diversify Their Functions via Sequence Modifications at the Ends** Shuo Gu, Ph.D., Center for Cancer Research, NCI
11:45 a.m.	**Long Noncoding RNAs: Hidden Treasures in the Cancer Genome** Nadya Dimitrova, Ph.D., Yale University
12:15 p.m.	**BREAK**
12:30 p.m.	**POSTER SESSION AND LUNCH BREAK** **Poster Session 1: RNA Therapeutics** **Poster Session 2: RNA Decay** **Poster Session 3: Non-Coding RNAs 1**
SESSION 3	Chair: Colin Wu, Ph.D., Center for Cancer Research, NCI
1:45 p.m.	**Mysteries of RNA Processing by IRE1** Peter Walter, Ph.D., University of California, San Francisco
2:45 p.m.	**BREAK**
3:00 p.m.	**Control of Protein Function Through Localized RNA Translation in Dynamic Cellular Systems** Stavroula Mili, Ph.D., Center for Cancer Research, NCI

| 3:30 p.m. | **Genome Regulation by Long Noncoding RNAs** |
| | Howard Chang, M.D., Ph.D., Stanford University |

| 4:00 p.m. | **Adjourn for the day** |

Thursday, April 15, 2021
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| SESSION 4 | Chair: Eugene Valkov, Ph.D., Center for Cancer Research, NCI |

| 10:00 a.m. | **Initiation of Translation in Bacteria and Eukaryotes** |
| | Venki Ramakrishnan, Ph.D., Cambridge University |

| 11:00 a.m. | **BREAK** |

| SESSION 5 | Chair: Eugene Valkov, Ph.D., Center for Cancer Research, NCI |

| 11:15 a.m. | **Post-Translational Modification Effects on Phase Separation to Regulate RNA Biology** |
| | Julie Forman-Kay, Ph.D., Hospital for Sick Children |

| 11:45 a.m. | **Mapping Protein/RNA Phase Separation in Living Cells** |
| | Clifford Brangwynne, Ph.D., Princeton University |

| 12:15 p.m. | **BREAK** |

12:30 p.m.	**POSTER SESSION AND LUNCH BREAK**
	Poster Session 4: RNA Processing
	Poster Session 5: RNA Modifications 1
	Poster Session 6: Non-Coding RNAs 2

| SESSION 6 | Chair: Pedro Batista, Ph.D., Center for Cancer Research, NCI & Natasha Caplen, Ph.D., Center for Cancer Research, NCI |

| 1:45 p.m. | **RNA Modifications Modulate Function of RNA Virus Genomes** |
| | Cara Pager, Ph.D., University of Albany-SUNY |

| 2:15 p.m. | **Living in the World of RNAi Therapeutics Using Biomimetic Chemistry** |
| | Muthiah Manoharan, Ph.D., Alnylam Pharmaceuticals |

| 2:45 p.m. | **BREAK** |

| 3:00 p.m. | **The Convergence of Nanoscience and Nucleic Acid Medicines: New Approaches for Tracking and Treating Disease** |
| | Chad Mirkin, Ph.D., Northwestern University |

| 3:30 p.m. | **Toward Personalized microRNA Therapeutics** |
| | Frank Slack, Ph.D., Beth Israel Deaconess Medical Center |

| 4:00 p.m. | **Adjourn for the day** |

Friday, April 16, 2021

SESSION 7	Chair: Thomas Gonatopoulos-Pournatzis, Ph.D., Center for Cancer Research, NCI
10:00 a.m.	**The Structural Dynamics of the Soliceosome** Holger Stark, Ph.D., Max Planck Institute
10:30 a.m.	**RNA in Genomic Medicine Diagnosing Rare Disease and COVID 19 Implications** Diana Baralle, M.D., MBBS, University of Southampton
11:00 a.m.	**BREAK**
SESSION 8	Chair: Phil Adams, Ph.D., National Institute of Child Health and Human Development, NIH
11:15 a.m.	**Networks of Regulatory RNAs Connecting Metabolism Adaptation, Metal Homeostasis, Stress Responses and Virulence in Staphylococcus Aureus** Pascale Romby, Ph.D., University of Strasbourg
11:45 a.m.	**Phase Dependent CRISPR Evolution** Blake Wiedenheft, Ph.D., Montana State University
12:15 p.m.	**BREAK**
12:30 p.m.	**POSTER SESSION AND LUNCH BREAK** **Poster Session 7: Translation** **Poster Session 8: RNA Modifications 2**
SESSION 9	Chair: Katherine McJunkin, Ph.D., National Institute of Diabetes and Digestive and Kidney Diseases, NIH and Jack Shern, M.D., Center for Cancer Research, NCI
1:45 p.m.	**Distinguished Self and Nonself dsRNA in Vertebrates and Invertebrates** Brenda Bass, Ph.D., University of Utah
2:45 p.m.	**BREAK**
3:00 p.m.	**The HNRNPH1-Dependent Maturation of the EWS-FLI1 Pre-mRNA Expressed in One-Third of Ewing Sarcomas** Natasha Caplen, Ph.D., Center for Cancer Research, NCI
3:30 p.m.	**How microRNA-Target Interactions Direct Argonaute Recycling and Turnover** Joshua Mendell, M.D., Ph.D., University of Texas, Southwestern Medical Center
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CRISPR/CAS13 EFFECTORS DIFFER SIGNIFICANTLY IN THEIR SPECIFICITY OF RNA TARGETING IN CELLS

AI Y[1], LIANG D[1], WILUSZ JE[1]

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CRISPR/Cas13 effectors have garnered increasing attention in recent years as easily customizable tools that can be used to knock down RNAs of interest in cells. In a manner analogous to how Cas9 is recruited to DNA, the Cas13 ribonuclease is brought to its target RNA in a highly specific manner via base pairing with the Cas13-bound guide RNA. Near perfect complementarity over a 22-30 nt region is needed to trigger structural rearrangements that activate Cas13 RNase activity, and thus Cas13 has been proposed to be more specific than RNAi-based approaches. However, once activated, the Cas13 RNase catalytic sites appear to be exposed on the protein surface, thus providing the opportunity for other RNAs to be promiscuously cleaved. It has been suggested that such collateral damage effects are minor or non-existent in cells, but we now provide clear evidence that the off-target effects of some (but, importantly, not all) commonly used Cas13 effectors can be as strong as the level of on-target knock down observed. Using fluorescent reporter genes or minigenes that can be alternatively spliced to yield a linear or circular RNA in *Drosophila* and mammalian cells, we find that activated RfxCas13d non-specifically cleaves many RNAs, including the mRNA encoding RfxCas13d itself. The extent of off-target effects is positively correlated with the target RNA expression levels, and collateral damage can be observed even after reducing RfxCas13d/guide RNA levels. On the other hand, we find that PspCas13b shows much better specificity in these assays and can knock down an RNA of interest with minimal off-target effects. These results underscore the need for caution when interpreting Cas13 experiments, while also suggesting that certain Cas13 effectors, especially PspCas13b, are most appropriate for use in cells.

AN RNA THERAPY FOR ADVANCED METASTATIC BREAST CANCER

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Despite significant advances in the treatment of breast cancer overall, there is still a gap in the ability to successfully treat patients with late-stage metastatic breast cancer, particularly in the absence of the hormone receptors for estrogen and progesterone or the absence of HER2. In the case of triple negative breast cancer (TNBC), a breast cancer that lacks all three of these receptors, treatments are limited to standard chemotherapy, Poly ADP Ribose Polymerase (PARP) inhibitors for those tumors carrying BRCA1/2 mutations, and PD-L1 checkpoint blockade for those tumors with infiltrating lymphocytes, or tumors that are PD-L1+. The development of novel therapeutic strategies is therefore critically important to improving the survival rates of patients with this disease. Our research has identified microRNA-22 (miR-22) as a potential therapeutic target to fill this treatment gap. miR-22 is an oncogenic miRNA whose up-regulation promotes epithelial-mesenchymal transition (EMT), tumor invasion, and metastasis in non-triple negative breast cancer cell lines. Here we show that overexpression of miR-22 in a metastatic TNBC model causes the cancer to become more aggressive and it promotes the EMT phenotype, as shown by the increased expression of notable EMT key genes. Furthermore, we propose a novel therapeutic approach, based on locked nucleic acid (LNA) technology, that can drastically reduce metastasis and prolong overall survival in a TNBC mouse model. LNA compounds have proven to be effective *in vitro*, in 2D and 3D systems, as well as *in vivo*. We demonstrate that LNA antagomirs suppress proliferation and revert the EMT phenotype triggered by miR-22 overexpression. Our leading antagomir compound designed to target miR-22 effectively represses metastatic spread and markedly prolongs survival in xenograft models of metastatic TNBC. Our study therefore not only demonstrates the essential role of miR-22 in TNBC but also identifies a novel RNA-based therapy that could serve as the basis of an important therapeutic option in the treatment of TNBC by slowing its metastatic spread.

MIR-22 REPRESENTS A KEY REGULATOR OF LIPID HOMEOSTASIS AND A THERAPEUTIC TARGET IN NAFLD AND OBESITY

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A.M.N.: Department of Nutritional Sciences & Toxicology, University of California, Berkeley, CA, USA.

Obesity is a growing public health problem, affecting almost 2 billion people worldwide. It is associated with increased risk of type 2 diabetes, cardiovascular disease, non-alcoholic fatty liver disease (NAFLD) and cancer. Here we identify microRNA-22 (miR-22) as an essential rheostat involved in the control of lipid and energy homeostasis as well as the onset and maintenance of obesity. Using miR-22 knockout and transgenic models we demonstrate that miR-22 inactivation protects against obesity and hepatic steatosis, while its overexpression promotes these phenotypes even when mice are fed a regular chow diet. Mechanistically, we show that miR-22 orchestrates multiple pro-lipogenic programs and adipogenesis through both direct and indirect targets. Genetic ablation of miR-22 suppresses these programs, favoring metabolic reprogramming that promotes higher energy expenditure and browning of white adipose tissue. Importantly, we have developed an effective Locked Nucleic Acid (LNA) oligonucleotide-based therapy for obesity treatment that pharmacologically inhibits miR-22 and reverses both obesity and NAFLD in diet-induced obese mice. Over the past decade the field has seen the development of several new therapeutic approaches to obesity, ranging from bariatric surgery to pharmacological therapies, however these were not proven methods and their efficacy is limited thence there is still a tremendous need for new, effective and safe therapies for obesity. Our findings identify a critical non-coding RNA target and an effective therapy for treatment of obesity and other metabolic disorders supported by the results of our rigorous studies.

THERAPEUTIC DNA/RNA HYBRID NANOPARTICLES

LORENA PARLEA, HANNAH PRENSKY, KAYLEE MOODY, ANUK DAYAPREMA, BRUCE A. SHAPIRO

RNA Biology Laboratory, National Cancer Institute, Frederick, Maryland, USA

Due to their unique scaffolding properties, nucleic acid constructs can be programmed to incorporate in one nanoparticle several functional moieties for therapeutic, diagnostic, and/or imaging purposes. Complementary hybrid DNA/RNA constructs can be designed to release embedded functional RNAs solely upon reassociation through their toeholds. By manipulating the DNA length, hybrids can be programmed to re-associate through either DNA or RNA toeholds, according to the desired purpose. Generally, these hybrid constructs present several advantages over double stranded RNA: the release of the active RNAs is conditional to the hybrids reassociation, and they present lower cytotoxicity and immunogenicity than either DNA or RNA. Here, we demonstrate the use of RNA/DNA hybrid constructs with RNA toeholds functionalized for therapeutic purposes. We validated the hybrids in vitro, in cell culture, and in vivo. Hybrid formation, reassociation, and release of functional RNAs, and stability in serum, were characterized in vitro by electrophoretic mobility shift assays. To study the potential of these hybrids as therapeutic agents, we incorporated Dicer substrate RNAs (DsiRNAs) targeting overexpressed genes in the apoptotic pathways: Polo-like kinase 1 (PLK1), B-cell lymphoma 2 (BCL2), and Survivin. The release of DsiRNAs knocked down gene expression, arrested cell cycle, and restored apoptosis. We chose human colorectal adenocarcinoma cell line HT29 as a cancer model for validation in cell culture and in vivo. Hybrid assembly, reassociation and degradation in serum were determined in vitro with electromobility shift assays. Hybrid toxicity and efficacy in HT29 cell culture were determined by flow cytometry, cell cycle assays and cell viability assays. We used xenograft mouse models to assess the hybrid toxicity and efficacy in vivo. Preliminary studies revealed that hybrids are not toxic at the studied doses and significantly slow down tumor growth compared to untreated mice. We confirmed the downregulation of targeted genes and induction of apoptosis through antibody staining and TUNEL assays ex vivo. We conclude that hybrids can successfully reduce tumor growth in vivo. The therapeutic potential of this technology can be explored further by incorporating a variety of therapeutic RNAs according to the overexpressed gene signature of any particular disease or cancer type.

