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RMaP

RNA Modification and Processing

4th Symposium on Nucleic Acid Modification

23rd – 25th September 2024

imb Institut für Molekulare Biologie

JOHANNES GUTENBERG UNIVERSITÄT MAINZ JG|U

Location addresses:

Institute of Molecular Biology (IMB)

Ackermannweg 4, 55128 Mainz

Citadel tour

Windmühlenstraße, 55131 Mainz

Eisgrub-Bräu

Weissliliengasse 1a, 55116 Mainz

Zenz

Bahnhofpl. 4, 55116 Mainz

Organised by:

Prof. Mark Helm (JGU, Mainz),

Dr. Julian König (IMB, Mainz),

Dr. Francesca Tuorto (UMM, Mannheim)

Sponsored by:

Monday, 23rd September 2024

12:00 – 13:00 Registration and lunch

13:00 – 13:15 Welcome (Mark Helm)

13:15 – 15:00 Session I Chair: Kristina Friedland

13:15 – 13:45 **Ramesh S. Pillai** (Department of Molecular Biology, University of Geneva, Switzerland)
RNA modifications in control of mammalian gene expression

13:45 – 14:15 **Rupert Fray** (University of Nottingham, United Kingdom)
Mutants of the plant m6A writer complex

14:15 – 14:30 **Miona Corovic** (Institute of Molecular Biology (IMB) Mainz, Germany)
m6A sites in the coding region trigger translation-dependent mRNA decay

14:30 – 14:45 **Francesca Aguilo** (Umea University, Sweden)
Unveiling the dual roles of METTL3 in breast cancer tumorigenesis

14:45 – 15:00 **Konstantinos Stellos** (Medical Faculty Mannheim of Heidelberg University, Germany
Adenosine RNA modifications in post-transcriptional regulation of gene expression

15:00 – 15:30 Coffee Break

15:30 – 17:15 Session II Chair: Nina Papavasiliou

15:30 – 16:00 **Kristin Koutmou** (University of Michigan, USA)
PUS7 cytoplasmic localization directs a pseudouridine-mediated cellular stress response

16:00 – 16:30 **Schraga Schwartz** (Weizmann Institute of Science, Rehovot, Israel)
mRNA metabolism governs m6A deposition and dynamics

16:30 – 17:00 **Vivian Cheung** (University of Michigan, USA)
Decoding RNA Modifications in an eRNA: The Role of Sequential m6A to Abasic Sites in Regulating APOE Expression.

17:00 – 17:15 **Michael Jantsch** (Medical University of Vienna, Austria)
Editing patterns in double-stranded RNAs camouflaging MDA5-activation

17:15 – 17:45 Coffee Break

17:45 – 18:45 Keynote Chair: Mark Helm

17:45 – 18:45 **Chuan He** (University of Chicago, USA)
Chromatin regulation by RNA methylation

18:45 – 20:00 Postersession Fingerfood and beer

Tuesday, 24th September 2024

09:00 – 10:55 Session III Chair: Christoph Dieterich

09:00 – 09:30 **Yuri Motorin** (Université de Lorraine, Nancy, France)
Challenges in RNA modification mapping by deep sequencing

09:30 – 10:00 **Eva Maria Novoa** (Center of Genomic Regulation, Barcelona, Spain)
Decoding the epitranscriptome at single molecule resolution: towards clinical applications

10:00 – 10:15 **Ann Ehrenhofer-Murray** (Humboldt-Universität to Berlin, Germany)
Detection of queuosine, pseudouridine and other tRNA modifications by direct RNA sequencing

10:15 – 10:30 **Laurence Drouard** (IBMP-CNRS, Strasbourg, France)
Acquisition of a new functional eukaryotic tRNA by hijacking a prokaryotic modification

10:30 – 10:45 **Stefanie Kaiser** (Goethe University Frankfurt, Germany)
Hunting for truth – towards more accurate RNA modification stoichiometry analysis

10:45 – 10:55 **Nicola Conci** (siTOOLs Biotech GmbH, Planegg, Germany)
rRNA Contamination in Ribo-Seq: Origin, Characteristics and Mitigation Strategies

10:55 – 11:30 Coffee break

11:30 – 12:30 Keynote Chair: Mark Helm

11:30 – 12:30 **Marina Rodnina** (Max-Planck-Institute for Physical Biochemistry, Göttingen, Germany)
Non-canonical Decoding by the Ribosome

12:30 – 14:00 Lunch break and Postersession

14:00 – 15:45 Session IV Chair: Matthias Soller

14:00 – 14:30 **Zoya Ignatova** (University of Hamburg, Germany)
N^6-methyladenosine modification in mRNA – the positions it may or may not affect mRNA utilization

14:30 – 15:00 **Tsutomu Suzuki** (University of Tokyo, Japan)
Expanding world of tRNA modifications in health and disease

15:00 – 15:15 **Klemens Wild** (University of Heidelberg, Germany)
Comprehensive analyses of rRNA modifications in a thermophilic eukaryote

15:15 – 15:30 **Jirka Peschek** (University of Heidelberg, Germany)
Structure of fungal tRNA ligase with RNA reveals conserved substrate binding principles

15:30 – 15:45 **Group photo**

16:00 – 16:30 **Departure excursion**

17:00 – 19:00 **Citadel tour**

19:00 – 23:00 **Dinner (Eisgrub-Bräu)**

Wednesday, 25th September 2024

09:00 – 10:45 Session V Chair: Clement Carré

09:00 – 09:30 **Janusz Bujnicki** (International Institute of Molecular and Cell Biology in Warsaw, Poland)
tba

09:30 – 10:00 **Valérie de Crécy-Lagard** (University of Florida, USA)
Common Purpose, Diverse Solutions: Queuosine Precursor Transporters Across Species and Kingdoms

10:00 – 10:30 **Carine Tisné** (Institute of Physico-Chemical Biology (IPBC), Paris, France)
Advances in the structural and functional understanding of m^1A modifications

10:30 – 10:45 **Zeynep Baharoglu** (Institut Pasteur, France)
tRNA queuosine modification reprograms translation in response to antibiotics in Vibrio cholerae

10:45 – 11:15 Coffee break

11:15 – 12:30 Session VI Chair: Alper Akay

11:15 – 11:45 **Stefan Bauer** (Philipps-University Marburg, Germany)
RNA methylation and RNA glycosylation in self tolerance

11:45 – 12:15 **Donal O'Carroll** (University of Edinburgh)
tba

12:15 – 12:30 **Rene Ketting** (Institute of Molecular Biology (IMB) Mainz, Germany)

A novel 5'end processing enzyme matures piRNA precursors in the nematode C. elegans

12:30 – 14:00 Lunch Break and Postersession

14:00 – 15:30 Session VII Chair: Alessia Ruggieri

14:00 – 14:30 **Danny Nedialkova** (Max Planck Institute for Biochemistry and Department of Bioscience Munich, Germany)
Context-dependent essentiality of RNA modifications in human cells

14:30 – 15:00 **Jeppe Vinther** (University of Copenhagen, Denmark)
Conserved 5'metabolite RNA capping among hepaciviruses

15:00 – 15:15 **Marc Graille** (Laboratory of Structural Biology of the Cell, Ecole Polytechnique, Institut Polytechnique de Paris, CNRS, Palaiseau, France)
RNA methyltransferase holoenzymes: implications in mRNA maturation and translation and in neurodevelopmental disorders

15:15 – 15:30 **Lukas Schartel** (Johannes Gutenberg-University Mainz (JGU), Germany)
Selective RNA pseudouridinylation using circular gRNA in designer organelles

15:30 – 16:00 Coffee break

16:00 – 17:30 Session VIII Chair: Jean-Yves Roignant

16:00 – 16:30 **Sebastian Glatt** (Jagiellonian University Krakow, Poland)
The molecular basis of tRNA selectivity by human pseudouridine synthase 3

16:30 – 17:00 **Olivier Duss** (European Molecular Biology Laboratory, Heidelberg, Germany)
How processes cooperate with each other: Real-time tracking of m6A RNA modification and co-transcriptional rRNA processing

17:00 – 17:15 **Marie-Luise Winz** (Johannes Gutenberg University Mainz (JGU), Germany)
Yeast elongation factor homolog New1 protects specific mRNAs from no-go decay, probably modulating ribosomal RNA modification

17:15 – 17:30 **Andres Jäschke** (Heidelberg University, Institute of Pharmacy and Molecular Biotechnology, Heidelberg, Germany)
NON-CANONICAL RNA CAPS – NEW CHEMISTRY, NEW FUNCTIONS

Closure: Mark Helm

19:00 **Dinner RMaP PI + Speaker + Chair (Zenz)**

Abstracts - Talks
(sorted according to schedule)

Monday, 23.09.2024

Ramesh S. Pillai (University of Geneva, Switzerland)

RNA modifications in control of mammalian gene expression

Ramesh S. Pillai

Department of Molecular Biology, University of Geneva, 30 Quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland.

N^6-methyladenosine (m^{6}A) is an essential internal RNA modification that is critical for gene expression control in most organisms. They are catalyzed by RNA methyltransferases 'writers' on specific targets, while protein 'readers' with a YTH domain recognize the m^{6}A marks to mediate molecular functions like RNA splicing, mRNA decay and translation control. The functional relevance of these marks is demonstrated by the ability of RNA demethylase 'erasers' to remove this mark, pointing to potential reversibility and regulation. I will report the recent studies from the lab on the writer human METTL16 to reveal its physiological roles and the mechanistic basis for target regulation. We use a combination of mouse genetics and biochemistry to decipher how the targets are engaged and how this impacts developmental programs. Our results highlight the pivotal role of an m^{6}A RNA methyltransferase in facilitating early developmental decisions via regulation of SAM availability

Rupert Fray (University of Nottingham)

Mutants of the plant m6A writer complex

Mi Zhang, Nathan Archer, Nigel Mongan, Rupert Fray

University of Nottingham

The formation of m6A in mRNA is catalysed by a large multiprotein complex, consisting of (at least) six components that are conserved between plants and animals. Knockout of some of these components abolishes methylation and is lethal, whilst knockout of other components is not lethal but reduces m6A levels. We have generated a set of knockout and hypomorphic plant lines in which m6A is reduced to as little as 10% of its normal level. These plants show severe growth and developmental defects. Using these low m6A lines, we have carried out suppressor screens that restore normal growth, and we have identified some of the mutations responsible. Suppressor mutants fall into two classes, those in which m6A levels have been partially restored through compensatory mutations in other m6A “writer” complex members, and those in which m6A levels remain low, but mutation of genes in pathways normally regulated my m6A has occurred.

Miona Corovic (IMB Mainz, Germany)

m6A sites in the coding region trigger translation-dependent mRNA decay

Miona Corovic[1]; You Zhou[2]; Dr. Peter Hoch-Kraft[1]; Dr. Julian König[1]; Dr. Kathi Zarnack[2]

[1] Institute of Molecular Biology (IMB), Mainz, Germany
[2] Goethe-Universität Frankfurt a. Main, Germany

N^6-Methyladenosine (m6A) is the predominant internal RNA modification in eukaryotic messenger RNAs (mRNAs) and plays a crucial role in mRNA stability. Here, we reveal that m6A sites in the coding sequence (CDS) trigger CDS–m6A decay (CMD), a novel mRNA decay pathway that is distinct from previously reported m6A-dependent degradation mechanisms. Importantly, CDS m6A sites act considerably faster and more efficiently than those in the 3' untranslated region, which to date have been considered the main effectors of m6A-mediated RNA decay. Mechanistically, CMD depends on translation whereby m6A deposition in the CDS triggers ribosome pausing and transcript destabilization. The target transcripts of CMD are recognized by the m6A reader protein YTHDF2, selectively enriched in processing bodies (P-bodies), and degraded via the decapping factor DCP2. Our findings highlight CMD as a previously unknown pathway for m6A-mediated decay, which is particularly important for controlling the expression of developmental regulators and retrogenes.

Francesca Aguilo (Umea University, Sweden)

Unveiling the dual roles of METTL3 in breast cancer tumorigenesis

Francesca Aguilo

Umea University, Sweden

N^6-methyladenosine (m6A) modification is the most prevalent internal modification of eukaryotic messenger RNAs (mRNAs) and plays a central role in gene expression regulation. Despite impaired m6A deposition in various cancers, the specific role of METTL3 in breast cancer remains unclear. In this study, we show that METTL3 enhances the breast cancer phenotype by a novel dual mechanism depending on its sub-cellular localization. Our results indicate that the canonical nuclear function of the m6A-methyltransferase complex, comprising METTL3 and METTL14, marks transcripts implicated in cell proliferation and migration, thus delineating a breast cancer-specific m6A-related gene signature. Furthermore, we observe that METTL3 is highly expressed in the cytoplasmic compartment of breast cancer cells from patients. Cytoplasmic METTL3 interacts with the exocyst complex, an evolutionary conserved octameric complex of proteins that mediates intracellular membrane trafficking, facilitating the targeting and tethering of post-Golgi secretory vesicles to specific membrane sites. Noteworthy, breast cancer cell lines depleted of METTL3 displayed less gelatinases activity and less invadopodia formation, supporting the role of METTL3 in cell invasion via exocytosis. Our findings unveil a novel non-canonical role for cytoplasmic METTL3 and its adverse impact on breast cancer growth and progression."