SPURIOUS ACTIVITIES OF T7 RNA POLYMERASE DURING *IN VITRO* TRANSCRIPTION

BIJOYITA ROY*, PETER WEIGELE, MATHILDE TOURIGNY, TIEN-HAO CHEN, MONICA WU

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The ability to transcribe genes of interest *in vitro* has led to the dissection of key biological steps that are involved in gene expression. In addition to their use as tools for the biochemical understanding of physiological processes, synthetic RNAs are being used in biopharmaceuticals, including RNA-based therapeutics, vaccines, and diagnostics. These applications have necessitated the development of enzymes for robust and efficient synthesis of homogeneous RNA populations that can be used both *in vitro* and *in vivo*. The technology used for the synthesis of these *in vitro*-transcribed (IVT) RNAs utilize phage-encoded single-subunit RNA polymerases (ssRNAPs). The most widely used ssRNAP is T7-RNAP because of its high processivity, specificity for small promoter sequence, and extensive characterization. However, T7-RNAP has spurious activities resulting in formation of by-products such as abortive transcripts during transcription initiation, heterogeneous 3' ends due to non-templated additions, and 3'-extended RNAs due to self-extension of the run-off product. In some cases, the spurious products can result in formation of dsRNA regions that have been identified as a trigger of cellular immune responses. It is critical to either eliminate these by-products from the mRNA preparations or minimize their formation. Thus, the discovery and characterization of ssRNAPs that can overcome the limitations of T7-RNAP and can generate IVT RNA devoid of unwanted by-products is highly desired.

Here, we discuss our multi-pronged approach to characterize the nature and source of dsRNA by-products generated by 3'-extension of the run-off product or by non-template strand transcription (antisense dsRNAs). These two classes of dsRNA are formed through distinct mechanisms. The 3'-extended RNA, which constitutes the bulk of dsRNA by-products, is formed in an RNA-dependent manner, whereas antisense dsRNA is formed by template switching in a DNA-template-dependent manner by T7 RNAP. We demonstrate that certain sequences at the 3' end of the DNA template have a higher propensity to promote non-template strand switching by T7 RNAP. We also present data on the propensity of other ssRNAPs that are similar and distant to T7 RNAP to form 3' extension of the run-off transcript.

We envision that understanding the mechanism of by-product formation in multiple ssRNAPs will provide insights into altering these activities in the RNAPs and generating more-homogeneous IVT RNA preparations.

GENOME EDITING WITH THIRD-GENERATION CHEMICALLY MODIFIED OLIGONUCLEOTIDES

WOOLF TW[1], RIVERA-TORRES N [2], HOGREFE R[3] AND KMIEC E[2]

[1]ETAGEN Pharma

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[3]TriLink Biotechnologies

Genome editing provides one of the few approaches that could in principle permanently cure genetic disorders. The overriding barrier to employing genome editing is safe and efficient delivery to the target cells *in vivo*. When donor DNAs for homology-directed genome editing are comprised of nuclease-stabilized, chemically-modified, single-stranded oligonucleotides we term them "ETAMERs™" (Gamper *et al.* 2000). Multiple treatments with ETAMERs result in accumulation of editing (Andrieu-Soler *et al.* 2005) which is projected to achieve editing levels sufficient to have a substantial therapeutic effect. Work by independent laboratories has demonstrated editing of various target genes in a number of target tissues *in vivo* with our proprietary 2nd generation ETAMERs design (for example, *Bertoni et al.* 2004).

ETAMERs have a number of advantages compared to methods of genome editing that require exogenous programmable nucleases, including:

- small size facilitating delivery *in vivo*
- non-immunogenic structure, allowing for multiple doses
- protein-free design allowing manufacture by chemical synthesis
- precise repair, permitting editing without insertions and deletions
- Reduced cost of manufacturing

Here we describe ETAGEN's 3rd generation ETAMERs with internal chemical modifications that are known to have reduced immune stimulation, increased serum nuclease stability, increased hybrid affinity and reduced chemical toxicity compared to 2nd generation ETAMER chemistries and unmodified donor DNA. These ETAMERs were screened for efficacy in our mutant GFP cell line, which becomes fluorescent when successfully edited *(Brachman et al. 2004)*. We propose self-delivering ETAMER designs that are the lowest molecular weight compounds that could permanently correct a point mutation without encapsulation in a delivery vehicle.

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ZCCHC8 IS A GLOBAL REGULATOR OF PERVASIVE TRANSCRIPTION

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The vast majority of mammalian genomes are transcribed as non-coding RNA in what is referred to as "pervasive transcription." Though the significance of pervasive transcription is unclear, it is obvious that cells must regulate these transcripts in order to protect against detrimental effects that may disrupt the finely tuned kinetics of gene expression and genome stability. While mechanisms of mRNA regulation are well-characterized, less is known regarding how cells regulate pervasive transcription. Recent studies have uncovered a particular family of non-coding RNA transcribed upstream of transcription start sites. These highly unstable promoter upstream transcripts, known as PROMPTs, are targeted for exosomal degradation by the nuclear exosome targeting complex (NEXT) consisting of the RNA helicase MTR4, the zinc-knuckle scaffold ZCCHC8, and the RNA binding protein RBM7. Rather than a specialized function of degrading PROMPTs, we hypothesized that ZCCHC8 and the NEXT complex may regulate pervasive transcription in a much more global manner. We performed a comprehensive and comparative genome-wide analysis of the effects of ZCCHC8 ablation on pervasive transcription. We used the CRISPR/Cas9 system to disrupt the *Zcchc8* gene in mouse salivary gland cells and then performed RNA-seq analysis to produce a global profile of pervasive transcription. Differential expression analysis of genomic regulatory features with non-overlapping genomic coordinates revealed for the first time that, in addition to PROMPTs, ZCCHC8 is required for the targeted degradation of pervasive transcripts produced at CTCF binding sites, enhancers, open chromatin regions, promoters, promoter flanking regions, and transcription factor binding sites. Further, our analysis revealed that many RIKEN cDNAs and predicted genes are pervasively transcribed, display all the hallmarks of PROMPTs, and are regulated by ZCCHC8 suggesting that they may not be authentic genes. To confirm our results, we analyzed publicly available RNA-seq data taken from E12.5 brains and ES cells derived from *Zcchc8* knockout mice. Despite highly differing gene expression profiles between salivary gland cells, brains, and ES cells, differential expression analysis confirmed ZCCHC8 is required for the targeted degradation of transcripts produced at these genomic regulatory features. Our results suggest that ZCCHC8, a key component of the NEXT complex, is a global regulator of pervasive transcription.

RNASE L MEDIATED ANTIVIRAL RESPONSE TRIGGERS TRANSLATION OF THE NON-CODING REGIONS OF MESSENGER RNAS

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Viral infections often trigger the activation of a ribonuclease, RNase L, which plays an important role in the innate immune response. During viral infection, oligo-adenylate synthase (OAS) produces a small molecule, 2'-5'-oligoadenylate (2-5A), which dimerizes and activates RNase L. The activated form of RNase L cleaves single stranded regions of viral and host RNAs and this activity is thought to promote clearance of the virus and apoptosis. Widespread cleavage of messenger RNAs (mRNA) leads to broad changes in their abundance but it remains unknown how this reduced pool of mRNAs is translated. Activation of RNase L was previously shown to trigger translation in the 3' untranslated regions (UTRs) of mRNAs in *in vitro* assays. To further investigate this phenomenon *in vivo*, we performed ribosome profiling experiments on RNase L activated A549 lung carcinoma cells. We found that RNase L activation leads to substantial accumulation of ribosomes in the 3'UTR, but not in RNase L KO cells, suggesting specificity for this pathway. In addition, we observed a relative increase in ribosomes in other non-coding regions of mRNAs, such as in the 5' UTRs and in alternate reading frames within protein coding regions. We also verified that ribosomes were actively translating in non-coding regions of mRNA using *in vitro* biochemical assays and by computationally dissecting positions of ribosomes. Analysis of published ribosome profiling data on viral infected cells showed that these unconventional translation events also occur during viral infections. Since translation of non-coding regions of mRNAs was dependent on the catalytic activity of RNase L, we favor a model where cleavage of mRNA by RNase L leads to the translation of mRNA fragments. While the function of the synthesized cryptic peptides is unknown, we propose that they could be presented by MHC-I molecules on the cell surface. These MHC I-peptide complexes are potentially recognized by the immune system as "non-self" and could therefore enhance clearance of the virus.

MONOCYTE CHEMOATTRACTANT PROTEIN-INDUCED PROTEIN 1 DIRECTLY DEGRADES VIRAL MIRNAS WITH A SPECIFIC MOTIF AND INHIBITS KSHV INFECTION

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Kaposi's sarcoma-associated herpesvirus (KSHV) is a member of γ-herpesvirus family and it is closely associated with Kaposi sarcoma (KS) and two rare lymphoproliferative disorders, primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD). Kaposi's sarcoma-associated herpesvirus (KSHV) expresses 12 pre-miRNAs during latency that are converted into 25 mature miRNAs. Host factors that are targeted by KSHV miRNAs play key roles in viral life cycle and cellular activities such as tumorigenesis, immune response, and apoptosis. Although KSHV miRNA sequences are highly conserved in cell lines and clinical samples, their expression levels vary. It is poorly understood how expression of KSHV miRNAs are regulated during KSHV infection. Our prior studies demonstrated that monocyte chemoattractant protein-induced protein 1 (MCPIP1), also known as Regnase-1 acts as a negative regulator of KSHV miRNA biogenesis by directly degrading KSHV pre-miRNAs. Interestingly, we also found that MCPIP1 degraded specific human, KSHV, and EBV pre-miRNAs with the different efficiencies. Here we further characterized MCPIP1 substrate specificity and its antiviral potential against KSHV infection. *In vitro* cleavage assay and binding assay showed that MCPIP1 cleavage efficiency is related to binding affinity. Motif based sequence analysis identified that KSHV pre-miRNAs that are well degraded by MCPIP1 have a 5-base-motif (M5 base motif) within their terminal loops and this motif region consists of multiple pyrimidine-purine-pyrimidine (YRY) motifs. We further demonstrated that this M5 base motif within terminal loop of pre-miRNAs positively affects MCPIP1-mediated enzymatic action. We also revealed that MCPIP1 has an antiviral effect against KSHV infection. Exogenous MCPIP1 expression inhibited KSHV infection while MCPIP1 depletion enhanced infection. Downregulated MCPIP1 expression and upregulated expression of Dicer were observed during KSHV *de novo* infection. We found that increased expression of MCPIP1 suppressed the levels of Dicer expression. Knockdown of Dicer inhibits *de novo* KSHV infection. Taken together, these results demonstrated that MCPIP1 inhibited KSHV infection and suppressed viral miRNA biogenesis by directly degrading KSHV pre-miRNAs and altering expression of miRNA biogenesis factor, Dicer. Antiviral function of MCPIP1 on KSHV infection suggests a potential novel therapeutic strategy to inhibit infection.

MULTIPLE CELLULAR SURVEILLANCE PATHWAYS MODULATE THE LEVEL OF COLLIDED RIBOSOMES

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Translation of aberrant eukaryotic mRNAs, such as those that are damaged, truncated or prematurely poly-adenylated, causes ribosomes to stall. Stalled ribosomes can collide with upstream, translating ribosomes, resulting a collision complex of two ("disome") or three ("trisome") ribosomes. A ribosome-associated quality control (RQC) system recognizes the collision, removes stalled ribosome and triggers degradation of the aberrant mRNA. Dysregulation of the RQC is associated with neurodegenerative disorders, suggesting that tight control of collisions is critical for cellular homeostasis. However, it has been unclear where collisions occur on non-aberrant, endogenous mRNAs and if these collisions are also targeted by the RQC pathway. To address this question in yeast, we used ribosome profiling technique, which is based on nuclease digestion of the mRNAs and next generation sequencing of ribosome-protected mRNA fragments. To obtain the genome-wide distribution of ribosome collisions in the cell, we specifically isolated disome/trisome footprints. Disome/trisome profiling showed that the collisions were enriched on stop codons and diverse sequence motifs known to slow translation. We also observed collisions at strategic locations of some mRNAs, which may potentially modulate co-translational events. Deletion of the E3 ubiquitin ligase and RQC factor, Hel2 (ZNF598 in mammals), or expression of an E3 ligase mutant of Hel2, decreased the relative occupancy of collisions. These findings show that Hel2 recognizes collisions and promotes their stabilization, possibly to allow a slower rescue process. Recognition by Hel2, however, did not trigger widespread degradation of collision-associated transcripts, indicating that endogenous collisions may not always lead to mRNA decay. Interestingly, we found that loss of Hel2 triggered the integrated stress response (ISR), which is a major signaling pathway that senses and combats stress. These data suggest the existence of crosstalk between the RQC and ISR pathways, where ISR proteins recognize collisions in the absence of Hel2. Consistent with a model, where two pathways share substrates, amino acid starvation known to trigger the ISR also increased ubiquitination of disomes by Hel2. Overall, our disome/trisome profiling methodology provided a global snapshot of ribosome collision events in the cell and led to a model that multiple surveillance pathways, ISR and RQC, can sense endogenous ribosome collisions.

THE 3'-5' EXONUCLEASE DIS3L2 UNDERGOES CONFORMATIONAL DYNAMICS DURING STRUCTURED RNA DEGRADATION

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The 3'-5' exonuclease Dis3L2 plays a central role in cytoplasmic decay of poly-uridylated RNAs as part of the Dis3L2-mediated decay quality control pathway. Loss of Dis3L2 causes the congenital fetal overgrowth disorder Perlman Syndrome, and its dysregulation has been implicated in the pathogenesis of various cancers including hepatocellular carcinoma.

Dis3L2 is a homolog of the RNA exosome associated nucleases Dis3 and Dis3L and is structurally highly similar to them, containing two cold shock domains (CSD), an RNA binding domain (RNB) and a S1 domain. Unlike Dis3 and Dis3L which function predominantly as part of the RNA exosome, Dis3L2 acts independently. Direct comparison of these homologs has shown that Dis3L2 degrades structured RNA substrates much more efficiently.

We sought to determine the mechanism of structured RNA degradation by Dis3L2, given its unique functional characteristics among the Dis3 family nucleases. To this end, we combined single particle electron cryo-microscopy (cryoEM) studies and pre-steady state kinetic analysis to build a step-by-step model of structured RNA degradation. We will present a number of cryoEM structures that represent snapshots along the degradation pathway, as well an analysis of the elementary rate constants at each step of the degradation.

Together the structural and kinetic analysis has allowed us to reconstruct a detailed model of RNA degradation, revealing a novel conformational change and the site of strand separation. To our knowledge, these are the first structures of an RNase II/R family nuclease bound directly to double-stranded RNA.

THE PERLMAN SYNDROME DIS3L2 EXORIBONUCLEASE SAFEGUARDS ENDOPLASMIC RETICULUM-TARGETED MRNA TRANSLATION AND CALCIUM ION HOMEOSTASIS

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DIS3L2-Mediated Decay (DMD) is a surveillance pathway for certain non-coding RNA (ncRNAs) including ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), and RMRP. While mutations in DIS3L2 are associated with Perlman syndrome, the biological significance of impaired DMD is obscure and pathological RNAs have not been identified. Here, by ribosome profiling (Ribo-seq) we find specific dysregulation of endoplasmic reticulum (ER)-targeted mRNA translation in DIS3L2-deficient cells. Mechanistically, DMD functions in the quality control of the 7SL ncRNA component of the signal recognition particle (SRP) required for ER-targeted translation. Upon DIS3L2 loss, sustained 3'-end uridylation of aberrant 7SL RNA impacts ER-targeted translation and causes ER calcium leakage.

Consequently, elevated intracellular calcium in DIS3L2-deficient cells activates calcium signaling response genes and perturbs ESC differentiation. Thus, DMD is required to safeguard ER-targeted mRNA translation, intracellular calcium homeostasis, and stem cell differentiation.

ONCOGENIC LNCRNAS DRIVE HISTONE VARIANT MISLOCALIZATION IN CANCER CELLS

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Chromosomal instability is a major defining event of cancer progression. The histone H3 variant CENP-A (Centromere protein A) is normally restricted to centromeres where it plays a fundamental role in centromere structure, identity and function. However, in a variety of tumors, we have reported that CENP-A can hijack the H3.3 chaperone pathways to deposit ectopically, thus invading regions such as the chr8q24 locus. Other than the presence of the proto-oncogene MYC, this region is typically a gene desert; however, the existence of a large DNase I hypersensitive site has driven us to examine non-coding transcription from this locus. Intriguingly, several non-coding RNAs are transcribed from the chr8q24 locus, making this region an oasis for non-coding transcription.

Long non-coding RNAs (lncRNAs) have less or no protein-coding potential, are over 200 nt in length with diverse cellular functions ranging from genome organization to transcription regulation. Deregulation of lncRNAs is one of the potential hallmark features of cancer progression. Overexpression of chr8q24 locus derived lncRNAs is frequently reported in many cancers and correlates with poor therapeutic outcome and recurrence. Here, we hypothesize that chr8q24 derived oncogenic lncRNAs are unwitting players in altering the local chromatin landscape by recruiting incorrect chaperone-histone variant complexes.

We knocked down the top candidate lncRNAs at chr8q24 locus (PCAT1, PCAT2, CCAT1, CCAT2, and PVT1), to study ectopic CENP-A (eCENP-A) localization in metastatic SW480 colon cancer cells. Interestingly, using coIF-DNA-FISH, we found that disruption of chr8q24 lncRNAs significantly reduced eCENP-A. Releasing the cells from the knockdown treatment significantly rescued the eCENP-A level at this locus. Remarkably, knocking down H3.3 chaperones (HIRA and DAXX) following the lncRNA knockdown prevented the cells from acquiring eCENP-A at the chr8q24 locus. Levels of the CENP-A at centromeres were not affected by the chr8q24 lncRNA perturbation, which confirms that these non-coding transcripts specifically alter the local chromatin from where they were transcribed. Furthermore, we find that the colocalization of kinetochore proteins with eCENP-A at chr8q24 impacts the chromosomal architecture, resulting in an increase in chromosomal break within this region. These data suggest a novel epigenetic mechanism linking locus and cancer-specific lncRNAs to aberrant chromatin structures in cancer cells.

LINKING *MUNC* LNCRNA STRUCTURE TO ITS FUNCTION

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MUNC lncRNA is upregulated in murine skeletal muscles and was firstly described as a facilitator of *Myod1* function in myogenesis. There are two *MUNC* isoforms: spliced and genomic, and both have promyogenic abilities. *siMUNC* reduces myoblast differentiation, and stable over-expression stimulates promyogenic RNAs. *MUNC* is also one of the evolutionary conserved lncRNA and its homolog can be found in human muscles.

In this study we focus on the relationship between *MUNC* lncRNA structure and function. Although the functional aspects of various lncRNAs have been studied for over 30 years, their structure and especially the functionality of distinct structural domains remain widely unknown. Therefore, studies of lncRNA structure and subsequent elucidation of its function and the regulatory mechanisms are urgently needed.

To analyze the functional similarities and differences between the *MUNC* two isoforms, we performed RNA-seq on myoblasts overexpressing *MUNC* spliced or genomic in proliferation and differentiation conditions. We show that both *MUNC* transcripts can regulate different sets of genes that are important for the same promyogenic pathways. We also find that *MUNC* spliced has more prominent promyogenic functions than the other isoform. Next, we obtained the secondary structure using SHAPE-MaP and we found that *MUNC* spliced and genomic have six common structural domains and eight common protein protected sites. Knowing that both *MUNC* isoforms induce promyogenic genes, we systematically deleted the most structurally similar regions. We have identified domains which are required for induction of *Myod1*, *Myogenin* and *Myh3*. We have also found out a domain which is necessary for *Myod1* stimulation, but not *Myogenin*, proving again that *MUNC* can act both *in cis* and *in trans*. We characterized distinct structural features facilitating *MUNC* binding to specific genomic sites by ChIRP-qPCR followed by measurement of the change of expression of the adjoining gene by qPCR. We also establish the structural domains which are necessary for *MUNC* interaction with cohesin complex (SMC3 protein) via RIP-qPCR.

Using the data about *MUNC* functional domains in context of gene expression regulation during skeletal muscle differentiation we can now better characterize this lncRNA and describe its mechanism of function.

CIS-ACTING SUPER ENHANCER LNCRNAS AS DIAGNOSTIC MARKERS OF PROGRESSION TO EARLY STAGE BREAST CANCER

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Increased breast cancer screening over the past four decades has led to a substantial rise in the diagnosis of ductal carcinoma in situ (DCIS). Although DCIS lesions precede invasive ductal carcinoma (IDC), they do not always transform into cancer. The current standard-of-care for DCIS is an aggressive course of therapy to prevent invasive and metastatic disease resulting in over-diagnosis and over-treatment. Thus, there is a critical need to identify functional determinants of progression of DCIS to IDC to allow discrimination between indolent and aggressive disease.

Super-enhancers are regulatory regions of DNA that play critical roles in driving expression of genes that define cell fate decisions and importantly, their normal function can become co-opted during tumorigenesis. Recent studies have found that super-enhancers, in addition to promoting other gene transcription, are themselves transcribed producing super-enhancer associated long noncoding RNAs (SE-lncRNAs). These SE-lncRNAs can interact with their associated enhancer regions in cis and influence activities and expression of neighboring genes. Furthermore, they represent a novel, untapped group of therapeutic targets.

The MCF10A breast cancer progression series is composed of four cell lines that mimic the progression of normal cells (MCF10A) to atypia (AT1), to DCIS (DCIS), and finally, to IDC (CA1). With an integrative analysis of enhancer loci with global expression of SE-lncRNAs in the progression series and patient samples in the TCGA database, we have unveiled 31 clinically relevant SE-lncRNAs that potentially interact with their enhancer to regulate nearby gene expression. To complement SE-lncRNA expression studies, we conducted an unbiased global analysis of super-enhancers that are acquired or lost in progression.

Here we designate SE-lncRNAs RP11-379F4.4 and RP11-465B22.8 as potential markers of progression through regulation of the expression of their neighboring genes. Moreover, defining the super-enhancers that are gained and lost at each transition reveals critical pathways that change during progression. 383, 684, and 28 super-enhancers were newly acquired at the AT1, DCIS, and CA1 stages, respectively. Conversely, 173, 120, and 259 super-enhancers were lost. Comparison analysis of acquired regions with super-enhancer regions in 47 ER positive patients, 10 Triple Negative Breast Cancer (TNBC) patients, and 11 TNBC cell lines reveal critically acquired pathways including STAT signaling and NF-kB signaling. In contrast, protein folding and local estrogen production are identified as major pathways lost in progression.

Collectively, these analyses identify differentially expressed SE-lncRNAs and acquired/lost super-enhancers in progression of breast cancer important for promoting DCIS lesions to IDC.