Konstantinos Stellos (Medical Faculty Mannheim of Heidelberg University, Germany)

Adenosine RNA modifications in post-transcriptional regulation of gene expression

Konstantinos Stellos

Medical Faculty Mannheim of Heidelberg University, Germany

Background

More than 170 types of RNA modifications have been discovered so far that regulate many aspects of the mRNA life cycle, including pre-mRNA splicing, mRNA export, stability, and translation. N-methyl adenosine (m6A) RNA methylation and Adenosine-to-Inosine RNA editing are the most abundant internal RNA modifications. In many cases, RNA modifications function by altering how cellular machinery such as RNA binding proteins (RBPs) interact with RNA substrates. Indeed, most interactions between RBPs and RNA modifications have a complicated dependence on sequence context and binding modality. The RBP HuR may control the fate of several genes in a synchronized manner comprising an additional regulatory layer of gene expression. How HuR is regulated is yet to be shown.

Methods

Primary human vascular endothelial cells were used to evaluate the inosimone and methylome effects on HuR binding. Transcriptome-wide single nucleotide profiles were generated by array star and nanopore direct long RNA sequencing. Molecular studies involving RNA modification-specific RNA immunoprecipitation, transcriptomics, stability assays, RNAi and gain- and loss of function assays provided mechanistic insights. Cellular studies were applied under homeostatic and stress conditions.

Results

We first identified by iCLIP and DiCLIP experiments an interaction between the stabilizing RNA-binding protein Human Antigen R (HuR) and the 3'-untranslated Region (3'UTR) of several mRNAs. We then determined HuR interactome in absence or presence of m6A RNA methylation as well as in homeostatic (unstimulated) or TNF-alpha induced-pro-inflammatory conditions. We observed that in absence of m6A RNA methylation an increased of HuR binding to pro-inflammatory target genes, thereby increasing their expression, which was amplified in a cytokine-mediated inflammation environment. Regarding RNA editing, we found that HuR preferentially binds to the edited 3’ UTR transcript enhancing their stability. Inosinome and m6A RNA methylome are critical regulators of HuR binding to its targets adding a novel layer of post-transcritpional regulation of gene expression. Stress and environmental cellular changes modify the inosinome and m6A RNA methylome which subsequently dramatically alter the HuR-mediated RNA processing affecting cell resilience.

Conclusion

Collectively, these findings suggest the notion that RNA modifications are a master upstream regulator of HuR-mediated RNA abundance across the transcriptome. This mechanism was dependent of cell state and environmental conditions indicating that RNA modifications may have helped humans adapt to the constant environmetal stress and changes throughout evolution.

Kristin Koutmou (University of Michigan, USA)

PUS7 cytoplasmic localization directs a pseudouridine-mediated cellular stress response

Minli Ruan[1], Sean M. Engels[2], Matthew R. Burroughs[2], Dylan Bloch[3], Oleksandra Fanari[3], Stuart Akeson[3], Daniel E. Eyler[4], Xiaoyan Li[4], Chase A. Weidmann[1], Sara Rouhanifard[3], Miten Jain[3], Lydia M. Contreras[2], Kristin S. Koutmou[1,4]

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[2]University of Texas, McKetta Department of Chemical Engineering, Austin, TX 78712
[3]Northeastern University, Department of Bioengineering, Boston, MA 02120
[4]University of Michigan, Department of Chemistry, Ann Arbor, MI 48109

Pseudouridine (Y) is an abundant post-transcriptional modification found across all classes of RNA. Since its discovery in mRNAs, it has been widely speculated that Y might provide an avenue for cells to control post-transcriptional gene expression. Here we demonstrate that one of the principal mRNA pseudouridylating enzymes, pseudouridine synthase 7 (PUS7), exhibits a stress induced accumulation in the cytoplasm of yeast and human epithelial lung cells. The cytoplasmic localization of PUS7 promotes Y-incorporation into hundreds of mRNA sequences and increases cellular fitness under ROS and divalent metal ion stress. Quantitative proteomics reveal a reshaping of the proteome upon PUS7 relocalization under stress, with proteins that bind metal being particularly sensitive. Collectively, our data demonstrate that the post-transcriptional inclusion of Y into mRNA impacts protein production in cells. Furthermore, they suggest a conserved mechanism for a Y-mediated cellular stress response, whereby stressors relocalize PUS7 and modulate protein production from stress response mRNAs.

Schraga Schwartz (Weizmann Institute of Science, Rehovot, Israel)

mRNA metabolism governs m6A deposition and dynamics

David Dierks, Anna Uzonyi, Ran Shachar, Ronit Nir, Schraga Schwartz

Weizmann Institute of Science, Rehovot, Israel

m6A is the most widespread mRNA modification. Research over the past decade has primarily focused on how m6A controls mRNA metabolism. In my talk I will examine m6A through an opposite lens, asking how mRNA metabolism impacts m6A. I will address two fundamental questions pertaining to m6A biogenesis. First, what is the basis for the non-uniform distribution of m6A across the transcriptome? Second, what is the basis for changes in m6A levels, as can be observed across different subcellular compartments and across some stimuli? For each of these two questions, I will propose a simple model based on which these are governed by mRNA metabolism. For the first question, I will present our model wherein m6A is shaped by mRNA splicing, and specifically by the deposition of the exon junction complex that prevents the formation of m6A in the vicinity of splice sites. For the second question, I will propose a model wherein rather than being actively shaped, m6A levels are passively controlled via changes in mRNA metabolism. These two models establish a naïve baseline for guiding future research into m6A deposition and dynamics.

Vivian Cheung (University of Michigan, USA)

Decoding RNA Modifications in an eRNA: The Role of Sequential m6A to Abasic Sites in Regulating APOE Expression.

Vivian G. Cheung

MD Department of Pediatrics, University of Michigan, USA

The role of RNA modifications in gene regulation is an emerging frontier in biology. In this study, we focused on RNA abasic sites, an underexplored RNA modification generated by methylpurine glycosylase, which cleaves N-glycosidic bonds in total RNA including mRNA of yeast and human cells.

Through our investigation of RNA abasic sites, we discovered an enhancer RNA that undergoes sequential modifications, beginning with N6-methylation of adenosines followed by N-glycosidic cleavage, leading to the formation of abasic sites. This enhancer RNA, which we named APOE-activating noncoding RNA (AANCR), is a crucial regulator of APOE expression, a gene with strong allelic associations with Alzheimer's Disease. Before the identification of AANCR, the transcriptional regulation of APOE was primarily understood only in the context of lipid load, leaving a significant gap in our knowledge.

Our research shows that AANCR is governed by sequential RNA modifications that occur on R-loops. These modifications stabilize the R-loops formed by nascent AANCR RNA, effectively pausing its transcription and keeping it poised for rapid activation. This mechanism allows AANCR to promptly regulate APOE expression in response to cellular signals. In cells that express APOE, AANCR does not form R-loops and does not undergo modification; instead, it is transcribed into full-length enhancer RNA that activates APOE expression.

By studying RNA abasic sites, we not only identified AANCR but also uncovered a novel mechanism by which RNA modifications influence gene expression through the stabilization of RNA structures. This finding enhances our understanding of the cell-type-specific expression of APOE and its regulation in response to stress, highlighting the crucial role of RNA modifications in gene regulation.

Michael Jantsch (Medical University of Vienna, Austria)

Editing patterns in double-stranded RNAs camouflaging MDA5-activation

Rajagopa Varada[1]; Alina Leuchtenberger[2]; Cornelia Vesely[1]; Beata Kaczmarek[3]; Carrie Bernecky[4]; Andy Sombke[1]; Hamid Mansouri Khosravi[1]; Margret Eckhard[5]; Michael Jantsch[1]

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[2]University of Vienna, Austria
[3]Institute of Science and Technology Austria
[4]Institute of Science and Technology, Austria
[5]Medical University of Vienna, Austria

Pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) with high specificity. In RNAs, unique features such as cap structure, double-strandedness, base content, or nucleotide modifications help to distinguish self from non-self RNAs. Adenosine to inosine (A-to-I) deamination by ADARs is a very abundant type of RNA editing in metazoa. A-to-I RNA editing has been shown to play an essential role in mammalian embryonic development and tissue homeostasis, and is implicated in the pathogenesis of many diseases, including inflammatory diseases (1). In mammals, ADAR1 and ADAR2 are responsible for A-to-I editing, while ADAR3 is seemingly inactive. ADAR1 is constitutively expressed but also has an IFN-inducible isoform. Mice lacking the interferon inducible version of ADAR1 show embryonic lethality at day 12.5, accompanied by a strong IFN signature (2). A deletion of the cytoplasmic double-stranded RNA sensor MDA-5 can rescue a catalytic dead ADAR1 mutation, suggesting that inosines in endogenous RNAs edited by ADAR1 prevent their sensing through MDA5 (3).

Here we characterize endogenous mouse RNAs that need to be critically edited by ADAR1 to prevent the activation of MDA-5 signaling. We also show that inosines must be deposited at specific positions to prevent MDA5 activation, interferon stimulated gene expression (ISG) and stress granule formation (SG) indicating that a specific “inosine code” helps to distinguish self-from non-self RNAs.

Chuan He (University of Chicago, USA)

Chromatin regulation by RNA methylation

Chuan He

Department of Chemistry, Department of Biochemistry and Molecular Biology, Institute for Biophysical Dynamics, Howard Hughes Medical Institute, The University of Chicago, USA

Over 150 types of post-transcriptional RNA modifications have been identified in all kingdoms of life. We have discovered RNA demethylation and shown that reversible RNA modification could impact a wide range of biological processes. We have also characterized proteins that selectively recognize m6A-modified mRNA and affect the translation status and lifetime of the target RNA. I will present our recent discoveries on chromatin state regulation by chromatin-associated regulatory RNA (carRNA) methylation. We found that carRNAs contain different chemical marks which facilitate recruitment of chromatin factors to shape local and global chromatin state. Some of these carRNA methylation-dependent pathways explain oncogenic roles of well-known oncogenes, which provides potential new targets for future anti-cancer therapies.

Tuesday, 24.09.2024

Yuri Motorin (Université de Lorraine, Nancy, France)

Challenges in RNA modification mapping by deep sequencing

Yuri MOTORIN

Ingénierie Moléculaire, Cellulaire et Physiopathologie (IMoPA), UMR 7365 CNRS-Université de Lorraine, Nancy, France

RNA modifications found in almost all types of cellular RNAs are now recognized as key players of RNA metabolism, affecting all steps of RNA life : processing/maturation, folding, recognition by cognate proteins and RNP assembly, trafficking, translation and degradation. Numerous methods are now available for RNA modification mapping in a subset of stable RNA species or transcriptome-wide. Despite substantial efforts for already >10 years in the epitranscriptome field, the consensus map of RNA modifications is only achieved for a few model living species and mostly only for stable RNAs. The number and the exact location of RNA modifications, as well as their stoichiometries for mRNA and other scarce RNA species, are still under debate. Deep sequencing methods are undoubtedly the best suited for extensive mRNA analysis, but their application is not always straightforward, and every method has its own limitations and drawbacks. Newly appearing protocols involving nanopore sequencing are promising, but their application is still very far from routine RNA modification analysis. Most popular second-generation deep sequencing protocols and their application to whole transcriptome are discussed as well as possible general guidelines for protocol validation and application.

Eva Maria Novoa (Center of Genomic Regulation, Barcelona, Spain)

Decoding the epitranscriptome at single molecule resolution: towards clinical applications

Ivan Milenkovic[1,2], Sonia Cruciani[1,2], Laia Llovera[1], Morghan C Lucas[1,2], Rebeca Medina[1], Cornelius Pauli[3,4,5], Daniel Heid[3,4,5], Thomas Muley[6,7], Marc A. Schneider[6,7], Laura V. Klotz[8], Michael Allgäuer[9], Ruben Lattuca[10], Denis LJ LaFontaine[10], Carsten Müller-Tidow[3,4] and Eva Maria Novoa[1,2,*]

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[9] *Institute of Pathology, Heidelberg University Hospital, Heidelberg, Germany*
[10] *Université libre de Bruxelles (ULB), Biopark campus, B-6041 Gosselies, Belgium*

Ribosomes have been historically considered as uniform macromolecular structures that have identical composition across cell types, tissues and conditions. This view, however, has been challenged in the past few years, leading to a change of paradigm in which ribosomes are now surveyed as dynamic entities that can be heterogeneous in their composition. The heterogeneity of these 'specialized ribosomes' can arise from the use of ribosomal protein paralogs, distinct rRNA variants or differential rRNA modifications, among others.