LONG NON-CODING RNA HOTTIP REGULATES MIRNA-196B REPRESSES FAS SIGNALING IN LEUKEMOGENESIS

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MicroRNAs (miRNAs) may modulate more than 60% of human coding genes and act as negative regulators, while long non-coding RNAs (lncRNAs) regulate gene expression on multiple levels by interacting with chromatin, functional proteins, and RNAs such as mRNAs and microRNAs. Growing evidence indicates that non-coding RNAs, in particular lncRNAs and microRNAs, regulate one another and cooperate to influence the levels of target mRNAs in a cell-type specific manner. LncRNAs process, interact with, and regulate miRNAs at both transcriptional and post-transcriptional levels. LncRNAs can function as miRNAs sponges, acting as decoys to impair the functional interaction of a miRNA and its target mRNA, thereby preventing suppression of target gene expression. Additionally, lncRNAs can be precursors of miRNAs and regulate miRNA biogenesis at different points. Primary miRNAs (pri-miRNAs) are transcribed in the genome (i) either within the body of another gene and often their expression is linked to the expression of the parent transcript, or (ii) from independent miRNA genes, similar to mRNA, where transcription is primarily controlled by RNA polymerase II-driven promoters. However, the molecular mechanisms, particularly those mediated by lncRNAs, regulating miRNAs transcription remains elusive. Using combined integrated analyses of global miRNA expression profiling and state-of-the-art genomic analyses of chromatin such as ChIRP-seq., (genome wide *HOTTIP* binding analysis), ChIP-seq., and ATAC-seq., we found that miRNA genes are directly controlled by *HOTTIP*. Specifically, the HOX cluster miRNAs (miR-196a, miR-196b, miR-10a and miR-10b), located *cis & trans*, were most dramatically regulated and significantly decreased in *HOTTIP*$^{-/-}$ AML cells. *HOTTIP* bound to the miR-196b promoter, and *HOTTIP* deletion reduced chromatin accessibility and enrichment of active histone modifications at HOX cluster associated miRNAs in AML cells, while reactivation of *HOTTIP* restored miR gene expression and chromatin accessibility in the CTCF-boundary-attenuated AML cells. In particular, miR-196b, which is located adjacent to *HOXA9*, targets *HOXA9* and its cofactor *MEIS1*. It also targets proapoptotic factor *FAS*, suggesting a double-edged sword (miR-196b) that could simultaneously repress both the oncogenic and tumor suppressor target genes. Inactivation of *HOTTIP* or miR-196b promotes apoptosis by altering the chromatin signature at the *FAS* promoter and increase *FAS* expression. Transplantation of miR-196b knockdown MOLM13 cells in NSG mice increased overall survival compared to wild-type cells. Thus, remodels the chromatin architecture around miRNAs to promote their transcription, consequently repressing tumor suppressors and promoting leukemogenesis.

CHARACTERIZING EXPRESSION AND REGULATION OF HUMAN AND VIRAL CIRCULAR RNAS

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Multiple herpesviruses have been recently found to regulate human circular RNA (circRNAs) expressions and to encode viral circRNAs. Like cellular circular RNAs, these RNAs lack poly-A tails and their 5' and 3' ends have been joined, which confers protection from RNA exonucleases. The regulatory mechanism of circRNAs by viruses is largely unknown and viral circRNA expression profiles *in vivo* is limited.

We first examined the expression patterns of circular RNAs from Kaposi's sarcoma herpesvirus (KSHV) in various environments. We performed deep sequencing of circRNA-enriched total RNA from a KSHV-positive patient lymph node for comparison with previous circRNA-Seq results. We found that circvIRF4 is highly expressed in the KSHV-positive patient sample relative to both B cell lines and *de novo* infected primary vascular and lymphatic endothelial cells. Overall, this patient sample showed a viral circRNA expression pattern more similar to the pattern from B cell lines, but we also discovered new back-spliced junctions and additional viral circular RNAs in this patient sample. We validated some of these back-spliced junctions as circular RNAs with RT-qPCR utilizing RNase R and divergent primers, and amplicon sequencings.

Differential expression patterns of circular RNAs in different cell types led us to investigate what cellular factors might be influencing the ratio of viral linear mRNAs to circular RNAs. We found that repression of certain RNA-binding proteins shifted the balance between viral linear mRNAs and circular RNAs, suggesting possible mechanism by which KSHV regulate circRNA expression during infection. Taken together, examining viral circular RNA expression patterns may become useful tools for discovering their functions, the regulators of their expression, and determining the stage and cell types of infection in humans.

AUF1 LIGANT CIRCPCNX REDUCES CELL PROLIFERATION BY COMPETING WITH P21 MRNA TO INCREASE P21 PRODUCTION

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Mammalian circRNAs can influence different cellular processes by interacting with proteins and other nucleic acids. Here, we used ribonucleoprotein immunoprecipitation (RIP) analysis to identify systematically the circRNAs associated with the cancer-related protein AUF1. Among the circRNAs interacting with AUF1 in HeLa (human cervical carcinoma) cells, we focused on *hsa_circ_0032434* (*circPCNX*), an abundant target of AUF1. Overexpression of *circPCNX* specifically interfered with the binding of AUF1 to *p21* (*CDKN1A*) mRNA, thereby promoting *p21* mRNA stability and elevating the production of p21, a major inhibitor of cell proliferation. Conversely, silencing circPCNX increased AUF1 binding to *p21* mRNA, reducing p21 production and promoting cell division. Importantly, eliminating the AUF1-binding region of *circPCNX* abrogated the rise in p21 levels and rescued proliferation. Therefore, we propose that the interaction of *circPCNX* with AUF1 selectively prevents AUF1 binding to *p21* mRNA, leading to enhanced p21 mRNA stability and p21 protein production, thereby suppressing cell growth.

THE NASCENT RNA STRUCTURE-OME IS A HIGHLY PLASTIC EFFECTOR OF CO-TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL SPLICING

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Most splicing occurs on nascent pre-mRNAs. We identified structures in nascent transcripts by chemical and enzymatic probing, and determined how the "nascent RNA structureome" relates to co-transcriptional pre-mRNA splicing and transcription speed. Extensive RNA folding within introns and steep structural transitions at splice sites are associated with efficient co-transcriptional splicing. Nascent RNA structure is extensively remodeled into more highly folded conformations associated with increased A-I editing in response to slow pol II transcription. Those introns that become more folded get spliced more efficiently suggesting that RNA structure promotes rapid co-transcriptional splicing. Slow transcription altered folding of some intronic Alu elements and promoted their cryptic splicing and intron retention, an outcome that is mimicked by UV treatment which decelerates transcription. Slow transcription also altered RNA folding at 3' splice sites downstream of alternative exons and those re-modeled structures predict whether exon skipping or inclusion is favored even though these splicing reactions are predominantly post-transcriptional. Hence co-transcriptional RNA folding, which responds to transcription speed, can influence post-transcriptional alternative splicing. These results suggest that nascent RNA structures are highly plastic with widespread effects on splicing.

MASSIVE PARALLEL REPORTER ASSAY DECODES CD19 ALTERNATIVE SPLICING IN CART-19 THERAPY RESISTANCE

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B cell acute lymphoblastic leukemia (B-ALL) is the most common type of cancer in children. Several therapies have been developed for this disease, being CART-19 (Chimeric antigen receptor T) one of the most successful. For this therapy, modified T-cells express a CAR that recognizes the CD19 receptor in the surface of cancerous B-cells, excerpting its elimination. However, it has been estimated that up to ~40% of patients experience a relapse. In almost half of the cases the relapse could be linked to the loss of the CD19 receptor from the surface, often due to errors in splicing like is the case of the exon 2 in the *CD19* gene.

Bearing in mind that alterations in splicing can be detrimental, we aimed to dissect the splicing regulatory network controlling the alternative splicing of *CD19* exon 2. We developed a massive parallel reporter assay of more than 10 000 wild-type and mutated minigenes containing the first three exons of *CD19*. By measuring the RNA products of each minigene we identified mutations that influence the splicing of *CD19*. Some of these mutations lead to the generation of cryptic isoforms, many of which will not produce a functional CD19 receptor. Along these cryptic isoforms, we also detected mutations linked to the production of an intron 2 retention isoform, which has been observed in relapsed patients.

Given that the control of splicing is highly dependent on *trans*-regulators like RNA-binding proteins, we also complemented our results with data from TCGA, GTEx other B-ALL cohorts and state-of-the-art prediction tools to characterize potential RBP regulators. Together, this approach allowed us to clarify the mechanisms of splicing regulation in CD19 and could potentially contribute to predicting therapy success prior to treatment.

TARGETING THE EXPRESSION OF ANDROGEN RECEPTOR SPLICE-ISOFROM IN CASTRATION-RESISTANT PROSTATE CANCER

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Advanced prostate cancer (PCa) treated with androgen deprivation therapy (ADT) eventually develops into castration-resistant PCa (CRPC). Studies have confirmed that alterations in the androgen receptor (AR) signaling lead to CRPC progression. Among the most frequent alterations in CRPC, are direct alterations in the AR gene; including AR amplification, point mutations, and generation of constitutively active AR splice-isoform. This splice-isoform lacks the ligand-binding domain and therefore, is the ultimate target of androgen depletion and a prognostic biomarker of CRPC. Serine/arginine-rich splicing factor 1 (SRSF1) binds to the AR pre-mRNA driving its alternative splicing to generate the AR splice-isoform and therefore, may act as a potential therapeutic target in CRPC. Splice factor kinase is a key protein in SRSF1 activation cascade, and is highly upregulated in CRPC patients. Hence, we hypothesized that modulating the protein kinase would be beneficial in advanced PCa treatment. Here, we have utilized a variety of molecular cell biology tools to generate CRPC cell lines with regulated expression of the splice factor kinase and analysed its effect on expression of AR isoform. We have observed downregulation of AR splice-isoform at transcript as well as protein levels on splice factor kinase knockdown without affecting endogenous AR-FL levels. Similar results were obtained on treating CRPC cells with the splice factor kinase inhibitor. Moreover, we have observed retarded growth and migration ability of kinase knockdown cells in clonogenic and wound healing assays, suggesting a direct role of splice factor kinase in regulating oncogenic potential of CRPC cells.

RETROVIRAL RIBONUCLEOPROTEIN COMPLEXES AS BIOMOLECULAR CONDENSATES: BIOGENESIS IN THE NUCLEUS AT TRANSCRIPTION SITES

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Retroviruses have single-stranded RNA genomes that are reverse transcribed into proviral DNA with integration into the host cell chromosome. The proviral DNA is transcribed by RNA polymerase II to produce spliced and unspliced viral RNAs (vRNA). Unspliced vRNA (US-vRNA) serves two roles, as mRNA for the translation of Gag and Gag-Pol structural proteins or as the genome (gRNA) packaged by Gag into new virus particles. Retrovirus assembly is nucleated when Gag binds to gRNA. It was previously thought that the initial Gag-vRNA interaction occurred in the cytoplasm or at the plasma membrane; however, we have reported that the Gag proteins of HIV-1 and the oncoretrovirus Rous sarcoma virus (RSV) form discrete nuclear foci that co-localize with their respective US-vRNAs at the burst of vRNA transcription. Using live cell time-lapse microscopy, we demonstrated that RSV Gag binds US-vRNA in the nucleus to form punctate ribonucleoprotein complexes (vRNPs) that traffic through the nuclear pore into the cytoplasm. Mutations in the RSV Gag nuclear export signal result in accumulation of the protein in large phase-contrasted nuclear foci that resemble nuclear bodies. These observations led us to examine whether RSV Gag and vRNPs undergo liquid-liquid phase separation (LLPS), a process by which protein-nucleic acid complexes exhibit liquid-liquid demixing to form biomolecular condensates.

Proteins that undergo LLPS form biomolecular condensates possessing liquid-like characteristics that depend on multivalent interactions, contain intrinsically disordered regions, and are sensitive to phase disrupting agents. Importantly, RNA plays an important role in regulating LLPS in RNA binding proteins such as Gag. Using FRAP, we found that RSV Gag foci are highly dynamic, with molecules that rapidly exchange with the surrounding environment; they undergo fusion and fission in vitro and in cells; they are disrupted by 1,6-hexanediol; and recombinant Gag proteins form phase-contrasted droplets that colocalize with vRNA. In cells, formation of condensed nuclear RSV RNPs depends on the RNA binding domain of Gag and its target sequence in the gRNA. Formation of Gag nuclear foci is sensitive to actinomycin D, suggesting that active transcription plays a critical role in the genesis and maintenance of Gag foci. Further exploration of the mechanism of vRNA-driven aspects of retroviral RNP formation is ongoing. We are testing the hypothesis that RSV RNPs form biomolecular condensates in the nucleus with biophysical properties that help them remain intact as they traverse the nuclear pore, traffic through the cytoplasm, and bind the plasma membrane to release progeny virions.

NUSG IS AN INTRINSIC TRANSCRIPTION TERMINATION FACTOR THAT STIMULATES MOTILITY AND COORDINATES GENE EXPRESSION WITH NUSA

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NusA and NusG are transcription elongation factors that stimulate RNA polymerase pausing and termination in *Bacillus subtilis*. Intrinsic termination is one of the two mechanisms by which transcription can terminate in bacterial systems. This mechanism proceeds via the formation of a GC-rich RNA hairpin within the RNA exit channel of RNA polymerase (RNAP), which initiates the melting of an AU-rich RNA-DNA hybrid. While intrinsic termination is generally described as a factor-independent process, by conducting Term-seq, a functional genomics assay that can be used to comprehensively identify all 3' ends within a transcriptome on both wild type (WT) and NusA-depleted ($nusA_{dep}$) strains of *B. subtilis*, we previously found that depletion of NusA resulted in a termination efficiency decrease ($\Delta\%T$) of $\geq 25\%$ for ~30% of all intrinsic terminators. We categorized these terminators as being NusA-dependent. The role that NusG serves in this process was still unknown, and so to examine the individual and combinatorial roles that NusA and NusG play in intrinsic termination, we further applied Term-seq towards WT, $nusA_{dep}$, $\Delta nusG$, and $nusA_{dep} \Delta nusG$ strains of *B. subtilis*. Through these experiments, we determined that NusG functions as an intrinsic termination factor, with 25% of all intrinsic terminators being NusG-dependent. Interestingly, we found that NusG works cooperatively with NusA, with 88% of all intrinsic terminators being categorized as dependent in the $nusA_{dep} \Delta nusG$ strain. Thus, we found that intrinsic termination is generally a factor-mediated process in *B. subtilis*. Our results indicate that NusG stimulates a sequence-specific pause via its NGN domain and this pause provides additional time for the completion of suboptimal terminator hairpins with weak terminal A-U and G-U base pairs at the bottom of the hairpin stem. Moreover, we found that the loss of both proteins leads to one third of all genes expressed in the wild type strain becoming misregulated and that the loss of NusG results in flagella and swimming motility defects.

CLINICAL SIGNIFICANCE OF DIFFERENTIALLY EXPRESSED RNA BINDING PROTEINS IN CASTRATION-RESISTANT PROSTATE CANCER PROGRESSION

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RNA binding proteins (RBPs) are a group of proteins associated with RNA metabolism. They regulate the transcriptome and proteome by controlling post-transcriptional processes such as alternative splicing, mRNA stability, mRNA polyadenylation, mRNA localization. Alteration in their function generates abnormal protein phenotype leading to the progression of diseases such as cancer. Castration-resistant prostate cancer (CRPC) is one of the deadliest forms of cancer among males which develops upon resistance to androgen deprivation therapy (ADT). In this study, we analyzed publicly available data sets of CRPC patients from the cancer genome atlas (TCGA) to identify RNA binding proteins whose altered expression is associated with poor survival outcome in CRPC patients. Our comprehensive analyses and rigorous selection of candidates based on significant fold change and false discovery rate, provided us with impactful RNA binding proteins, which can be considered for novel therapeutics in CRPC treatment.

Keywords: CRPC, RBPs, TCGA, DGE analysis.

FINDING NOVEL REGULATORY ELEMENTS OF RNA MODIFYING PROTEINS IN CANCER METABOLISM

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N6-methyladenosine (m6A) is a modification found on mRNA that regulate downstream events such as alternative splicing, localization, as well as transcript stability. Levels of m^6A are determined by the opposing activities of methylases and demethylases. Global disruptions of either the methylases or demethylases of m6A can result in a dysregulated cellular state such as cancer. Levels of methylation can be perturbed in response to metabolic rewiring, one of the hallmarks of cancer. As an example, high levels of the metabolites fumarate, succinate, and 2-hydroxylglutarate have been shown to competitively inhibit alpha-ketoglutarate (aKG) dioxygenase enzymes, a family that includes the RNA demethylases FTO and ALKBH5. RNA binding proteins (readers) are often responsible for determining the function of an m^6A mark, however, correlating these readers and the downstream events can be influenced by the sequence, position, and even the structure of the surrounding regions of the RNA. Complemented with how reader proteins can bind to multiple targets, there is a lack of understanding in the molecular and cellular dependencies when trying to associate m^6A readers to a phenotype. A screening approach to target each of these readers can be useful in determining a global role these reader proteins have on particular functions. Cancer's rewiring of gene expression induced by changes in metabolism can be dictated by different readers involved in m6A processing. In order to uncover cellular details about the relationship between RNA modification readers and cancer phenotype, we are exploring hereditary leiomyomatosis and renal cell carcinoma (HLRCC), which is defined by a mutation in the fumarate hydratase (FH) gene resulting in high levels of fumarate, as a model in CRISPR screens. Previously, our lab has established that accumulation of fumarate in HLRCC results in mRNA hypermethylation. In our present work, we aim to screen for different dependencies between cells with and without fumarate accumulation using an isogenic model of an FH mutant and FH wildtype. We performed a CRISPR KO screen, targeting around 300 RNA modification related proteins. In order to assay tumorigenic potential of HRLCC, we optimized the CRISPR screen with a Matrigel invasion assay to discover RBP dependencies based on cancer metabolism.

INVESTIGATING THE MOLECULAR AND CELLULAR ROLES OF METABOLISM ON RNA EPITRANSCRIPTOMICS IN RENAL CELL CARCINOMA

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Metabolites play key roles in cellular homeostasis by acting as secondary messengers and enzyme effectors. In cancer, metabolic reprogramming promotes tumorigenesis by facilitating proliferation, invasion, and other transformative processes. Mutations in genes encoding the tricarboxylic acid (TCA) cycle can lead to an accumulation of the metabolites 2-hydroxyglutarate, succinate, or fumarate. One manner by which these "oncometabolites" are proposed to promote tumorigenesis is by inhibiting alpha-ketoglutarate (aKG)-dependent dioxygenases. In particular, accumulation of oncometabolites has been shown to inhibit the aKG-dependent demethylases FTO and ALKBH5, which are critical regulators of RNA methylation. N6-methyladenosine (m6A) is the most abundant internal modification in mRNA and is thought to influence all aspects of mRNA metabolism, including splicing, stability, and translation. An emerging theme in RNA biology is that dysregulated metabolism can disrupt the balance of RNA post-transcriptional modifications thereby altering RNA processing within the cell, leading to altered gene expression programs and driving cancer. Hereditary leiomyomatosis and renal cell carcinoma (HLRCC) is a rare, highly metastatic cancer characterized by germline mutation of the TCA enzyme fumarate hydratase (FH) and provides an ideal model to examine the effects of fumarate accumulation on m6A methylation, and its downstream role in gene expression and tumorigenesis. In cells with loss of FH activity, we observe an increase in both the global and transcript-specific levels of m^{6}A. Analysis of mRNA half-life reveals shorter mRNA half-life in cells with fumarate accumulation, supporting the hypothesis that fumarate accumulation disrupts RNA processing. To assess the role of RNA methylation in cellular identity and tumorigenesis, we used CRISPR-Cas9 to disrupt the expression of m6A writers or erasers in several HLRCC cell lines. Our current work focuses on the use of these knockout cell lines to assess the role of m6A-dependent pathways in tumorigenic phenotypes, including cellular proliferation, migration, and invasion, while future work will focus on testing targets of interest in a mouse xenograph model. Together, eukaryotic cells use multiple mechanisms to regulate genetic programs and the completion of this work will increase our understanding of the mechanisms by which metabolism and RNA modifications interact to influence gene expression programs in cancer.

LOSS OF ADAR1 INCREASES T CELL MIGRATION AND PROLONGS SURVIVAL IN OVARIAN CANCER

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Novel therapies are urgently needed for ovarian cancer (OC), the fifth deadliest cancer in women. OC is generally characterized by an immunosuppressive tumor microenvironment (TME) and less than 10% of patients respond to immune checkpoint blockade (ICB) therapy, though better OC prognosis is associated with high tumor-infiltrating CD8 T cells. DNA methyltransferase inhibitors (DNMTis) remove methylation and activate transcription of double-stranded (ds)RNA in OC. These dsRNAs are sensed in the cytoplasm and trigger the induction of type I IFN and also the transcription of interferon-stimulated genes (ISGs). Adenosine deaminase 1 (ADAR1) is an ISG that edits mammalian dsRNA with an A-to-I nucleotide change. These edited dsRNAs cannot be sensed by dsRNA sensors, and thus ADAR1 can inhibit this type I IFN response. Our preliminary RNA-seq analysis from human OC cell lines indicates that RNA editing by ADAR1 is increased after DNMTi treatment.

To test the hypothesis that Adar1 loss will amplify the DNMTi-induced IFN response in OC, we implanted mice with syngeneic ID8 murine OC Adar1 knockdown (KD) cells or control cells and treated with DNMTi. We show that DNMTi treatment reduces tumor burden and extends survival in this OC model. Loss of Adar1 significantly prolongs survival in this model, which is further improved with DNMTi treatment. DNMTi treatment of murine OC cells combined with Adar1 KD increases T cell migration in transwell migration assays and also enhances expression of chemokines CCL5, CCL2, and CXCL10. Furthermore, immunophenotyping analyses of the TME show increased significantly frequencies of NK cells, significantly increased frequencies of M1 macrophages as well as significantly decreased frequencies of M2 macrophages in the Adar1 DNMTi-treated group compared to mice implanted with control cells and receiving mock treatment. These studies thus describe a role of Adar1 in the DNMTi-induced immune response in OC.

IN-SILICO AND PHENOTYPIC CHARACTERIZATION OF GENTIC VARIATION IN THE M6A METHYLTRANSFERASE COMPLEX

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The most prevalent of all mRNA modifications, N6-methyladenosine (m^{6}A) has been associated with a growing number of pathologies including cancer. While dysregulated expression of proteins responsible for the incorporation of this chemical mark on RNA targets has been observed in a number of cancers, less attention has been given to the small nucleotide polymorphisms in these genes, which may also contribute to carcinogenic phenotypes. Despite this, a number of such genetic variants can be found across genes of the m^{6}A pathway in the general and cancer populations. In this study, genetic variation in methyltransferase-like 3 (METTL3) and 14 (METTL14), which make up the core of the m^{6}A methyltransferase complex, was evaluated across the population-wide germline, cancer germline, and cancer somatic tissue sequencing databases. A domain of interest within the METTL14 coding sequence was chosen for a high-throughput investigation into how the identified variants within this domain contribute to proliferation, migration, and invasion in cancer cells. Results from the in-silico characterization of variation in these genes will help elucidate the prevalence of potentially pathogenic variants in the m^{6}A pathway in both cancer and non-cancer populations. The functional variant screen will identify those variants conferring a carcinogenic advantage and provide models for further in vitro and in vivo studies of the m^{6}A pathway.