While the rRNA modification landscape has been previously characterized for some species, most studies and rRNA databases do not take into account the tissue and/or cell type of origin in their annotations. While the different types of rRNA modifications are likely interconnected, detailed maps of all rRNA modification patterns are lacking.

In this context, direct RNA nanopore sequencing (DRS) has emerged as a promising technology that can overcome these limitations, as it is in principle capable of mapping all RNA modifications simultaneously, in a quantitative manner, and in full-length native RNA molecules. Notably. previous works have already shown that rRNA modifications can be identified using DRS.

Here, I will first present our latest work on how we can use DRS to study the mammalian rRNA epitranscriptomic landscape across tissues, cell types, developmental stages and cancer types. We identify rRNA modification 'signatures' that are characteristic and distinct across tissues, cell types and developmental stages, including several previously unannotated rRNA sites, which we validate using orthogonal methods. Moreover, we show that upon cancer, these signatures vary, thus constituting novel promising biomarkers that could be further exploited by future diagnostic approaches. We propose that this approach could be used in the future to identify tissue-of-origin of cancer samples, as well as to predict cancer.

Ann Ehrenhofer-Murray (Humboldt-Universität to Berlin, Germany)

Detection of queuosine, pseudouridine and other tRNA modifications by direct RNA sequencing

Ann Ehrenhofer-Murray[1]; Yu Sun[1]; Franziskus Hauth[1]; Bhargesh Patel[1]; Michael Piechotta[2]; Isabel Naarman-de Vries[2]; Christoph Dieterich[2]

[1] Humboldt-Universität to Berlin, Germany
[2] University Hospital Heidelberg, Germany

tRNAs undergo extensive chemical modifications that are crucial for regulating their three-dimensional structure, stability, and decoding capacity during mRNA translation. Our research focuses on the queuosine (Q) modification of tRNAs, which is a 7-deazaguanine derivative with a cyclopentene diol moiety. Q modification is positioned at the Wobble site 34 in four NAC/U-decoding tRNAs and plays a pivotal role in ensuring equal decoding efficiency between C- and U-ending codons. Our prior research has shown that Q modification enhances Wobble base-pairing with U. The absence of Q leads to mild ribosomal stalling on U-ending Q codons, resulting in protein misfolding. In S. pombe, Q deficiency particularly impacts transcripts encoding proteins required for mitochondrial function, consequently leading to mitochondrial deficiencies.

The study of queuosine has been challenging due to the limited availability of high-throughput sequencing methods for its detection and analysis. We therefore have adapted direct RNA sequencing (Nanopore sequencing, Oxford Nanopore Technology, ONT) to detect modifications on tRNAs. To obtain end-to-end base calling, adapters were ligated to the 5' and 3' end of tRNAs that were either obtained by in vitro transcription, or were isolated from the yeast S. pombe and from Escherichia coli. Alignment of synthetic and biological as well as Q-modified and non-modified tRNAs revealed base miscalling, deletions and insertions at and around the Q position, as detected with the JACUSA2 framework. The analysis of tRNAs from E. coli strains with defects in queuosine biosynthesis allowed us to distinguish between Q and its precursors preQ1 and preQ0. Furthermore, comparison of synthetic tRNAs with those obtained from S. pombe indicates that other tRNA modifications in principle are detectable with this method.

In more recent work, we have expanded Nanopore sequencing to detect pseudouridine modifications in S. pombe tRNAs by evaluating Nanopore sequencing runs of tRNAs extracted from strains lacking pseudouridine synthetases. This has allowed us not only to generate a full pseudouridinylation map of S. pombe, but also to determine the interdependence of pseurouridine with other tRNA modifications. Overall, direct RNA sequencing is a powerful tool to detect tRNA modifications as well as to establish networks of modifications that jointly regulate tRNA function.

Laurence Drouard (IBMP-CNRS, Strasbourg, France)

Acquisition of a new functional eukaryotic tRNA by hijacking a prokaryotic modification

Christina Berrissou; Anne-Marie Duchêne; Laurence Drouard

IBMP-CNRS, Strasbourg, France

In eukaryotes, reading the three isoleucine codons requires two isoacceptor tRNAs, tRNAIle UAU for decoding AUA and tRNAIle AAU for decoding AUU and AUC thanks to the post-transcriptional modification, inosine, present at the wobble position of the anticodon. While this situation exists in all photosynthetic organisms, we provide evidence that no tRNAIleUAU is expressed from the nuclear genome of the early vascular plant, Selaginella kraussiana. Instead, we identified 7 nucleus-encoded tRNA genes, phylogenetically related to the 19 tRNAIleAAU genes, but possessing a CAU anticodon. Mim-tRNAseq analysis shows that both types of tRNAs are expressed. At the gene level, a CAU anticodon corresponds to a tRNAMet and enables the reading of the AUG codon. Since nucleus-encoded initiator and elongator tRNAMet genes are expressed in S. kraussiana, no other tRNA with a CAU anticodon is required to ensure cytosolic translation. However, this situation is reminiscent of that found in bacteria and plant organelles where tRNAs encoded by genes with a CAU methionine anticodon are converted into tRNAIle thanks to the modification, during tRNA processing, of the C34 into Lysidine (L) in eubacteria or its derivative in plant organelles. Importantly, this single modification converts both the reading of the codon (from AUG to AUA) and the amino acid specificity (from methionine to isoleucine) of the tRNA. Analysis of mim-tRNAseq data shows that two marks characterize this modification in A. thaliana mitochondrial and plastidial tRNAIle LAU. Strikingly, the same two features are visible on the eukaryotic nucleus-encoded S. kraussiana tRNAs possessing a CAU anticodon at their gene level. Additionally, although both mitochondrial and plastidial tRNAIle LAU have been lost in the course of evolution in S. kraussiana, we show the existence of a functional bacterial-like nucleus-encoded tRNA isoleucine lysidine synthase that is no longer targeted to organelles. Overall, our data strongly support the replacement of nucleus-encoded tRNAIle UAU by tRNAIle CAU via the acquisition of a prokaryotic-type modification of C34 to Lysidine by a bacterial enzyme hijacked from its original substrates.

Stefanie Kaiser (Goethe University Frankfurt, Germany)

Hunting for truth – towards more accurate RNA modification stoichiometry analysis

Stefanie Kaiser

Goethe University Frankfurt, Germany

Understanding life requires firm knowledge on the qualitative and quantitative number of players involved in a biological process. The same is true for RNA biology, where RNA modifications dictate the function of the RNA and thus the outcome of the biological process the RNA is involved in. From the qualitative perspective, the chemical structures of RNA modifications are best studied using the powerful combination of liquid chromatography (LC) and mass spectrometry (MS). Similarly, LC-MS is also ideally suited for quantitative analysis of RNA modifications if some analytical rules are followed. Due to high sample-to-sample variability (e.g. salt load) MS signals do not always respond to analyte concentration linearly. By spiking in stable-isotope-labeled-internal-standards (SILIS), these fluctuations can be easily cancelled, and precise analysis becomes fairly simple. Thus, addition of a SILIS allows comparison of one sample with another and the increase or decrease of the modification of interest can be easily calculated and plotted as a fold change.

Yet, fold-changes can be misleading. For example, in all cases where a modification is only present in extremely low stoichiometry, e.g. only 0.01 mol of modification per mol RNA, a fold change might imply a biological importance, that is not supported by the stoichiometric abundance of the modification. Thus, external calibration of precisely weighed standard nucleoside powders is needed to assess the absolute abundance of a modification and determine the biological relevance of sample-to-sample changes.

But absolute quantification remains challenging. We recently identified three classes of errors that may distort quantitative analysis of RNA modifications, with some not limited to LC-MS analysis but also sequencing. These classes comprise (I) errors related to chemical instabilities, (II) errors revolving around enzymatic hydrolysis to nucleosides, and (III) errors arising from issues with LC separation and/or subsequent MS analysis. In addition, we question the synthetic purity of the commercially available nucleoside standards by using qNMR spectroscopy for accurate and precise quality control. Last but not least, we want to raise awareness to the chemical stability of aqueous solutions of nucleoside standards.

All in all, we are confident that we systematically assess all hindrances of absolute RNA modification quantification which will pave the way towards robust and significant RNA modification research.

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Nicola Conci (siTOOLs Biotech GmbH, Planegg, Germany)

rRNA Contamination in Ribo-Seq: Origin, Characteristics and Mitigation Strategies

Nicola Conci

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Ribosome profiling (Ribo-Seq) is a technique based on the sequencing of ribosome protected fragments (RPF), mRNA regions of ca. 30 nucleotides enclosed by ribosomes. It allows to determine the position of ribosomes on mRNAs and can thus provide insight into several aspects of protein translation and its regulation mechanisms. Since its first development in yeast, several Ribo-Seq workflows have been established and applied to a variety of organism including animals, bacteria and plants. A key step of RiboSeq workflows is nuclease digestion, which degrades mRNAs regions not protected by ribosomes and allows the isolation of RPFs. However, nucleases also cause the partial degradation of ribosomal RNA (rRNA), causing rRNA fragments to be co-isolated alongside RPFs. The presence of significant rRNA amounts in Ribo-Seq libraries is in fact a common occurrence and often limits sequencing depth. Here, we discuss the characteristics of rRNA contamination in Ribo-Seq experiments, how it arises during sample preparation, the factors that can influence the nature and abundance of rRNA contaminants, and possible depletion strategies to mitigate it.

Marina Rodnina (Max-Planck-Institute for Physical Biochemistry, Göttingen, Germany)

Non-canonical Decoding by the Ribosome

Marina V. Rodnina

Max Planck Institute for Multidisciplinary Sciences, Göttingen, Germany

Dynamic mRNA modifications regulate key steps of cellular mRNA metabolism including translation. We have studied how two modifications, m6A and m5C, modulate mRNA decoding by the ribosome using a combination of rapid kinetics, smFRET and single particle cryo-EM. We show the modifications favor alternative, non-canonical conformations of the codon-anticodon complex, thereby either delaying the recognition of the cognate codon (m6A) or stabilizing near-cognate codon-anticodon pairs (m5C). These results highlight how modifications outside the Watson-Crick edge can interfere with codon-anticodon base pairing on the ribosome, thereby modulating the translational efficiency of modified mRNA and transiently altering the proteome composition of the cell.

Zoya Ignatova (University of Hamburg, Germany)

N^6-methyladenosine modification in mRNA – the positions it may or may not affect mRNA utilization

Zoya Ignatova

Institute of Biochemistry and Molecular Biology, University of Hamburg, Hamburg, Germany

Reversible N^6-methyladenosine (m^{6}A) modifications on mRNA emerge as prevalent phenomena in RNA metabolism, but is installed non-stoichiometrically across transcripts. We use an integrative approach, including functional, structural, single molecule, and computational approaches, to elucidate the position-specific function of m^{6}A along with its thermodynamic contribution to translation dynamics. Comparing the m6A-modification pattern under homeostatic growth conditions and following stress exposure, we identified regions that are important and others that may not affect mRNA utilization at all.

Tsutomu Suzuki (University of Tokyo, Japan)

Expanding world of tRNA modifications in health and disease

Tsutomu Suzuki

Department of chemistry and biotechnology, University of Tokyo, Japan

RNA molecules are frequently modified post-transcriptionally, and these modifications are required for proper RNA functions. To date, approximately 150 types of chemical modifications have been identified in various RNA molecules across all domains of life. There are still a number of novel modifications to be discovered. RNA modifications appear to confer chemical diversities to simple RNA molecules basically composed of four letters, to acquire a greater variety of biological functions. These modifications play critical roles in stability and functions of RNA molecules. The physiological importance of RNA modification is highlighted by human diseases caused by aberrant RNA modification. We previously reported a severe reduction in the frequency of tRNA modifications in mitochondrial disease patients, like MELAS and MERRF. These findings provided the first evidence of RNA modification disorder. We call “RNA modopathy” as a new category of human diseases.
We are engaged in a project to identify novel RNA modifications from various sources, and have reported nine modifications so far. Taking advantage of mass spec analysis of RNA modifications, we identified more than 50 genes responsible for tRNA modifications, rRNA modifications as well as mRNA modification. In my talk, I will report the latest progress in our project.