DECOUPLING CO-EXPRESSION OF ADAR1 P150 AND P110 ISOFORMS

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ADAR1 (adenosine deaminase acting on RNA 1), an A-to-I (adenosine-to-inosine) RNA editing enzyme, is an essential protein that regulates how cells respond to endogenous and exogenous RNA. Mutations in ADAR1 that abolish its editing ability cause devastating autoinflammatory diseases such as Aicardi-Goutières syndrome. On the other hand, inhibition of ADAR1 in certain malignancies remarkably reduces tumor growth. ADAR1 binds and edits RNA through two isoforms: p150 (150 kDa) and p110 (110 kDa), but the specific functions and RNA targets of each isoform are incompletely known because of the challenges in expressing the two isoforms independently, and specifically, expressing p150 by itself. We report that the canonical p150-encoding mRNA can co-express p150 and p110 because of leaky ribosome scanning and translation initiation downstream of the p150-AUG. The presence of a strong Kozak consensus context surrounding the p110-AUG enhances translation initiation, and the lack of other in-frame start codons between the p150-AUG and p110-AUG suggests the mRNA is optimized to express only two discrete protein isoforms. This finding raises the importance of investigating the biological significance behind coupled expression of p150 and p110. To this end, we present a genetic strategy to decouple p150 and p110 expression that introduces synonymous mutations in the coding region between the p150-AUG and p110-AUG, aiming to reduce leaky ribosome scanning and translation initiation at the p110-AUG. Cells expressing p150 constructs with these synonymous mutations produce wild-type p150 with significantly reduced levels of p110, allowing for determination of RNA editing events when p150 is the dominant A-to-I editor. Editing analysis of total RNA from ADAR1 knockout cells reconstituted separately with p110 and modified p150 sequences reveals that the majority of A-to-I edit sites are selectively edited by p150, but many sites can also be edited individually by either p150 or p110. Our method of isoform-selective ADAR1 editing analysis, making use of the modified p150 sequence, has the potential to be adapted for investigating the biological significance of coupled p150 and p110 expression in diverse cell types.

ELUCIDATING THE N6-METHYLADENOSINE LANDSCAPE OF VIRAL LNCRNA IN THE CONTEXT OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS REPLICATION

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The complexity of Kaposi's sarcoma-associated herpesvirus (KSHV) transcriptome expands far beyond the coding RNAs and includes numerous non-coding transcripts with polyadenylated nuclear (PAN) RNA leading in abundance and significance for viral replication.

PAN RNA is a long non-coding transcript involved in regulating KSHV gene expression, modulation of host immune response, and nuclear export of late viral mRNAs. Ablation of PAN RNA results in loss of late lytic gene expression and, consequently, reduces progeny virion release. We have previously shown that PAN RNA has a dynamic secondary structure and protein binding profiles, which change depending upon the biological context[1]. This work constituted the most extensive structural characterization of viral lncRNA and its interactome inside the living cells and virions, providing a broad framework for understanding PAN's roles in KSHV infection.

The 6-methyladenosine (m^6A) is one of the most abundant signatures found in viral RNA genomes and virus-encoded RNAs. Here, we have addressed dynamics of the m^6A landscape of PAN RNA expressed during latent and lytic stages of KSHV replication by applying the 2nd and 3rd (Nanopore) generation RNA sequencing analyses. We have shown that PAN RNA is the most extensively modified at the late lytic stages of KSHV replication, in contrast to most of its epitranscriptome. Using a newly developed method, termed Selenium-modified deoxythymidine triphosphates (SedTTP)-RT and Ligation Assisted PCR analysis of m^6A (SLAP), we have dissected the fraction of modification at each site, showing that residues near expression and nuclear retention element (ENE) involved in PAN triple helix formation undergo extensive methylation during the late lytic stages of KSHV infection. Proteomic approaches, including RNA antisense purification with mass spectrometry and immunoblotting, have allowed identifying the specific readers, writers, and erasers that facilitate the modification of PAN RNA and its phenotypic effect. By applying the selective 2'-hydroxyl acylation analyzed by primer extension and mutational profiling (SHAPE-MaP) in KSHV-positive cells with ablated expression of these enzymes, we have shown that m^6A influences not only the local but also global folding of PAN RNA. To our knowledge, this study provides the first comprehensive insight into the m6A status of specific viral lncRNA, reveals the sophisticated interplay that exists between a viral RNA and cellular epitranscriptomic machinery, and creates a paradigm for future studies in the field of host-pathogen interactions.

[1]Kaposi's sarcoma-associated herpesvirus polyadenylated nuclear RNA: a structural scaffold for nuclear, cytoplasmic and viral proteins. Nucleic Acids Res. 2017 Jun 20;45(11):6805-6821.

NON CODING RNA SYNTHESIS AT DNA LESIONS

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Our group has previously reported that DNA double-strand breaks (DSBs) trigger the synthesis by RNA polymerase II of damage-induced long non-coding RNA (dilncRNA) that can be processed into shorter DNA damage response RNAs (DDRNAs). Such transcripts are essential for full DDR activation and their inhibition by antisense oligonucleotides (ASO) allows site-specific inhibition of DNA damage signalling and repair (Francia et al Nature 2012, Michelini et al Nature Cell Biology 2017, D'Alessandro et al Nature Communications 2018).

We recently discovered that such transcriptional events depend on the assembly

of seemingly fully functional transcriptional promoters that include a complete RNA polymerase II preinitiation complex (PIC), MED1 and CDK9. Absence or inactivation of any of these factors causes a reduction in the activation of the DNA damage response (DDR) both in cells and in an in vitro system that reconstitutes DDR activation events on nucleosomes.

Importantly, dilncRNAs drive molecular crowding of DDR proteins, such as 53BP1, into globular structures that exhibit liquid–liquid phase-separation condensate properties (Pessina et al Nature Cell Biology 2019).

Telomeres, the ends of linear chromosomes, progressively accumulate DNA damage during physiological and pathological aging. We recapitulated the above-described events at damaged telomeres (Rossiello et al. Nature Communications 2017) and demonstrated that, in independent animal models of accelerated aging, specific DDR inhibition at telomeres by ASO improves aging's detrimental phenotypes and extends lifespan (Aguado et al. Nature Communications 2019).

ABERRANT GERMLINE GENES IN-ACTION?

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PIWI-interacting RNAs (piRNAs) ensure animal fertility by silencing transposable elements (TEs). During germline development, piRNA precursor transcripts are processed into small RNA fragments (24-31-nt) and bound by PIWI proteins to form a piRNA-induced silencing complex. Expression of core piRNA pathway genes is restricted to germ cells in mammals. Intriguingly, some of these genes are aberrantly expressed in various cancers - a phenomenon known as 'cancer/testis gene expression.' Owing to their restricted physiological and broad pathological expression, these aberrant genes are promising candidates for diagnosis and therapy. In this study, we set out to describe the expression of aberrantly expressed piRNA pathway genes in cancers and characterize their functional impacts on cancer cell biology.

A comprehensive analysis of gene expression data from The Cancer Genome Atlas (TCGA) revealed a mosaic expression of select piRNA pathway genes in almost all cancer types. Lack of co-expression of essential pathway components suggests that ectopic reactivation of piRNA silencing is unlikely. To directly probe the function of aberrantly expressed piRNA pathway genes, we identified cancer cell lines that recapitulated the aberrant expression profiles. First, we examined the expression of PIWIL1 in a colon cancer cell line. We used CRISPR to tag PIWIL1 endogenously and confirmed that the protein was expressed. Immunoprecipitation experiments revealed PIWIL1 did not associate with piRNAs in the aberrant context. Knockdown and knockout experiments of PIWIL1 confirmed that PIWIL1 does not impact gene expression or transposon transcripts. Next, we examined the aberrant expression of the piRNA biogenesis factor MAELSTROM (MAEL) in the lung cancer culture model. Our biochemical and genome-wide studies show that MAEL binds and regulates target RNAs. We are currently focused on elucidating the function of MAEL on cancer cell biology in more detail.

Our candidate approach results indicate a broad spectrum of involvement of aberrant piRNA genes in cancer. In the future, we aim to expand the repertoire of tested aberrant piRNA pathway genes to increase the repertoire of potential cancer diagnostic or therapeutic targets.

CLASSIFYING SMALL RNA SORTING IN ARGONAUTE PROTEINS USING CONVOLUTIONAL NEURAL NETWORKS

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Cells co-opt small RNAs (sRNAs) for a number of functions, including mRNA destruction through deacetylation or cleavage, transcriptional regulation, and heterochromatin formation. A subset of these sRNAs, microRNAs (miRNAs) and endogenous-siRNAs (endo-siRNAs), are the main players of the RNAi pathway, alongside the Argonaute subfamily of proteins. In *Drosophila melanogaster*, two distinct pathways exist in which miRNAs bind almost exclusively to Ago1 and endo-siRNAs bind to Ago2. In mammals, there are four Argonaute proteins (Ago1-4) that interact with miRNAs and one (Ago2) that is thought to interact with endo-siRNAs, though it remains unclear whether endo-siRNAs interact exclusively with Ago2. Unlike the sRNAs in *Drosophila*, early studies suggest that there is no loading bias in vertebrates when observing sorting of miRNAs and exogenous-siRNAs (exo-siRNA) onto the Argonaute proteins. However, more recent studies reveal that there are exceptions to this rule, as miR-451 binds exclusively to Ago2 and isomiRs, nucleotide-level variants of miRNA loci, are suggested to preferentially load onto distinct Ago proteins in humans. This implies that a nucleotide-specific sorting mechanism might be at play here. Surprisingly, sorting of endo-siRNA has thus far not been investigated in vertebrates. Machine learning tools have been utilized to predict and classify several different RNA-related features, including miRNA-mRNA target sites, lncRNA-miRNA target sites, and pre-miRNA classification. In particular, deep learning has been a versatile tool used to classify RNAs by the use of nucleotide-level features. Indeed, convolutional neural network (CNN) and recurrent neural network (RNN) tools have been constructed for their automatic feature extraction to answer such biological questions. Recently published papers have utilized such tools to classify canonical and non-canonical pre-miRNAs; however, no deep learning model to date has been constructed to predict sorting of sRNA onto the Ago proteins. Here, I configured a nucleotide-level CNN tool that predicts sRNA association towards Argonaute protein isoforms.

A DUAL-ACTIVITY TOPOISOMERASE COMPLEX INTERACTS WITH PIRNA MACHINERY TO PROMOTE TRANSPOSON SILENCING AND GERM CELL FUNCTION

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Topoisomerase 3 beta (Top3b) is the only dual-activity topoisomerase in eukaryotes that can change topology for both DNA and RNA. Emerging evidence suggests that Top3b is required for multiple RNA and DNA metabolisms including transcription, replication, and resolving DNA/RNA hybrid (R-loop) in cells. Furthermore, Top3b forms a complex with RNA binding proteins Tudor domain containing 3 (TDRD3) and Fragile X Mental Retardation Protein (FMRP) to regulate mRNA translation. *Top3b* mutation in mice exhibits reduced lifespan and fertility, chromosomal abnormality, and abnormal neurodevelopment while *Top3b* mutation in human has been linked to schizophrenia, autism, epilepsy, and cognitive impairment. Such evidence indicates that Top3b has an important role in aging and mental health. However, the mechanism of how Top3b maintains normal life-span and mental health remains largely unclear.

We have recently shown that the Top3b-TDRD3 complex interacts with the siRNA machinery to facilitate heterochromatin formation in fly heads. Heterochromatin maintains and represses transcription of genes and transposable elements (TEs) within its regions, and loss of heterochromatin results in de-repression of TE. TEs are mobile genetic elements that can cause genomic instability by uncontrolled expression and transposition. Additionally, mobilization of TEs has been shown as a driver for aging, age-associated inflammation, and neurodegeneration. Therefore, one mechanism by which Top3b and TDRD3 functions in aging and neuronal function could be through the regulation of TEs via the siRNA machinery.

In addition to siRNAs, PIWI-interacting RNAs (piRNAs) is the other major class of small RNAs that mediate suppression of TEs in gonads. Here, we present evidence that the Top3b-TDRD3 complex interacts with piRNA machinery to promote ovary development and silencing of TEs. First, Top3b and TDRD3 form stable complexes with the piRNA machinery, including PIWI, in gonads of both mouse and *Drosophila*. Second, mutation of either *Top3b* or *Tdrd3* results in reduced fertility due to defective oogenesis and embryogenesis, as well as de-silencing of multiple transposons in ovary. Third, *Top3b* and piRNA nuage components *maelstrom (mael), vasa, aubergine (aub)* and *ago3* genetically interact to suppress expression of TEs and promote ovarian functions. Notably, the double mutants exhibit defective piRNA biogenesis in dual strand piRNA clusters. Together, our data reveal a novel role of the Top3b-TDRD3 complex: regulation of TE silencing and germ cell function via interaction with piRNA machinery.

A NOVEL BACTERIAL SILENCER OF SMALL RNA FUNCTION, ACETYLTRANSFERASE YHBS, TARGETS THE RNA CHAPERONE HFQ IN *E. COLI*

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Bacterial small regulatory RNAs (sRNAs) play important roles in regulating gene expression in response to various stress conditions. In the past few decades, their molecular mechanisms of action have been extensively studied. Mechanistically, sRNAs regulate the translation and/or stability of their target mRNAs through direct base-pairing. In *Escherichia coli* and many other species, this process is associated with the RNA chaperone Hfq, which binds sRNAs and their target mRNAs on different faces. A search for novel post-transcriptional regulators of sRNA signaling pathways has been carried out using a fluorescence-based genetic screen in this laboratory. One of the identified genes encoded YhbS, an uncharacterized GNAT family acetyltransferase.

Overproduction of YhbS was initially identified by its ability to suppress regulation of one target, *sodB*, by the sRNA RyhB; this activity depends on the predicted active site of YhbS. However, YhbS was found to generally suppress all tested Hfq-dependent regulatory events, including one in which Hfq directly represses *mutS* mRNA translation without the need for an sRNA. In addition, the RNA chaperone Hfq accumulates upon YhbS overexpression, suggesting the failure of Hfq autoregulation. Both *mutS* repression and Hfq autoregulation have been shown to depend upon the distal face of Hfq, where many mRNAs bind. This observation led to the hypothesis that YhbS catalyzes an acetylation reaction on the distal face of Hfq, which in turns disrupts the ability of sRNA/mRNA binding to Hfq. Consistent with this model, mutations in a conserved lysine on the distal face, K31, blocked the ability of YhbS to disrupt sRNA regulation. Taken together, our results strongly suggest that YhbS is a universal sRNA silencer, likely functioning by acetylating the RNA chaperone Hfq. While the work here was done with overexpressed YhbS, YhbS is rapidly induced upon anaerobic shock, implicating this regulatory effect in modulating how Hfq functions under anaerobic conditions. Other than YhbS, there are another 25 acetyltransferase encoding genes in *E. coli*, targeting proteins, RNAs or small molecule under various conditions, raising the possibility that other inducing conditions may also modify Hfq-dependent signaling via acetylation. This work expands our knowledge on the regulation of sRNA signaling at the post-transcriptional level.

MIRGE3.0: A COMPREHENSIVE MICRORNA AND TRF SEQUENCING ANALYSIS PIPELINE

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MicroRNAs and tRNA fragments (tRFs) belong to a group of small non-coding RNAs that act as regulators of gene expression and modulators of disease. Understanding the miRNA and tRF levels in individual cell types and tissues will assist in establishing basal expression and can help identify drug targets for disease states. The growing big data repositories such as NCBI-SRA, host numerous datasets enabling cellular level expression of miRNAs and tRFs. However, a small RNA processing workflow is required to accurately annotate small RNA types and efficiently handle high-throughput next-generation sequencing (NGS) data. We developed a robust small RNA annotation and alignment pipeline, miRge3.0, to be a more efficient tool than our previously developed miRge and miRge2.0 sequence aligners. miRge3.0 is developed in python3 and several python packages such as pandas, concurrent.futures, numpy are incorporated to be memory efficient and support parallel processing. Further, this tool incorporates JSON, JavaScript, HTML, CSS, NodeJS and Electron to deliver an interactive graphical user interface (GUI). Besides annotation and prediction of novel miRNAs, new features of miRge3.0 includes advanced data quality control, processing of Unique Molecular Identifiers (UMIs) to account for PCR duplicates, reporting of isomiRs in GFF3 format, and differential expression analysis. miRge3.0 was benchmarked to miRge2.0, Chimira and sRNAbench demonstrating increased speed. We conclude miRge3.0, our 3rd generation tool, is user friendly and supports the latest technological advancements with improvements in speed, versatility, and functionality over previous iterations.

INVESTIGATING THE DYNAMICS OF TRANSLATION READTHROUGH IN LIVE CELLS

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A certain fraction of ribosomes fails to terminate translation at stop codons, leading the ribosome to "read through" the stop codon and translate the 3' untranslated region (UTR) of mRNAs. This can lead to undesirable C-terminal extension of proteins that can cause mis-localization, instability, or aggregation. In addition, a substantial portion of genetic disease is caused by premature stop codons in protein coding regions, leading to truncated protein and the decay of the message by a quality control pathway known as nonsense-mediated decay. One proposed approach to treat this class of disease is by inducing stop codon readthrough by treating cells with aminoglycoside antibiotics. However, the effectiveness of aminoglycosides is greatly affected by the type of nonsense mutation and the surrounding context. Thus, a better understanding of the contexts and mechanisms that lead to stop codon readthrough are needed. We developed single-molecule reporters that report on the appearance of nascent chains encoded by both the main open reading frame (ORF) and the 3'UTR on single mRNAs to monitor translation readthrough in live mammalian cells. We demonstrate that different stop codon sequence contexts lead to varied levels of readthrough, as expected from bulk studies. Our single-molecule methodology allows us to further examine the time-dependent dynamics of this process. We also show treatment with aminoglycoside antibiotics increases stop codon readthrough. Finally, we knocked down ABCE1, involved in removing the large ribosomal subunit during translation termination, and eRF3, involved in peptide release, and find that both proteins are critical to stop codon recognition and prevention of readthrough in a context dependent manner. We are currently developing methods to investigate the precise dynamics of stop codon readthrough to determine how these proteins respectively contribute to the mechanism of stop codon readthrough. Additionally, we plan on investigating more stop codon contexts, including those that are known to regulate programmed readthrough events on genes such as VDR and AGO1, to determine how stop codon context can be used to precisely regulate protein function.

PARTIAL SPONTANEOUS INTERSUBUNIT ROTATIONS IN ACTIVELY TRANSLATING RIBOSOMES

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 Protein translation is a critical step in gene expression. The prokaryotic 70S ribosome is composed of two subunits: the 30S small subunit and the 50S large subunit. During translation, the 30S subunit rotates relatively to the 50S subunit by ~8 degrees. Intersubunit rotations are essential for catalytic activity, movement over mRNA, and are part of translational recoding events. However, when and why the ribosome alternates between two rotational conformations remains unclear, with two well supported, but conflicting, hypotheses. Ribosomes may undergo a single cycle of forward and backward rotations per codon read, where forward rotation is triggered by peptidyl transfer, while translocation is accompanied by reverse rotation. Alternatively, ribosomes may undergo multiple full spontaneous rotations per codon. This discrepancy precluded understanding of mechanism of translation.

We used high-speed single-molecule TIRF microscopy to follow translation in real-time. Ribosome undergoes not full, but partial, spontaneous rotations between at least four different states. These novel rotational states are critical intermediates of initiation and translocation reactions. Our results bridge the two hypotheses together and provide a new insight into ribosome dynamics related to protein translation.

DIRECT CELL TO SINGLE MOLECULE: A VERSATILE PLATFORM TO ANALYZE PROTEINS IN SINGLE-MOLECULE TIRFM

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The inner workings of cells are driven by dynamic interactions between biological molecules. Studying these interactions at the single-molecule level provides the advantages of revealing reaction pathways that are usually hidden by averaging in ensemble experiments. Application of single molecule methods requires site specific attachment of fluorophores and immobilization handles to the studied proteins. The resulting need for extensive purifications and modifications greatly hamper the application of single-molecule fluorescence methods.

To overcome these limitations, we have developed a strategy that allows direct immobilization of proteins of interest from total cell lysate thus allowing immediate single-molecule imaging without any purification. This **D**irect **C**ell to **S**ingle **M**olecule (DCSM) approach is based on the isopeptide bond formation between Lys31 and Asp117 of the split fibronectin binding protein i.e., the SpyTag-SpyCatcher chemistry.

 As proof-of-concept we used DCSM strategy to immobilize purified Spy-fusion proteins, and then demonstrated the immobilization from cell lysates. We further showed that this strategy is compatible with different fluorescent tags and dyes (SNAP, ybbR and Halo), and could be used for pull-down assays as well as for single-molecule experiments. We are currently applying the DCSM strategy to explore the conformational dynamics of eIF2 by itself and during translation initiation since protein biosynthesis is a highly dynamic process that requires precise temporal and spatial coordination of biomolecules, namely ribosomes, mRNAs, tRNAs and translation factors. However, due to the ease of use and the stable and specific binding we believe that DCSM is widely applicable to study RNA-protein and protein-protein interactions at the single molecule level.

CPEB2B INTERACTS WITH HNRNPR TO REGULATE TRANSLATION OF TWIST1 IN TNBC

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Triple-negative breast cancer (TNBC) is an aggressive and deadly form of breast cancer linked to higher metastatic rates and low 5-year survival rates. Recently, our lab reported a role for which an alternatively spliced isoform of cytoplasmic polyadenylation element-binding protein 2 (CPEB2), CPEB2B (exon 4 inclusion), promotes translation of TWIST1 and contributes to TNBC metastasis. This contrasted with the CPEB2A isoform (exon 4 exclusion), which decreased TWIST1 translation. In this study, the mechanism for the opposing roles of CPEB2 translational regulation of TWIST1 was examined. CPEB2A (exon 4 excluded) has been reported to inhibit translation of TWIST1 via 3'UTR CPE binding and poly-A tail extension. Here we report CPEB2B (exon 4 included) decreased binding to the 3'UTR CPE region of TWIST1 resulting in decreased poly-A tail length. We also report that heterogeneous nuclear ribonuclear protein R (hnRNPR), a multifunctional RNA binding protein, strongly associates with CPEB2B via exon 4 but not with CPEB2A (exon 4 excluded). This novel mechanism of CPEB meditated translational regulation, whereby an increase in the alternative spliced isoform CPEB2B increases translation of TWIST1 via reduced mRNA 3'UTR poly-A tail length and association with hnRNPR, promotes metastatic transformation in TNBC.

MEDULLOBLASTOMA-ASSOCIATED DDX3X MUTATIONS ALTER TRANSLATION OF A SUBSET OF HUMAN mRNAS

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DDX3X is a member of the DEAD-box RNA helicases family that is involved in the translation of mRNAs with highly structured 5′ UTRs. Despite being implicated in several neurodevelopmental disorders and human cancers, its precise mechanism of action in normal cells and its malfunction in diseased cells is poorly understood. Using an *in vitro* translation assay, I determined that medulloblastoma-associated DDX3X mutations lead to decreased translation initiation on the 5'UTR of several genes. I will perform SHAPE on these 5'UTRs to dissect the structural requirements of DDX3X sensitivity. Additionally, it is unknown whether interaction with the ribosome is necessary for DDX3X activity. I will tile mutations across DDX3X to find mutants that retain helicase activity but lose RNA-independent co-precipitation with ribosomes and use these mutants to determine if DDX3X-sensitive translation is dependent on ribosome binding. Since alteration of DDX3 is associated with diverse cancers, developmental disorders, and virus infections, this understanding will have far-reaching impacts on diverse diseases.

CRYPTIC TRANSLATION EVENTS TARGET YEAST ncRNA TRANSCRIPTS FOR NONSENSE-MEDIATED DECAY

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The nonsense-mediated mRNA decay (NMD) pathway targets mRNAs undergoing premature translation termination for rapid degradation, including nonsense alleles associated with severe forms of human disease. One therapeutic approach for such cases involves stabilization of the mRNA via suppression of NMD and treatment with drugs that induce stop codon readthrough. In order to utilize NMD as a drug target, it is important to understand what the natural role of NMD is in the cell and whether it is safe to disrupt it. The *up frameshift* (*UPF*)*1-3* genes are required for NMD and their loss causes stabilization of targeted mRNAs. RNA-seq of yeast *upf* deletion strains surprisingly revealed that many genes targeted by NMD (88%) appear to lack premature termination codons[1]. One possible explanation for this targeting is that translation of cryptic ORFs results in widespread premature termination events. To test this hypothesis, we developed a combined approach using RNA-seq to identify the transcript isoforms that are targeted by NMD and ribosome profiling to find cryptic translation events. In particular, we used 80S footprint density to assess translation efficiency and 40S footprint peaks on start and stop codons to demarcate the boundaries of cryptic ORFs with high sensitivity.