Keywords: tRNA modification, epitranscriptomics, ribosome, protein synthesis

Sebastian Glatt (Jagiellonian University Krakow, Poland)

The molecular basis of tRNA selectivity by human pseudouridine synthase

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Pseudouridine (Ψ), the isomer of uridine, is ubiquitously found in RNA, including tRNA, rRNA and mRNA. Human pseudouridine synthase 3 (PUS3) catalyzes pseudouridylation of position 38/39 in tRNAs. However, the molecular mechanisms by which it recognizes its RNA targets and achieves site-specificity remain elusive. Here, we determine single particle cryo-EM structures of PUS3 in its apo form and bound to three tRNAs, showing how the symmetric PUS3 homodimer recognizes tRNAs and positions the target uridine next to its active site. Structure-guided and patient-derived mutations validate our structural findings in complementary biochemical assays. Furthermore, we deleted PUS1 and PUS3 in HEK293 cells and mapped transcriptome-wide Ψ sites by Pseudo-seq. Although PUS1-dependent sites were detectable in tRNA and mRNA, we found no evidence that human PUS3 modifies mRNAs. Our work provides the molecular basis for PUS3-mediated tRNA modification in humans and explains how its tRNA modification activity is linked to intellectual disabilities.

Klemens Wild (University of Heidelberg, Germany)

Comprehensive analyses of rRNA modifications in a thermophilic eukaryote

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rRNA modifications stabilize ribosomes and cluster around functionally important sites. Changes of the modification pattern impact in gene expression, translational control and thermophily. RNA modifications alter stability, folding space, surface propensities and the interaction network of RNA molecules. In general, only a limited set of chemical modifications is found in rRNA. Here, we investigate on rRNA modifications for the thermophilic fungus Chaetomium thermophilum (Ct), a unique biochemical / biotechnological tool and model system for eukaryotic thermophily. Using state-of-the-art orthogonal NGS and NNGS RNA-seq methods, we qualitatively and quantitatively assign all Ct rRNA modifications and model them in our completely re-refined high-resolution 2.3 Å cryo-EM structure of the idle Ct 80S ribosome retaining nascent chains (unpublished). Data are comprehensively compared with plant, yeast and human modifications and analysed in context of phylogeny and thermophily. The ligand network including magnesium and potassium ions as well as spermine/spermidine molecules is detailed and systemized

Jirka Peschek (University of Heidelberg, Germany)

Structure of fungal tRNA ligase with RNA reveals conserved substrate binding principles

Sandra Köhler; Jürgen Kopp; Jirka Peschek

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RNA ligases play a vital role in RNA processing and maturation including tRNA splicing, RNA repair and the unfolded protein response (UPR). In fungi and plants, the tRNA ligase Trl1 catalyzes the joining of splicing endonuclease (TSEN)-cleaved pre-tRNA exon halves and Ire1-cleaved HAC1 mRNA during the UPR. Trl1 is a tripartite enzyme, consisting of an RNA ligase (LIG) and two end-modifying modules. The ligase reaction progresses via three steps. First, ATP-dependent ligase activation yields a covalent LIG-AMP intermediate. Subsequently, the bound AMP is transferred to the 5'-P end of the 3' exon resulting in a 5'-5' RNA-adenylylate intermediate. Finally, the LIG domain catalyzes the nucleophilic attack on the RNA-adenylate by the 3'-OH to form a phosphodiester bond, thereby releasing AMP. The spatial arrangement of the RNA exon ends during the ligation reaction has remained elusive by the general lack of ligase-RNA structures. Here, we report the crystal structure of Trl1-LIG in complex with an activated tRNA-derived substrate and define the conserved substrate binding interface. The underlying enzyme-substrate interplay reveals a general substrate binding principle for adenylyltransferases. Moreover, we identify the determinants of RNA end specificity as well as the specific roles of Trl1-LIG's subdomains during ligase activation, substrate binding and phosphoryl transfer. Guided by our structural data, we could pinpoint the unique requirement of fungal tRNA ligases for a terminal 2' phosphate to a pair of conserved arginine residues within its C-terminal subdomain. Since Trl1 is often discussed as potential target for antifungal therapy due to the fundamental difference compared to the human tRNA ligase complex, this study provides additional insights on how to potentially perturb RNA binding. In summary, our study uncovers conserved RNA binding modes between nick-sealing and single strand-end joining RNA ligases, indicating how different RNA ligase families evolved from a common principle to perform their specific cellular tasks.

Janusz Bujnicki (International Institute of Molecular and Cell Biology in Warsaw, Poland)

tba

Valérie de Crécy-Lagard (University of Florida, USA)

Common Purpose, Diverse Solutions: Queuosine Precursor Transporters Across Species and Kingdoms

Valérie de Crécy-Lagard

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Queuosine (Q) is a modification of the wobble base in tRNAs with GUN anticodons, playing a crucial role in decoding accuracy and efficiency. Its synthesis involves multiple enzymatic steps and several intermediates that can be salvaged. Depending on the organism, various precursor bases can be transported, including intermediates like preQ0 (7-cyano-7-deazaguanine) and preQ1 (7-aminomethyl-7-deazaguanine), as well as the final base, queuine (q). Some organisms can even transport the complete ribonucleoside Q. Until recently, the genes encoding these transporters were unknown. However, by combining comparative genomics and experimental approaches, we identified a diverse range of transporter families capable of transporting Q precursors in both bacteria and eukaryotes. This work highlights the concept of transporter plasticity and illustrates how organisms adapt to the availability of Q precursors in their environments.

Carine Tisné (Institute of Physico-Chemical Biology (IPBC), Paris, France)

Advances in the structural and functional understanding of m^{1}A modifications

Carine Tisné

IBPC, Laboratory of Microbial Gene Expression, UMR 8261 CNRS/University of Paris Cité, Paris, France

RNA modification is a co- or post-transcriptional process by which specific nucleotides are chemically altered by enzymes after their initial incorporation into the RNA chain, expanding the chemical and functional diversity of RNAs. Our understanding of RNA modifications has changed dramatically in recent years. In the last decade, RNA methyltransferases (MTases) have been highlighted in numerous clinical studies and disease models, modifications were found to be dynamically regulated by de-modification enzymes, and significant technological advances have been made in the field of RNA sequencing, mass spectrometry and structural biology. The mono-methylation of adenine at position N1 (m^{1}A) are frequently occurring methylations in RNAs. Among RNAs, transfer RNAs (tRNAs) exhibit the greatest diversity and density of post-transcriptional modifications, which allows for potential cross-talks and regulation during their incorporation. The N1-methyladenosine (m^{1}A) modification is found in tRNAs at position 9, 14, 16, 22, 57 and 58, depending on the tRNA and organism. My team has used and developed a large panel of tools to decipher the different mechanisms used by m^{1}A tRNA MTases to recognize and methylate tRNA. Recent results will be presented.

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Zeynep Baharoglu (Institut Pasteur, France)

tRNA queuosine modification reprograms translation in response to antibiotics in Vibrio cholerae

Zeynep Baharoglu

Institut Pasteur, France

Antimicrobial resistance develops as a major problem in infectious diseases treatment. Starting with a high-density Transposon insertion library in Vibrio cholerae and following its evolution by TN-seq in the presence of sub-inhibitory concentrations of antibiotics, we have confirmed 23 tRNA and rRNA modification enzymes' link with specific responses to various antibiotics in V. cholerae. We further studied the molecular mechanisms underlying this specificity in the absence of tgt/queuosine (Q) modification. Tgt is responsible for the Q modification of tRNAs with GUN anti-codon (Tyr/Asp/asn/His) at the anti-codon loop wobble position. We have identified tgt as being vital for growth in presence sub-lethal concentrations of aminoglycoside.

We used molecular reporters for translation fidelity and efficiency as well as a proteomics to further characterize molecular mechanisms behind the aminoglycoside sensitive phenotype.

We show that (i) the absence of Q impacts tyrosine codon decoding and leads to translational reprogramming in response to antibiotic stress. (ii) A protein's codon usage bias can influence its translation in a Q modification dependent way. (iii) Candidate transcripts subject to modification tunable translation can be identified in silico based on their codon content in bacteria. Our results highlight an epitranscriptomic and translational control of the bacterial response to antibiotic stress in V. cholerae.

Stefan Bauer (Philipps-University Marburg, Germany)

RNA methylation and RNA glycosylation in self tolerance

Nathanael B. Kegel & Stefan Bauer

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The innate immune system recognizes foreign nucleic acids based on localization (e.g. cytoplasmic DNA, endosomal RNA) and structure (e.g.
dsRNA, CpG motifs). However, cellular stress and even normal organelle formation may introduce self-derived RNA and DNA to innate immune receptors. Thus, mechanisms that prevent self-recognition are required. Defects in such mechanisms are associated with autoimmunity and cancer. Here we pursued the question if RNA modifications can act as a "self"-label for endogenous RNAs. We previously identified 2'O-methylation of bacterial tRNA by the methyltransferase trmH to modulate activation of TLR7. We hypothesize that the human methyltransferase TARBP1 may catalyse an analogous reaction and thus label endogenous tRNA as "self". In addition, RNA glycosylation of small RNAs has been recently introduced, the immunological role of glycoRNA however is unknown. This work aims at dissecting the roles of 2'O-methylation and RNA glycosylation in preventing self-recognition of endogenous tRNA / RNA.

Donal O'Carroll (University of Edinburgh)

tba

Rene Ketting (Institute of Molecular Biology (IMB) Mainz, Germany)

A novel 5'end processing enzyme matures piRNA precursors in the nematode C. elegans

Rene Ketting

Institute of Molecular Biology (IMB) Mainz, Germany

In germ cells small RNA pathways are key players in the control of transposable elements. In animals, the Piwi-piRNA pathway takes this role, and is essential for germ cell survival. The specificity of the Piwi-piRNA pathway is set by the identity of the small RNA molecules (piRNAs) that are used by the Piwi proteins to identify their targets. In the nemaotode C. elegans the piRNAs are transcribed from mini-genes that each produce a short, capped transcript of about 30 nucleotides. This transcript needs to be processed at its 5' and 3' ends to become fully functional. We identified a novel enzyme, consisting of multiple Schlafen-fold proteins that specifically can process the 5' ends of the piRNA precursors in C. elegans, fully dependent on the 5' cap and its methylation status. The composition of this enzyme, as well as its interactions with other piRNA biogenesis factors will be discussed.

Danny Nedialkova (Max Planck Institute for Biochemistry, Munich, Germany)

Context-dependent essentiality of RNA modifications in human cells

Danny Nedialkova[1,2]

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RNA molecules are modified with a variety of chemical groups that can regulate their structure and function, and RNA modification defects often elicit neurodevelopmental disorders and cancer via mechanisms that are poorly understood. To define them, we compared the essentiality of genes encoding RNA modifiers in human induced pluripotent stem cells (hiPSC) and hiPSC-derived neural and cardiac cells by focused CRISPR interference screens. We found that while rRNA-modifying enzymes are essential in nearly all cellular contexts, the loss of mRNA or tRNA modifiers yields a broad spectrum of cell context-dependent phenotypes. Ablating tRNA modifications destabilizes specific subsets of the tRNA transcripts that contain them, but tRNA destabilization is often insufficient to elicit ribosome pausing at their matching codons in endogenous mRNAs. Our findings highlight the complexity of predicting the molecular functions of individual RNA modifications in metazoans.

Jeppe Vinther (University of Copenhagen, Denmark)

Conserved 5'metabolite RNA capping among hepaciviruses

Helena S. Larsen, Anna V. Sherwood, Lizandro Rivera-Rangel, Paula H. Faller, Cathrine B. Vågbø, Kenn Holmbeck, Sonja Fernbach, Line A. Ryberg, Eva Jakljevič, Caroline E. Thorselius, Raphael Wolfisberg, Matthew J. Kennedy, Louise Nielsen, Amal Al-Chaer, Santseharay Ramirez, Jens Bukh, Troels K. H. Scheel, Jeppe Vinther

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We recently demonstrated that the cellular metabolite flavin adenine dinucleotide (FAD) is used as a noncanonical initiating nucleotide by the hepatitis C virus (HCV) RNA-dependent RNA polymerase resulting in a 5'FAD cap on the HCV RNA. This is the first demonstration of 5'metabolite capping of a virus RNA but it remains unknown whether 5'FAD RNA capping is conserved to related viruses in the hepacivirus genus.

The Norway rat hepacivirus (NrHV) is related with HCV and shares the ability to cause chronic liver infections in its host. As observed for HCV, the NrHV RNA genome was found to be 5'metabolite capped, but surprisingly not with FAD. Instead, the analysis of NrHV RNA with a set of decapping enzymes indicate that NrHV is 5'NAD capped. This finding was supported by the mass-spectrometry based detection of NAD released from NrHV RNA from up-concentrated viral particles. In addition, NrHV replication was attenuated in absence of NAD precursor, but not FAD precursor, and NrHV infection in culture was reduced by FK866, an inhibitor of the NAD biosynthesis enzyme NAMPT. In contrast, for equine hepacivirus (EqHV), which is a hepacivirus more closely related to HCV, the genome was found to be 5'FAD capped. In vitro replication assays with recombinant viral polymerases reflected the observed capping pattern, with the HCV and EqHV polymerases preferably initiating replication with FAD, whereas the polymerases from NrHV and related viruses such as hepatitis GB virus B (GBV-B) and bovine hepacivirus (BoHV) most efficiently initiated with NAD but accepted a broader range of metabolites.

In conclusion, 5'metabolite RNA capping is conserved across hepaciviruses, but interestingly the two major branches in the genus differ with respect to their cap preference. HCV and EqHV are 5'FAD capped, whereas our data indicates that NrHV and related viruses, such as GBV-B and BoHV, are capped with NAD.

Marc Graille (Laboratory of Structural Biology of the Cell, Ecole Polytechnique, Institut Polytechnique de Paris, CNRS, Palaiseau, France)

RNA methyltransferase holoenzymes : implications in mRNA maturation and translation and in neurodevelopmental disorders

Marc Graille

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Modified nucleotides in non-coding RNAs, such as rRNAs, tRNAs and snRNAs, represent an important layer of gene expression regulation through their ability to fine-tune mRNA maturation and translation. Dysregulation of such modifications and the enzymes installing them have been linked to various human pathologies including neurodevelopmental disorders and cancers. Several methyltransferases (MTases) are regulated allosterically by human TRMT112. The complete interactome of this regulator and the substrates of its interacting MTases have been only recently characterized, confirming that all these TRMT112-MTase complexes modify factors (snRNAs, tRNAs, rRNAs and proteins) involved in the mRNA maturation and translation processes. Indeed, the TRMT112-BUD23 and TRMT112-METTL5 complexes catalyze the formation of N7-methylguanosine (m7G) and N6-methyladenosine (m6A) on the 18S rRNA and participate in 40S ribosomal subunit biogenesis pathway. The TRMT112-HEMK2 complex modifies the translation termination factor eRF1, which is a tRNA mimicry critical for the release of the newly synthesized proteins. The TRMT112-ALKBH8, TRMT112-TRMT11 and TRMT112-THUMPD3 complexes contribute to translation elongation by modifying tRNAs. Finally, the TRMT112-THUMPD2 complex methylates U6 snRNA, the core component of the major catalytic spliceosome and hence is important for optimal splicing of weak splice sites. Recent results obtained on the functional characterization of eight homozygous METTL5 missense mutants identified in patients suffering from intellectual disability and microcephaly with varying degrees of penetrance will be presented. This work brings decisive information on the importance of this 18S rRNA m6A methylation, in normal brain development. Considering that the modification is adjacent to the ribosomal decoding site, it suggests that its absence rewires translation with important consequences in maturing neurons.

Lukas Schartel (Johannes Gutenberg-University Mainz (JGU), Germany)

Selective RNA pseudouridinylation using circular gRNA in designer organelles

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RNA modifications play a pivotal role in the regulation of RNA chemistry within cells. Several technologies have been developed with the goal of using RNA modifications to regulate cellular biochemistry selectively, but achieving selective and precise modifications remains a challenge. Using designer organelles, we can modify mRNA with pseudouridine in a highly selective and guide-RNA-dependent manner. These designer organelles are based on the principle of phase separation, a central tenet in developing artificial membraneless organelles in living mammalian cells. In addition, we used circular guide RNAs to markedly enhance the effectiveness of targeted pseudouridinylation. Our studies offer spatial engineering by means of optimized RNA editing organelles (OREO) as a complementary tool in using targeted RNA modification to expand potential avenues for future investigation.

Štěpánka Vaňáčová (Masaryk University, Brno, Czech Republic)

Protein-protein interactions reveal new functions for m^{6}A RNA demethylases

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Fat Mass Obesity Obesity (FTO) and ALKB homologue 5 (ALKBH5) are human proteins that posses demethylase activity towards N6-methyladenosine (m^{6}A) in RNA. FTO, in addition can demethylate m^{1}A in tRNAs and the N6-methyl group from m^{6}A$_m$ found in extended 5' caps of mRNAs and several other RNA Polymerase II noncoding transcripts. The high abundance of m^{6}A in eukaryotic mRNAs and the presence of an m^{6}A demethylase attracted much attention because of the predicted dynamic nature of the RNA modification. However, several follow-up studies have shown that the m6A pattern on mRNAs is rather stable once mRNAs leave chromatin. The role of FTO and ALKBH5 in mRNA and cellular metabolism remained to be elucidated. Multiple studies identified only smal numbers of mRNA targets for either demethylase in a type/disease specific manner. To date, it is unknown how FTO and ALKBH5 demethylate specific mRNAs in the large m^{6}A-modified transcriptome. We aimed to address this question by looking for transient and stable interactions of both proteins. ALKBH5 shows physical and genetic interactions with a number of pre-mRNA processing factors and interacts stably with the mRNA export machinery. In contrast, FTO's most prominent physical and genetic interactions are with DNA replication and repair processes. I will present our current results following up these findings for FTO and ALKBH5 and discuss possible models for their cellular mechanisms.

Olivier Duss (European Molecular Biology Laboratory, Heidelberg, Germany)

How processes cooperate with each other: Real-time tracking of m6A RNA modification and co-transcriptional rRNA processing

Anastasiia Chaban, Simone Höfler, Nusrat Shahin Qureshi, Olivier Duss

European Molecular Biology Laboratory, Heidelberg (Germany)

In the cell, various molecular processes are interconnected but it is challenging to dissect how they functionally cooperate. Our strategy is to reconstitute complex but active molecular systems and simultaneously track the dynamics of various multi-step processes in real-time using multi-color single-molecule approaches to provide a quantitative molecular understanding of the entire system.

In the first part, we will look into the dynamic process of how RNA is modified to m6A by the human Mettl3/14 enzyme and then dynamically recognized by the YTHDC1 reader protein. Simultaneously detecting specific Mettl3/14 and YTHDC1 binding to the same target RNA molecule and at the same time following RNA folding, we see that upon modification, the transient and mostly unproductive Mettl3/14 binding events are replaced by a dynamic YTHDC1 binding pattern suggesting a dynamic m6A-mediated interactome switch. Our data also suggests that modification and reading can occur on structured RNAs requiring only local structural rearrangements.

In the second part, I will discuss how transcription of bacterial rRNA, its co-transcriptional folding and initial processing by RNase III are functionally coupled. First, we have tracked and can provide a model of how the complete rRNA transcription antitermination complex (rrnTAC) is assembled onto an active transcription elongation complex. Then, we focus on the role of the rrnTAC in chaperoning the co-transcriptional formation of the long-range helix separating the 16S rRNA precursor (~1600 nt) from the single precursor transcript (~4500 nt) as the first co-transcriptional rRNA processing step. By directly tracking rrnTAC assembly and co-transcriptional RNase III cleavage in real-time, we show how the presence of the completely assembled rrnTAC facilitates RNase III cleavage by bringing 5'- and 3'-end of the rRNA spatially close, thereby chaperoning the long-range RNase III RNA helix substrate. This is the first direct experimental evidence of coupling between rRNA transcription and processing in bacterial ribosome assembly, mediated by long-range rRNA looping. Our data suggest that co-transcriptional RNA looping may provide a more general mechanism for the coordination of co-transcriptional processes as we recently showed for bacterial transcription-translation coupling (Qureshi & Duss, bioRxiv, 2023) or was proposed for co-transcriptional splicing (Zhang & Cramer, Science, 2021).

Marie-Luise Winz (Johannes Gutenberg-University Mainz (JGU), Germany)

Yeast elongation factor homolog New1 protects specific mRNAs from no-go decay, probably modulating ribosomal RNA modification

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New1 is a homolog of fungal translation elongation factor 3 (eEF3) in Saccharomyces cerevisiae. Its absence has previously been described to lead to cold sensitivity, ribosome biogenesis defects, and ribosomal queues upstream of Stop codons on selected mRNAs encoding C-terminal Lysine or Arginine. Here, we find that lack of New1 induces no-go decay (NGD) on specific mRNAs, leading, as a consequence, to decreased mRNA and protein levels of such genes. Our data show that the effect does not depend on the C-terminally encoded amino acid, but on the codon identity at this position. In total, more than 10% of yeast coding sequences end with the affected codons. We have confirmed NGD and subsequent downregulation at the protein level for the metabolic enzymes Pgk1, Gpm1, and Adh1, translation elongation factors EF-1 alpha and EF-1 beta (gamma-subunit), ribosomal protein Rps5, as well as ribosome biogenesis factor Nop1 and adenosine kinase Ado1. While Nop1 is part of the rRNA processome, and is crucial for 2'-O-methylation of pre-ribosomal RNA, Ado1 is required for the utilization of S-adenosylmethionine (SAM), suggesting consequences on RNA methylation. Indeed, in our preliminary data, rRNA 2'-O-methylation is significantly reduced in the absence of New1. These findings may explain the reported ribosome biogenesis defect. We speculate that regulation of New1 level and/or its RNA binding, e.g., under different stress conditions can fine-tune the yeast transcriptome, epitranscriptome, and ultimately proteome, by modulating NGD for a subset of highly important mRNAs

Andres Jäschke (Heidelberg University, Institute of Pharmacy and Molecular Biotechnology, Heidelberg, Germany)

NON-CANONICAL RNA CAPS – NEW CHEMISTRY, NEW FUNCTIONS

Andres Jäschke

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RNA capping has long been regarded as a hallmark of eukaryotic and viral gene expression. However, over the past decade, numerous novel non-canonical caps have been discovered across all domains of life. The most extensively characterized non-canonical RNA cap to date is the ubiquitous redox cofactor NAD, with detailed biochemical insights into its biosynthesis and degradation pathways, as well as a limited understanding of its function. Other non-canonical caps have been identified via mass spectrometry, but for most of them, the specific RNA sequences they modify remain elusive due to the lack of methods for selective enrichment and sequencing.
In this presentation, I will present our work on unraveling biological roles of NAD-caps, leading to the discovery of new bioconjugation strategies employed during viral takeover of the host's genetic machinery, and to a novel decapping mechanism in thermophilic archaea. I will furthermore share how our efforts to identify RNAs capped with the aminosugar nucleotide UDP-GlcNAc led to the unexpected discovery of a GlcNAc-derived modification at an internal position of a prokaryotic tRNA.

Abstracts - Poster
(in alphabetical order)

1 Alper Akay (University of East Anglia, Norwich, United Kingdom)

Alternative splicing regulation by U6 snRNA m6A modification

Tim Pearson; Aykut Shen; John D. Williams; Katarzyna Hencel; Alper Akay

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Pre-mRNA splicing is essential for the accurate expression of genes in many organisms. Cis-splicing of RNA removes intronic sequences and joins exons in coding and non-coding RNAs. In addition to cis-splicing, many organisms, including nematodes, use spliced leader (SL) trans-splicing to replace the 5' ends of their pre-mRNAs with a non-coding SL sequence. An important biological question is how the spliceosome recognises the correct 5' and 3' splice sites across thousands of sequences. In cis-splicing, 5' splice sites are recognised first by U1 snRNA and then by U5 and U6 snRNAs, whereas the U2AF proteins recognise 3' splice sites together with the branch site recognition by SF1 and U2 snRNA. SL trans-splicing requires the same snRNAs except for U1.

All U snRNAs carry multiple RNA modifications across species, but the biological roles of these modifications still need to be fully understood. Mutations of the U6 snRNA m6A methyltransferase METT-10 / METTL16 cause developmental defects, sterility and germ cell arrest (1–3). We recently showed that the absence of U6 snRNA m6A methylation in mett-10 mutant animals causes global cis- and trans-splicing defects in C. elegans and cis-splicing defects in human cells (4). U6 snRNA m6A modification is required to accurately and efficiently recognise 5' splice sites (SS) with adenosine at +4 position (+4A). This 5' SS +4A recognition mechanism by the U6 snRNA m6A is conserved between yeast (5), plants (6), nematodes and humans (4). In addition to alternative 5' SS usage, the absence of U6 snRNA m6A modification leads to alternative splicing of weak 3' SSs and trans-splice sites (4).

The non-random distribution of alternative splice sites in the absence of U6 snRNA m6A modification and the preservation of open reading frames in a large number of alternatively spliced transcripts raises the possibility that U6 snRNA m6A modification could be involved in alternative splicing and a regulated process. We will discuss intriguing examples of splice site usage dependent on the U6 snRNA m6A modification status.

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2 Nicolò Alagna (Institute of Human Genetics, University Medical Center Mainz, Germany)

ModiDeC: a multi-RNA modification classifier for direct nanopore sequencing

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RNA chemical modifications play a key role in altering the structure, stability and interactions of RNA with proteins, thus influencing various stages of the RNA life cycle. These modifications influence cellular processes such as gene transcription, RNA processing, and localization, which are essential for protein production and cell functionality. Chemical modifications commonly occur due to external stress stimuli, such as DNA damage or oxidative stress, and can trigger rapid degradation of RNA nucleotides, leading to changes in the cell's proteome and gene transcription. This can lead to an abnormal accumulation of RNA modifications, causing cellular malfunctions and diseases.