Using this approach, we identified novel translation events that likely trigger NMD on approximately three quarters of the targets that initially appeared to lack premature stop codons. Interestingly, the majority of these cryptic events occur on inhibitory ncRNA isoforms, for e.g., long un-decoded transcript isoforms (LUTIs)[2]. Transcription of these regulatory RNAs begins at an upstream promoter that represses use of the canonical gene promoter through the deposition of repressive chromatin marks. We found LUTIs generally encode multiple 5' upstream ORFs (uORFs) that appear to trigger NMD. We also identified a related class of transcripts that initiate transcription from a promoter within the coding sequence of the gene which lack the canonical start codon to initiate translation. Instead, ribosomes translate short, out-of-frame ORFs internal to the annotated coding sequence (iORFs) and appear to trigger NMD at associated premature stop codons. Some of these internal promoters are bi-directional, resulting in the production of antisense transcripts that may inhibit the canonical gene promoter. Here we show that inhibitory ncRNAs are a novel class of NMD substrates. Furthermore, NMD is critical for removing these repressive regulatory mRNAs from the cell, perhaps to prevent unproductive translation and the synthesis of potentially toxic peptides.

[1] Celik *et al*. (2017) RNA 23:735-748

[2] Chen *et al*. (2017) eLIFE 6:e27417

HAC-SEQ: A M[3]C-SPECIFIC SEQUENCING TECHNIQUE FOR NUCLEOTIDE-RESOLUTION PROFILING OF M[3]C METHYLOME ON RNA

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Objective

3-methylcytidine (m^3C) modification is a poorly studied RNA modification. Methods for the specific mapping of m^3C throughout the transcriptome are lacking. The objective of this study is to develop a m^3C-specific sequencing technique to profile the m^3C methylome at single-nucleotide resolution.

Methods and Results

We make use of an old m^3C-specific chemical reaction, hydrazine treatment followed by aniline-induced cleavage of RNA chain, to detect m^3C on RNA. HPLC-MS/MS analysis showed that hydrazine/aniline treatment was able to decrease m^3C levels on RNA. Additionally, northern blot analysis supported that hydrazine/aniline treatment was able to generate one or two cleaved 3' fragments of the correct sizes for several known m^3C-modified tRNAs. We further found that demethylation treatment by AlkB was able to diminish the hydrazine/aniline induced cleavage on tRNA. All demonstrate that hydrazine/aniline treatment can specifically cleave tRNAs at m^3C modification sites.

In order to explore the global m^3C RNA methylome we then coupled this hydrazine/aniline-induced chemical cleavage method with next generation sequencing to establish a Hydrazine-Aniline Cleavage sequencing (HAC-seq) technique for the identification of m^3C modification sites on RNAs at single-nucleotide resolution. rRNA-depleted total RNAs were randomly fragmented and end-repaired. The fragmented RNAs were treated with 10% hydrazine with 3M NaCl followed by aniline to induce the cleavage of the RNA backbone at the m^3C modification sites. The 5' fragment generated by HAC contains a damaged 3' end without the correct 3'-OH group which prevents the adaptor ligation in the library preparation step. Only the full length and 3' cleaved fragments can be subsequently sequenced. After the bioinformatic data analysis, m^3C-modified sites are determined by calculating the Cleavage Ratio at single nucleotide resolution. HAC-seq revealed that tRNAs are the predominant m^3C-modified RNA species, with 17 different m^3C sites on 11 cytoplasmic and 2 mitochondrial tRNA isoacceptors. We found no evidence for m^3C-modification of mRNA or other non-coding RNAs at comparable levels to tRNAs. In addition, the cleavage ratio calculated from HAC-seq can be used to estimate m^3C levels on RNA. Overall, cytoplasmic and mitochondrial tRNA-Thr species are the highest-m^3C modified tRNAs.

Conclusions

HAC-seq provides a novel method for the unbiased, transcriptome-wide identification of m^3C RNA modification at single-nucleotide resolution. The cleavage ratio calculated from HAC-seq can be used to estimate m^3C levels on RNA.

TOWARDS ELUCIDATION OF THE MECHANISM GUIDING PSEUDOURIDYLATION OF KAPOSI SARCOMA-ASSOCIATED HERPESVIRUS LONG NON-CODING PAN RNA

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Long non-coding RNAs (lncRNAs) are an important class of regulatory RNAs, that fulfill their function via structure-mediated interactions with effectors. Recently, post-transcriptional modifications to RNA have been shown to govern RNAs structure, function, and metabolism. Kaposi's sarcoma-associated herpesvirus (KSHV) encodes polyadenylated nuclear (PAN) lncRNA that governs lytic reactivation of viral genes and modulates cellular immune response. We found that during the latent-to-lytic reactivation of KSHV replication, specific uridines on PAN RNA undergo isomerization to pseudouridine (Ψ). Using CMC-RT (N-Cyclohexyl-N′-(2-morpholinoethyl) carbodiimide (CMC) reverse transcription) and Ligation Assisted PCR analysis (CLAP), we identified the position and frequency of Ψs and showed that the PAN pseudouridylation status is dynamic during KSHV replication, with some sites being modified with higher frequency than others. We performed the RNA antisense purification and mass spectrometry to affinity capture PAN-protein complexes. We identified three candidate proteins that can guide PAN pseudouridylation, with pseudouridine synthase 7 (PUS7) further confirmed by immuno-Western blotting to associate with PAN expressed during lytic stages of KSHV replication. The confocal microscopy analysis in KSHV-infected BCBL-1 cells verified the temporal and spatial co-localization of PAN RNA and PUS7 in the nucleus, during the lytic stage of viral replication. Ongoing research focuses on dissecting 1) the mechanistic aspects of PAN pseudouridylation, i.e., kinetics of the process, 2) the effects of Ψ modifications on PAN RNA biology, i.e., stability, structure, interactions with effectors, 3) the influence of PAN Ψs on KSHV replication, viral gene expression, progeny virion production, and infectivity. Our studies contribute to the more in-depth understanding of viral lncRNA plasticity and will contribute to an underrepresented body of knowledge regarding the host-pathogen interaction.

NUCLEOTIDE RECOGNITION OF A SOLUBLE APOBEC3G VARIANT STUDIED BY USING MICROSCALE THERMOPHORESIS (MST)

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Human APOBEC3 proteins belong a deoxycytidine deaminase family that specifically deaminates cytidine nucleotides to uridine in single stranded DNA (ssDNA) during the viral replication. Of seven APOBEC3 proteins in human, APOBEC3G(A3G) can restrict HIV-1 replication by deaminating cytidines in negative strand ssDNA of viral genome. A3G consists of two domains, a catalytically active C-terminal domain (CTD) that involves in deamination and catalytically inactive N-terminal domain (NTD) through which A3G strongly binds to RNA for its recruitment into HIV-1 virion. Although all APOBEC3 proteins deaminate deoxy-cytidine in ssDNA, they are different in recognition of hotspot sequence for their deamination activities. Unlike A3A and A3F that prefers 5′-TC sequence, A3G deaminates 3′ cytidine of a 5′-CC motif with higher efficiency to 5′-CCC. Here, we presented the binding kinetics of the C-terminal catalytic domain of A3G with substrate and non-substrate nucleotides using a technique called MicroScale thermophoresis (MST). We speculated that sugar conformation of the deamination target cytidine is important for substrate selection and non-substrate exclusion. To differentiate these and the role of 2′-endo sugar pucker conformation for binding and deamination, we have replaced the hydroxyl group by fluorine of the target cytidine; the first containing a fluorine substituted for the C2′ hydroxyl of the ribose (2′-deoxy-2′-fluororibonucleic acid, 2′-F-RNA) and the second containing an arabinose sugar with the C2′ hydroxyl substituted for fluorine (2′-deoxy-2′-fluoroarabonucleic acid, 2′-F-ANA). Difference in their binding constants and catalytic efficiencies indicated that the role of sugar pucker in the selection of deoxy-cytidine and exclusion of ribo-cytidine as the deamination target.

ELUCIDATING THE MODIFICATION STATUS OF TRNAS IN RESPONSE TO ENVIRONMENTAL AND METABOLIC STRESS

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RNA modifications extend the functionality of transcripts. While over 170 RNA modifications have been identified so far, determining the function and location of many of these has been limited by available reagents and technologies. 4-Thiouridine (s4U) is a naturally occurring RNA modification found in bacteria and archaea tRNAs, and functions as a near-UV sensor and a regulator of cell growth, in response to which levels of specific tRNAs have been reported to change. Here, we have adapted a chemical approach to capture and identify s4U-containing RNAs in a high-throughput manner using Next-Generation Sequencing. With this method we can observe enrichment for tRNAs known to be modified, amid potential depletion of tRNAs that lack this modification. This method allowed us to ask several questions regarding whether and how s4U modifications are influenced by environmental conditions. Bacterial cells were cultured in different types of media and RNA was collected from cells at different stages of growth. While the enrichment of specific tRNAs did not vary significantly between different stages of growth in the same media, we identified a few tRNAs, such as alanine, tyrosine and threonine, where s4U levels did respond to growth stages. Enrichment of these s4U-containing tRNAs varied mostly between bacteria grown in different types of media. These results suggest that nutrients provided by the extracellular environment, known to impact metabolic pathways, can influence the levels of s4U-modification in tRNAs within the cell. Altogether, a high-throughput approach to map and quantify s4U-containing tRNAs has elucidated how environmental nutrients can impact the levels of tRNA modifications in bacteria and can be applied to identify s4U modified RNAs in any sample.

MISREADING ERRORS IN THE YEAST SACCHAROMYCES CEREVISIAE

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Post-transcriptional modification of tRNAs modulates the behavior of tRNAs in several ways. Anticodon loop modifications alter details of codon-anticodon interactions either stabilizing or destabilizing interactions with the codon. These modifications can alter the rate of cognate codon recognition and the frequency of misreading of near-cognate codons. Modifications outside the anticodon stem-loop have been shown to alter the stability of the tRNA to degradation (Alexandrov et al., 2006). We are interested in whether these second set of modifications also modulate misreading error. This study aims to use *S. cerevisiae* as a system to understand the role of modification of the body of tRNA. We have exploited a set of misreading reporter systems developed in our laboratory to measure the effect on in vivo frequency of errors of the lack of modifications to the core region of the tRNA. Since the modifications are distant from the anticodon loop their effect on misreading suggests that they stabilize the three-dimensional structure of the tRNA increases its fidelity. I present data showing that in the presence of several modification mutations the frequency of misreading either increases or decreases. I show loss of various posttranscriptional modifications affects first position U•U, second position U•G, and wobble position U•U and U•C mismatches by $tRNA^{Lys}_{UUU}$ and $tRNA^{Glu}_{UUC}$. These results support the idea that the tRNA body modifications are important for regulating decoding during translation. Also, by performing northern blotting of the wild type and mutant strains lacking the modification, I showed that the abundance of tRNAs is not reduced in the absence of body modification. So, the stability of tRNA is not affected by the lack of unessential core modifications at the permissive temperature. Also, I will test the aminoacylation status of the tRNAs lacking body modification and retrograde transport of those tRNAs to the nucleus, to analyze all the possible pathways that the deletion of body modification may affect the availability of tRNA. Additionally, I am analyzing the global role of tRNA modification on inducing error during protein synthesis across the proteome by mass spectroscopy.

CHARACTERIZATION OF piRNA 3'-ENDS

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Piwi-interacting RNAs (piRNAs) are essential in protecting the genome against active transposons. piRNAs are typically seen in germ cells where they interact with PIWI proteins to form piRNA-induced silencing complexes (piRISC). These complexes target transposons for silencing either through epigenetic modifications in the nucleus, or post-transcriptional RNA cleavage. Primary biogenesis of piRNAs is carried out by the successive definition of 5' and 3'-ends by the endonuclease Zucchini. 3' ends can be further trimmed by an exonuclease. The position of the mature 3' end is variable. Occupancy by the PIWI protein and sequence preferences have been suggested to guide the position of the final 3' end, but the exact process is unknown. Here we show that both length and sequence preferences determine the position of the 3' end with different priority. Our results elucidate conserved rules for 3' end formation and show that every piRNA start has a single preferred end.