To date, over 200+ distinct chemical modifications have been identified, highlighting the complexity of the epitranscriptome. Notable examples include N6-methyladenosine (m6A), inosine (I), pseudouridine (Ψ), 2'-O-methylguanosine (Gm), and N1-methyladenosine (m1A), which are associated with eukaryotic cell growth, development, and human diseases. Various methods, such as MeRIP-Seq, m6ACE-Seq, Pseudo-seq, miCLIP, and GLORI, have been developed to explore these modifications using next-generation sequencing (NGS) technologies. However, short-read sequencing often falls short in capturing the full diversity of RNA modifications.

Direct RNA Sequencing (DRS) by Oxford Nanopore Technology (ONT) has revolutionized the field, enabling the identification of individual RNA modifications at single-nucleotide resolution in long reads by comparing expected and detected nucleotide current signal that cross the pore. This has led to the development of methods for both comparative and de novo detection of RNA modifications. Comparative methods like xPore and DRUMMER show promising performance but have limited applications. De novo methods such as m6Anet, nanom6A, DENA, mAFiA, and Penguin leverage deep neural networks trained on labelled datasets to achieve single-base resolution for specific RNA modifications.

Despite these advancements, analyzing multiple types of RNA modifications at the same time using DRS remains challenging. To address this, we have developed ModiDec, a deep neural network trained on a hybrid dataset comprising in vitro epitranscriptome and synthetic sequences. ModiDec can identify five different modifications (m6A, I, Ψ, Gm and m1A) across

40+ sequence motifs at single-base resolution, quantifying modifications in long reads. ModiDec was able to detect and classify the mentioned modifications at the single nucleotide level and quantify both the amount of modification types in the dataset and the frequency of occurrence of each modification. We also validated ModiDec's performance in several scenarios, including biological datasets obtained by direct RNA nanopore sequencing.

3 **Michael Amponsah-Offeh** (Medical Faculty Mannheim of Heidelberg University, Germany)

Primary microRNA editing controls Drosha processing

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[2] National and Kapodistrian University of Athens School of Medicine, Greece
[3] Newcastle University, United Kingdom

Introduction:

Immune cell trafficking is a crucial part of the body's response to tissue damage, but the role of inherent RNA nucleotide changes in this process is still unclear. Adenosine-to-inosine (A-to-I) RNA editing is an epitranscriptional regulator of RNA metabolism, catalyzed by the adenosine deaminases acting on RNA-1/-2 (ADAR1/2). RNA editing plays a key role in fine-tuning gene expression and maintaining cellular homeostasis by altering the coding potential of mRNAs, affecting splicing as well as influencing RNA stability and localization. Here, we examine how ADAR2 controls gene expression via microRNA biogenesis in vascular endothelial cells (ECs) and its biological relevance.

Methods:

Molecular biology and transcriptome-wide studies were performed in primary human vascular EC to assess EC cell responses to inflammatory stimuli. Deep RNA sequencing, target-specific microRNA (miRNA) editing studies, RNA-immunoprecipitation, miRNA/plasmid silencing/overexpression and luciferase reporter assay were performed to identify targets controlled by ADAR2 as well as their underlining mechanisms. The preclinical and translational value was confirmed in an experimental atherosclerosis model (double ADAR2 and ApoE knockout mice with Western diet intake for 20 weeks) and in 533 individuals at risk for or with established atherosclerotic cardiovascular disease.

Results:

ADAR2 is required for IL-6-signal transducer (IL6ST) expression, the co-receptor of IL-6, and thus, IL-6 trans-signaling, evidenced by STAT3-phosphorylation and downstream adhesion molecule expression. ADAR2-deficient transcriptome revealed a selective upregulation of a conserved group of miRNAs (such as miR-199a-5p and miR-335-3p) targeting the IL6ST mRNA. At a single-nucleotide level, ADAR2-induced RNA editing of the stem loops of the primary miR-199a1/2 and miR-335 directly disrupted the Drosha recruitment to both and inhibited their maturation process. Multilayered rescue studies using miR-inhibitors restored IL6ST levels in

ADAR2 depleted ECs. Unbiased transcriptome-wide analysis revealed ADAR2 controls hyper IL-6 (HIL-6) induced pan cytokine signalling in ECs. Moreover, silencing of ADAR2 in ECs reduced endothelial secretion of several HIL-6-induced pro-inflammatory cytokines and chemokines. Additionally, TNFα-induced innate immune responses were suppressed in ADAR2-deficient vascular ECs. ADAR2 depletion in mice reduced atherosclerotic plaque burden and decreased immune cell recruitment into atherosclerotic plaques. In humans, increased ADAR2 expression at baseline was prospectively associated with systemic inflammation, the extent of plaque buildup in the walls of the arteries and accelerated progression of peripheral arterial atherosclerosis.

Conclusion:

Inhibition of the microRNA maturation process by ADAR2-mediated RNA editing represents a novel layer of vascular RNA metabolism acting as a checkpoint of the vascular endothelial cell immunomodulatory capacity.

4 Elli-Anna Balta (Department of Neuroanatomy, Institute of Anatomy and Cell Biology, Faculty of Medicine, University of Freiburg, Freiburg, Germany)

rTMS stimulation induces changes in m6A-modified synaptic transcripts

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An estimated 3.8% of the global population experience depression, with 30% of these cases meeting the criteria for treatment-resistant depression. Repetitive transcranial magnetic stimulation (rTMS) is increasingly used to treat this condition. Despite its clinical success, the molecular mechanisms of rTMS-induced plasticity are not fully understood. N6-methyladenosine (m6A) modification, the most abundant RNA modification within the human transcriptome, is a reversible regulator of gene expression and plays a crucial role in synaptic plasticity. Using m6A-arrays, meRIP-seq and directRNA-seq post-rTMS, we found that the most differentially methylated transcripts were hyper-methylated and linked to synaptic plasticity. Single-cell recordings, immunostainings and electron microscopy confirmed changes in excitatory neurotransmission highlighting the important role of m6A mRNA modification in rTMS-induced synaptic plasticity.

5 Dan Bar Yacoov (Ben-Gurion University of the Negev, Israel)

A-to-I mRNA editing in bacteria can control disulfide bond formation to induce bacterial death

Dan Bar Yaacov

Ben-Gurion University of the Negev, Israel

Adenosine-to-inosine (A-to-I) mRNA editing and protein disulfide bonds can each, independently, affect the structure and function of proteins. However, the ability of A-to-I mRNA editing to control disulfide bond formation was never shown.

Here, we show that A-to-I mRNA editing constitutes a novel mechanism to control disulfide bond formation, which can affect bacterial viability and growth. We demonstrated that A-to-I mRNA editing recodes a tyrosine to a cysteine in the self-toxin HokB, increasing its toxicity in Escherichia coli. Subsequently, we show that edited HokB can induce bacterial death and early entrance to the stationary phase. We demonstrated that DNA-coded cysteines in HokB, and in vivo disulfide bond formation are essential to the toxicity of edited HokB. Finally, we show by structural modeling that editing-dependent disulfide bonds could stabilize HokB pores in the bacterial membrane.

Our work suggests that A-to-I mRNA editing can dictate disulfide bond formation across different proteins with functional consequences in bacteria.

6 Thomasz Bartosik (Lodz University of Technology, Poland)

Organocatalytic, diastereoselective synthesis of biologically relevant (R)- and (S)-methoxycarbonylhydroxymethyluridine (mchm5U)

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Lodz University of Technology, Poland

(R)- and (S)-5-methoxycarbonylhydroxymethyluridines (mchm5U) represent a very unique example of diastereomeric pair of modified tRNA nucleosides identified at the wobble position of mammalian tRNAArg(UCG) and tRNAGly(UUC), respectively.[1] (S)-5-Methoxycarbonylhydroxymethyluridine also occurs in tRNAsGly(UCC) from insects, worms and plants.[2] The ALKBH8 enzyme was found to play a bifunctional role in (S)-mchm5U biosynthesis, acting as a methyltransferase in the cm5U→mcm5U conversion and a oxygenase in stereoselective hydroxylation of mcm5U.[1] ALKBH8 deficiency caused by biallelic mutational events in the human ALKBH8 gene results in the absence of (R)- and (S)-5-methoxycarbonylhydroxymethyluridine in total tRNA (at the same level as in ALKBH8 knockout mice) and was associated with intellectual disabilities.[3] In addition, the silencing of ALKBH8 has been shown to induce apoptosis in human urothelial carcinomas and significantly suppressed the invasion, angiogenesis and growth of bladder cancer in vivo.[4]

The biological role of the wobble (R)-mchm5U and (S)-mchm5U in translation processes as well as the pathogenesis of human diseases remains elusive. Therefore, the efficient, reliable and stereoselective methods of their synthesis is needed to facilitate and support the biochemical, biological and medical studies.

This work presents a pioneering approach to the synthesis of (R)- and (S)-methoxycarbonylhydroxymethyluridine (mchm5U) using organocatalysis. The synthesis starts from chemically available 5-formyluridine which was protected at the 5'-hydroxyl group with tert-butyldimethylsilyl chloride.[5] Fully blocked formyluridine was subjected to organocatalytic cyanosilylation using trimethylsilyl cyanide and selected organocatalyst. Diastereoisomerically enreached cyanohydrins of 5-formyluridine were transformed into the corresponding Pinner salts via the Pinner reaction, which, upon hydrolysis and 18C RP HPLC separation furnished pure (R)- or (S)-methoxycarbonylhydroxymethyluridine (mchm5U).

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7 Maximilian Berg (Goethe University Frankfurt, Germany)

Impact of 5-fluorouracil on RNA modification in human cell culture

Maximilian Berg; Chengkang Li; Stefanie Kaiser

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RNA modifications play pivotal roles in various cellular processes and gained significant attention due to their involvement in diseases. Of particular interest is the connection between modifications and translation, where modifications within the anticodon loop of tRNA finely regulate protein synthesis. Environmental stressors, including chemical agents like arsenite and hypochlorite, can induce reprogramming of tRNA modifications. Drug treatment can be considered a chemical stress to humans but a comprehensive understanding of a drug's impact on epitranscriptome–proteome interactions remains elusive. Here, we present a detailed investigation into the effects of 5-fluorouracil (5-FU) on the epitranscriptome and its consequential alterations in tRNA modification patterns and codon-biased translation in HEK293T cells. Our study reveals significant alterations in the stoichiometry of 5-substituted uridine modifications upon 5-FU treatment, with exclusive effects observed on newly transcribed tRNA transcripts. Furthermore, our Northern Blot analysis identifies an upregulation of tRNALysUUU. These results in combination with shotgun proteomic analysis indicate that elevated uridine modification abundance may lead to codon-biased translation. Our results underscore the utility of nucleic acid isotope labeling coupled mass spectrometry (NAIL-MS) in elucidating the mechanisms of drug-induced tRNA modification reprogramming. We anticipate that elucidating the mechanistic basis of drug-induced tRNA modification re-programming will shed light on potential epitranscriptome-related adverse drug effects.

8 Pauline Böhnert (University Kassel, Institute of Biology, Germany)

Phosphorylation of Elp1 and its effect on Elongator function

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Transfer RNAs (tRNAs) undergo extensive modification at multiple positions. Carboxymethylation of uridines in the wobble position (U34) of anticodons is catalyzed by the protein complex Elongator. The complex consists of six different subunits, Elp1-Elp6, and is conserved from yeast to humans. Wobble uridine modifications are crucial and Elongator dysregulation is linked to neurodegenerative diseases and cancer.

In Saccharomyces cerevisiae, Elp1 has been identified as a tRNA binder and as a target for dynamic phosphorylation. This raises the question whether reversible phosphorylation of Elp1 might serve as a regulatory mechanism to coordinate tRNA binding and U34 modification output of Elongator.

Here we investigate the role of confirmed phosphorylation sites clustered close to the tRNA binding region of Elp1 through amino acid substitutions that mimic or ablate phosphorylation. Our results show that phospho-mimetic substitutions at multiple phosphorylation sites decrease tRNA binding in vitro, whereas phospho-ablative substitutions do not. Interestingly, Elongator related phenotypes in vivo do not necessarily correlate with tRNA binding, suggesting a more complex mechanism behind Elongator regulation than anticipated.

9 Milena Bors (Lodz University of Technology, Lodz, Poland)

Novel Post-Synthetic Strategy Of Formyl Group Incorporation Into RNA Oligomers

Milena Bors; Agnieszka Dziergowska; Grażyna Leszczyńska

Lodz University of Technology, Lodz, Poland

5-Formylcytidine (f5C) occurs in cellular RNA nucleic acids, where it acts as either static or dynamic modification. A stable and static f5C was discovered at the wobble position of the anticodon loop of mammalian mitochondrial mt-tRNAMet, including human (hmt-tRNAMet).[1] The modification affects the architecture of the anticodon loop enabling tRNAMet to decode both AUG and AUA codons during translation. The absence of f5C in tRNAMet impairs translation and respiratory function of mitochondria.[2] In mammalian mRNA, f5C participates in the oxidative demethylation cycle of 5-methylcytidine (m5C), alongside 5-hydroxymethylcytidine (hm5C) and 5-carboxylcytidine (ca5C).[3] Although the exact biological role of f5C in RNA is still unclear, its dynamic nature suggests potential regulatory functions. Therefore, developing new synthetic methods for f5C-modified oligonucleotides is essential to investigate their biological significance.

Incorporating 5-formylcytidine into RNA using standard solid-phase phosphoramidite synthesis is challenging due to the susceptibility of the formyl group to acidic conditions, oxidizers, and ammonia/amine treatments. These conditions can lead to N-glycosidic bond cleavage, formyl oxidation, and imine formation, respectively. Since commonly used protecting groups for the formyl group are incompatible with phosphoramidite chemistry, only the cyclic g-acetal group has been successfully used in the synthesis of f5C-RNA so far. [4, 5] A convenient alternative to the standard phosphoramidite method is a post-synthetic strategy, which involves the oxidation of a precursor RNA containing 5-(1,2-diacetoxyethyl)cytidine with $NaIO_4$. [6]

Herein, we present a novel synthon for the post-synthetic modification of RNA into 5-formyl pyrimidine-containing RNAs (f5U/ f5C -RNA) within a 5'-O-DMTr-2'-O-TBDMS protection system. This method uses 5-cyanohydrin moiety, which can be converted into a formyl group under mildly acidic conditions. This strategy encompasses the synthesis of 5-cyanohydrin-containing pyrimidine nucleosides [7] as well as the formation of formyl-modified RNA oligomers.

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Funding: This research was funded by the National Science Centre (NCN) OPUS-22: UMO-2021/43/B/ST4/01570."

10 Julia Brechtel (Institute of Pharmaceutical and Biomedical Sciences, Johannes Gutenberg-University Mainz, Germany)

Interactions of mitochondrial mRNA modifications and ribosome associated-quality control mechanisms – a novel approach to regulate mitochondrial function

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Mitochondria are often referred to as the powerhouse of the cell. In humans, over 1500 proteins are needed to maintain mitochondrial function. However, only 13 of the mitochondrially required proteins are encoded in the circular mitogenome (mtDNA), including many subunits of the respiratory chain complexes. All other proteins are determined externally by deoxyribonucleic acid (DNA) in the nucleus and are imported into mitochondria through the translocase of the outer mitochondrial membrane (TOMM) and the inner mitochondrial membrane (TIMM). On the one hand, the mRNA could be translated on the ribosomes of the cytoplasm and the precursor protein then imported post-translationally, on the other hand, up to a third of the mitochondrial proteins can be transported co-translationally. In this case, the synthesis synchronizes with the import at the mitochondrial outer membrane (MOM). Due to the direct localization to the mitochondrion, translation may be susceptible to changes within the organelle, such as reduced mitochondrial membrane potential (ΔΨm). Recently, our group identified a new mitochondrial mechanism that regulates the function of complex I of the respiratory chain and the mitochondrial membrane potential. Hereby, the translation of the mitochondrially encoded subunit NADH dehydrogenase subunit 5 (ND5) is significantly reduced by the mRNA modification N1-methyladenosine (m1A). Via incorporation of a methyl group in adenosine, the writer enzyme tRNA methyltransferase 10 homolog C (TRMT10C) interferes with Watson-Crick base pairing of mRNA and tRNA. Since the production of the ND5 subunit is correspondingly reduced, we aim to investigate the effect of m1A on other subunits of the respiratory chain in an tetracycline-inducible human embryonic kidney cell (HEK) model. Especially those that were transported co-translationally could be disturbed by the altered membrane potential. Together with other metastable proteins, the non-imported subunits can aggregate. If cellular stress persists, the body's ribosome associated-control mechanism (RQC) can no longer eliminate it, leading to apoptosis.

Therefore, we will first use Western blotting to detect changes in the protein levels of complex I subunits such as NDUFS1, NDUFS3 and NDUFB8. Additionally, we will investigate subunits of other complexes, including SDHA of complex II, COXIV of complex IV and ATP5A of complex V. Based on these results, we will then analyze possible changes at the RNA level using qPCR. This novel approach may provide insights into potential therapeutic targets for preventing or mitigating the progression of Alzheimer's disease.

11 Tamer Butto (Johannes Gutenberg University Mainz (JGU)), Germany

NanoRibolyzer unravels the rRNA Biogenesis Pathway using Nanopore Sequencing

Stefan Pastore[1]; Lioba Lehmann[2]; Ludivine Wacheul[3]; Beat Lutz[4]; Mark Helm[1]; Susanne Gerber[2]; Denis Lafontaine[3]; Tamer Butto[1]

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The ribosomal biogenesis pathway is a pivotal cellular process crucial for the precise regulation of ribosome functionality and the production of efficient ribosomal machinery. While the biological synthesis of ribosomal RNA has been a subject of study for decades, a gap in information persisted due to the absence of long-read and direct RNA sequencing results. Here, we present NanoRibolyzer, a novel tool specifically designed to facilitate the investigation of ribosomal biogenesis intermediates arising within the intricate ribosomal biogenesis pathway using ONT long-read Nanopore sequencing. NanoRibolyzer allows a quantification of known rRNA fragments and performs a similarity-based clustering of fragments to facilitate the investigation of novel non-canonical fragments. Additionally, the tool extracts polyA-tail lengths and aligned raw signal on a single molecule level and associates it via read ID to computed clusters. The interplay of cluster analysis and processing properties offers new insights on the ribosomal biogenesis pathway. In combination with different experimental extraction techniques, NanoRibolyzer allows a spatio-temporal analysis of the ribosomal biogenesis pathway including processing-steps and detection of distinct RNA modification in different physiological conditions. Ultimately, NanoRibolyzer allows an interactive exploration of rRNA on a multi-dimensional level, fostering a comprehensive understanding of its formation and potential regulation.

12 Adrian Chan (University Hospital Heidelberg, Germany)

psi-co-mAFiA - Concurrent Single-Molecule Detection of m6A and pseudouridine with direct-RNA Sequencing

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We use Oxford Nanopore Sequencing to simultaneously detect m6A and pseudouridine at single-base resolution in single RNA molecules. The method is validated by comparing its stoichiometric prediction across different sites on the human transcriptome with values previously reported by chemical assays, including GLORI (m6A), BID-Seq (psi), and PRAISE (psi). The orthogonal comparisons show excellent quantitative agreement, with correlation reaching above 90% across the overlapping sites. We then apply the method to HEK293 cell lines with TRUB1 overexpression or knock-down condition. Altering the expression of this psi writer specifically raises or lowers pseudouridation levels in the sequence motif GUUCN, leaving other locations largely unperturbed. Our method is able to quantify such subtle differences. Moreover, we observe localized effects on m6A deposition due to changes in a near-by psi site. While an exact mechanistic explanation is yet to be found, the technology opens up the exciting prospect of studying the intricate interaction among multiple RNA modifications.

13 Hsuan-Ai Chen (University of Würzburg, Germany)

Structure and catalytic activity of alkyltransferase ribozymes

Hsuan-Ai Chen; Claudia Höbartner

University of Würzburg, Germany

The ability of RNA folding into complex 3D structures renders them versatile functions in nature as well as in the laboratory. Nature utilizes structured RNAs for specific cellular tasks. Two well-investigated examples are riboswitches and ribozymes. While riboswitches sense the abundance of cognate metabolites and regulate gene expression accordingly, natural ribozymes heavily engage in nucleolytic reactions e.g. splicing or cleavage. Artificial ribozymes have been developed through in vitro selection and have expanded their catalytic spectra covering diverse chemical reactions, among which alkyltransferase ribozymes are of special interest due to their high efficiency and selectivity to install RNA modifications, as recently shown for the first methyltransferase ribozyme MTR1.[1] Ribozymes are easily accessible as biotechnological tools, for example, for site-specific labeling of RNA.[2] Moreover, ribozymes are speculated players in bridging the gap between the putative primordial RNA world and the modern protein-based biology.

Structural investigation of natural ribozymes and riboswitches has revealed important insights into the catalytic mechanisms as well as the ligand specificities. In contrast, only limited structures of artificial ribozymes have been reported. We report the 3D structure of a novel RNA-alkylating ribozyme to a resolution of 2.8 Å. The crystal structure represents the post-catalytic state, harboring the reacted cofactor in the catalytic core. The overall structure adopts an architecture of 4 parallel layers, where the cofactor and the reaction site were drawn into proximity. The core structure is stabilized by the continuous p-stacking and the hydrated magnesium ions. According to the recognition pattern, different cofactor analogues were synthesized to evaluate the impact of each functional groups, which, combing with the kinetic assessment and the in-line probing results, allows us to optimize the cofactor structure and hence improve the cofactor stability. Furthermore, the comparison between the alkylation ribozyme and natural riboswitches provides insights into the strategies used by natural and artificial RNAs for non-covalent ligand binding in order to avoid undesired reactions, or for active recruitment as a cofactor to yield modified RNA.

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14 Georgia Ciliberti (Medical Faculty Mannheim of Heidelberg University, Mannheim, Germany)

ADAR1-mediated adenosine-to-inosine RNA editing links dsRNA metabolism links with MDA5 dependent innate immunity

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Long double-stranded RNAs (dsRNAs) are recognised as danger-associated molecular patterns by the cytosolic RNA innate immune sensors inducing thus innate immune responses. RNA sensing by the RNA innate immune sensors is determined by the enzyme adenosine deaminase acting on RNA-1 (ADAR1) which catalyses the deamination of adenosine residues in double-stranded RNA molecules, a process called adenosine-to-inosine RNA editing. We have recently reported that ADAR1 is the main RNA editor in endothelial cells (ECs) but the role of ADAR1 in dsRNA metabolism.

Genetic deletion strategies were employed by crossing mice carrying a conditional (floxed) Adar1, Ifh1 or Tlr3 allele with a tamoxifen-inducible VE-Cadherin-CreERT2 mouse line. Primary human and murine vascular endothelial cell culture assays, dsRNA metabolism studies, gene-silencing and expression techniques, immunohistochemistry and confocal microscopy were used to assess the ADAR1-MDA5 axis specific effects.

Inducible endothelial ADAR1 ablation in adult mice triggered a sudden premature death within 8-12 days after the first tamoxifen injection. Post-mortem autopsy and histopathology examination revealed the presence of large exudate pleural effusions compressing the lungs as well as the formation of pulmonary oedema and extravasation of albumin in lung parenchyma, reflecting a compromised EC barrier function. Mechanistically, silencing of endothelial ADAR1 disrupted long-to-short dsRNA metabolism witnessed by the striking accumulation of cytoplasmic long dsRNAs, inducing the activation of the RNA innate immune sensor MDA5. Subsequently, the cytoplasmic accumulation of endogenous long dsRNAs induced aberrant levels of interferon-β and activation of innate immune system. Induction of MDA5 signalling resulted into the dissociation of β-catenin from VE-cadherin at EC junctions, while co-ablation of ADAR1 and the MDA5 in mice was sufficient to restore the integrity of the ADAR1-deficient endothelium and fully rescue the premature lethality. Co-ablation of ADAR1 and another dsRNA sensor, TLR3, increased the lifespan of the mice only for few days failing to rescue the lethal phenotype of endothelial ADAR1-deficient mice, indicating that MDA5 is the main dsRNA sensor in ECs. In human ECs, silencing of endothelial ADAR1 led to a striking

accumulation of cytoplasmic long dsRNAs and loss of junctional β-catenin and VE-cadherin, while co-silencing of ADAR1 and MDA5 restored endothelial integrity and activation of innate immune system.

Endogenous suppression of double-stranded RNA-induced MDA5 autoinflammatory signaling by the RNA editor ADAR1 is essential for the maintenance of vascular integrity in adult mice."

15 **Jan Felix Dalwigk** (Goethe University Frankfurt am Main, Germany)

Investigation of the effects of small molecular therapeutics on the human epitranscriptome

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Protein expression can be regulated in a myriad of different ways, one of which is mediated by tRNA modifications that fine-tune translation through codon-anticodon interactions. Changes in tRNA modifications can be induced by cell stress and modulate translation of codon biased transcripts. Among the most relevant chemical stressors are small molecular drugs but whether and how they affect the human epitranscriptome is understudied. Therefore, we investigate how small molecular therapeutics affect tRNA modifications using nucleoside mass spectrometry and map proteomic changes induced by drug treatment using shotgun proteomics. To connect changes on both levels of gene expression we subject the up- and downregulated proteins to co-don bias analysis, revealing potentially codon biased regulation. Part of this study focuses on compounds that are known to interact with RNA modifying machinery. In this effort we investigate how incorporation of 6-thioguanine in absence and presence of queuine exerts an effect on the expression of codon biased transcripts differentially decoded under queuine incorporation. However, we also study therapeutics not known to affect tRNA modifications at all. Doing so we have uncovered an increase in 3-methylcytidine modification in response to ibuprofen treatment of a HEK293T cell model and are working to unravel the mechanism underlying it.

16 Jasmin A. Dehnen (Institute of Molecular Biology (IMB), Mainz, Germany)

FIBO-seq for accurate f5C mapping in RNA

A two-dimensional method to map 5-formylcytosine indicates that f5C is absent in mRNA.

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5-formylcytosine (f5C) is an RNA modification in the anticodon of mitochondrial tRNA Methionine (mt-tRNAMet) and is produced by ALKBH1 from 5-methylcytosine (m5C). However, beyond tRNA, the distribution and function of 5fC in the mammalian transcriptome remains obscure. We employed a previously reported 5fC mapping technique, pyridine-borane-assisted sequencing (PB-seq) (Wang et al., 2022, ACS Chem. Biol.) to map 5fC in mRNA. Unexpectedly, we found that most PB-seq hits were artefacts, either resulting from the reaction of pyridine borane with ac4C or with unmodified cytosines in a CTCCA RNA sequence context. To allow for accurate detection of f5C sites, we developed a modified method, FIBO-seq, combining immunocapturing and PB-seq. Using this approach, we found no evidence for f5C in mRNA. Instead, ALKBH1-dependent f5C was only identified in the anticodon of mt-tRNAMet. The results suggest that unlike in DNA, in mRNA f5C is largely absent.

17 Kimberly Diaz Perez (University of Michigan, Ann Arbor, USA)

Sequence Composition Mediates Pausing of Elongating RNA Polymerase II

Kimberly Diaz Perez; Alan Bruzel; Vivian Cheung

University of Michigan, Ann Arbor, USA

RNA Polymerase II (RNAPII) is essential for transcribing DNA into RNA, a process tightly regulated to ensure proper gene expression. RNAPII does not synthesize RNA continuously, rather it pauses during transcription. While pausing during transcription initiation is well-studied, pausing of the RNAPII complex during elongation is less characterized. Here, we show that RNAPII pauses during transcription elongation, and identify sequence factors that mediate these pauses.

Using precision run-on sequencing in B-cells from 11 individuals, we mapped actively transcribing RNA Polymerases at single-nucleotide resolution. In the same cells, cDNA sequencing was performed to measure gene expression levels. We found that RNAPII pauses during transcription elongation. The positions of these pauses are remarkably consistent across individuals, indicating that the pausing of elongating RNAPII is highly regulated.

Our results reveal that RNAPII pauses during transcription elongation of a third of the genes in B-cells (35%), leading to lower expression levels of these genes ($P << 10E-10$). RNAPII pauses significantly more in exons than introns ($P < 10E-50$). By comparing pausing indices, we found that RNAPII pauses 2.5-fold more in exons than introns. Notably, 64% (1,776 out of 2,785) of exonic RNAPII pauses occurred within the first exon, with 3.7-fold more RNAPII pausing in the first exons than other exons.

Since the NELF and DSIF protein complexes that mediate RNAPII pausing during initiation are not found in gene bodies, we investigated whether the sequence composition might mediate elongating RNAPII pausing. We identified a sequence motif enriched around RNAPII pause sites ($P < 10E-72$), characterized by high GC content (~15% over background), high G-skew, and a cytosine before the pause site.

Our analysis found that GC content significantly correlates with the extent of RNAPII pausing ($R2=0.8$). Exons where RNAPII pauses more frequently are more GC-rich ($P < 10E-34$), with the first exon showing higher GC content (~67%) than subsequent exons (~53%). To assess how the sequence composition affects pausing, we measured their stability. The GC-rich regions upstream of the pauses have significantly higher melting temperatures than regions where RNAPII does not pause ($P << 10E-10$). Additionally, the sequences with cytosines before the pause have significantly higher melting temperatures than sequences without those cytosines ($P < 10E-06$).

In this presentation, I will describe the pattern of RNA Polymerase II pausing during transcription elongation and demonstrate how sequence composition critically regulates RNA synthesis and gene expression.

18 Laurence Ettwiller (Ipswich, MA, USA (New England Biolabs))

A Metagenomic Genome-Phenome Association (MetaGPA) Study Reveals the 2,6 Aminopurine (dZ) Biosynthetic Pathway in Unculturable Phage Metagenomes

Laurence Ettwiller; Shuang-Yong Xu; Weiwei Yang

Ipswich, MA, USA (New England Biolabs)

The study of phage-bacteria interactions has recently gained interest due to their striking resemblance to eukaryotic systems, their potential for antimicrobial therapy, and their capacity to serve as sources of novel enzymes with considerable biotechnological applications. One common strategy phages use to evade host restriction is the complete modification of their genomes. Identifying the various types of DNA modifications carried by phages and understanding how these modifications are synthesized and maintained within genomes hold great potential for applications, particularly in the modification of DNA and RNA molecules.

In this study, we applied our previously established metagenomics genome-phenome association (metaGPA) framework to analyze native viromes containing 2,6 aminopurine (dZ) from environmental samples. dZ is an interesting nucleobase analog of adenine that forms three hydrogen bonds with thymine when incorporated into double-stranded DNA, thereby altering the physical properties of nucleic acid molecules, such as melting temperature and helix conformation. MetaGPA enabled the identification of numerous novel dZ-containing phage genomes directly on native viromes without the need of isolation. This approach not only allowed us to recapitulate the known phage biosynthetic pathway for dZ but also to uncover novel components, thereby expanding our understanding of phage biology and enhancing the potential for innovative biotechnological applications.

19 Adriano Fonzino (Università di Bari "Aldo Moro", Bari, Italy)

NanoSpeech: transforming native RNA squiggles for direct inosine detection

Adriano Fonzino; Bruno Fosso; Grazia Visci; Carmela Gissi; Graziano Pesole; Ernesto Picardi

Università di Bari "Aldo Moro", Bari, Italy

Epitranscriptome modifications are now emerging as important factors in fine-tuning gene expression and regulation. Among the over 170 known RNA chemical modifications, A-to-I RNA editing by ADAR enzymes plays crucial biological roles and has been linked to several human diseases. Detecting A-to-I editing, distinguishing real events from sequencing noise or artifacts, remains still challenging. Oxford Nanopore Technologies (ONT) allow the direct sequencing of native RNAs, offering the unique opportunity to map RNA modifications. Several computational tools based on ionic current perturbations or alignment glitches have been developed to profile ribonucleotide modifications and detect inosines in direct RNA sequencing (dRNA) experiments. These tools involve a preliminary base calling step by programs such as Guppy that cannot directly identify modified bases, leading to fragmented results and hampering the inosinome profiling at the transcriptomic and read level. To fill this gap, we have developed NanoSpeech, the first “inosine-aware” base caller to identify inosines in native RNA sequences using raw currents measurements. Harnessing dRNA assays from different organisms and in-vitro transcribed synthetic constructs in which inosine locations were a priori known, we have collected millions of raw electric signals and their corresponding k-mers. Next, a transformer model with an expanded dictionary (A, C, G, U, I) was trained to detect both canonical ribonucleotides and inosines, employing electric measurements only. Our results on an independent set of transcripts from synthetic constructs with or without inosines indicate that NanoSpeech can efficiently identify inosines with a meager amount of false positives, although further optimizations and specific tests on real datasets are needed. Our tool is unique in detecting inosines during the base calling, providing a punctual map of A-to-I RNA editing at the single-molecule level. Interestingly, direct deciphering of inosines remarkably improved the mappability of I-rich synthetic transcripts compared to state-of-the-art programs able to exclusively recognize canonical bases.

20 Ariane F. Frey (Institute of Pharmaceutical and Medicinal Sciences, Johannes Gutenberg University Mainz, Mainz, Germany)

Nanomole Scale Libraries for Medicinal Chemistry of RNA Methyltransferases

Ariane F. Frey, Valerie Kadenbach, Annabelle C. Weldert, Laurenz Meidner, Robert Zimmermann, Mark Sabin, Zarina Nidoieva, Sabrina N. Hoba, Tanja Schirmeister, Fabian Barthels

Institute of Pharmaceutical and Medicinal Sciences, Johannes Gutenberg University Mainz, Mainz, Germany

The speed of drug development is increasing, driven by the need for new inhibitors and modulators of medicinal-relevant enzymes. Nanomole-scale chemistry coupled with high-throughput screening enables the fast discovery of unexpected ligand chemotypes and the development of novel inhibitors that may not be anticipated through rational drug design strategies.

This strategy could be employed for drug discovery on RNA-modifying enzymes. Dysregulation of RNA modifications has been linked to various disease phenotypes while RNA-modifying enzyme inhibitors have recently emerged as a cutting-edge topic in drug discovery, including the first-in-class clinically investigated drug STC-15 targeting METTL3/14.[1–4] Our focus includes the development of fluorescent probes for high-throughput microscale thermophoresis (MST) and Fluorescence Polarization (FP)-displacement assays and the identification of new selective inhibitors for RNA-methyltransferases.

Yet, a few drug development campaigns have focused on the 5-methylcytosine (m5C)-modification, introduced by the DNA methyltransferase 2 (DNMT2).[5] A high-throughput screening with DNA-encoded library libraries (DEL) resulted in the identification of novel selective allosteric-site binders and inhibitors with a low-micromolar affinity for the DNMT2 enzyme. The crystallographic structure shows that the DEL ligand occupies a cryptic allosteric pocket and inhibition is explained by the reorganization of an active site loop, and thus indirectly influences the function of DNMT2.

Furthermore, a combinatorial alkyne/azide nanoSAR approach could be harnessed for the development of selective fluorescent probes for in vitro characterization of methyltransferase ligand binding. The MST- and FP-assays enable the opportunity for fast and easy discrimination between non-binders and binders and the determination of ligand binding affinities.[6] This approach could be used for a high-throughput drug screening campaign which led to the identification of the first-in-class inhibitor for METTL1, an m7G RNA-methyltransferase that is found to play a crucial role in the oncogenesis of various types of cancer.[6–8]

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21 Pablo Gonzalez Jabalera (Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Germany)

Identification of FAD-capped RNAs: the FAD captureSeq protocol

Pablo Gonzalez Jabalera; Christina Julius; Andres Jäschke

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RNA presents a vast functional diversity, ranging from regulation to even cellular defense. This fact evidences that some of these roles rely only on the molecule's sequence and structure, but also on RNA modifications, which are now considered to be important regulators of RNA function and metabolism. However, of the many RNA modifications known to date, few are found at the 5' terminus of RNA, with the canonical eukaryotic m7G as the paradigmatic example. In the search for new RNA modifications, the coenzyme NAD was first discovered as a new RNA conjugate in bacteria. Although this was originally regarded as prokaryotic-specific, noncanonical NAD capping of RNA was also found in several eukaryotes, such as yeast, plant, and human cells. Apart from NAD, other nucleotide analogs, such as the riboflavin-derived coenzyme FAD were also found to cap RNA. Even though FAD-capped RNAs have already been detected and quantified in vivo, the identification of the specific RNAs carrying a FAD-cap still needs new methodologies that can selectively isolate these molecules. Here, several affinity-based enrichment strategies are proposed to develop a new FAD CaptureSeq protocol which will enable the study of RNAs bearing the FAD-cap. The study of new noncanonical caps has helped to unveil a new paradigm in epitranscriptomics, stimulating future efforts to investigate new regulatory processes, which may find a new link between cellular metabolism and gene expression.

22 Katharina Görlitz (University Kassel, Institute for Biology, Germany)

Different tRNA modifications inhibit fluoropyrimidine induced tRNA decay in yeast

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The pyrimidine analogs 5-fluorouracil (5-FU) and 5-fluorocytosine (5-FC) are respectively used in cancer therapy and for treatment of severe fungal infections. One mechanism of action for both drugs is the incorporation of 5-fluorouridine triphosphate (FUTP) into RNA of targets cells. In the yeast Saccharomyces cerevisiae, defects in various tRNA modification genes lead to enhanced sensitivity to fluoropyrimidines at elevated temperature. Previous works showed a rapid destabilization of tRNAValAAC in the double mutant trm4 trm8, lacking 5-methylcytosine and 7-methylguanosine, when cells were exposed to elevated temperature. We demonstrate that tRNAValAAC decay in trm4 trm8 also occurs upon exposure to fluoropyrimidines. In the single mutant trm8, tRNAValAAC is already degraded after fluoropyrimidine stress paired with heat stress. This decay is mediated by the exonuclease Xrn1 and sets in after the incorporation of FUTP into tRNA. Quantification of tRNA modification levels of fluoropyrimidine stressed WT cells revealed a decrease in pseudouridine and 5-methyluridine modifications, suggesting that loss of tRNA methylation in addition to drug induced loss of uridine modifications activates the decay of tRNAValAAC. In addition, the double mutant trm44 tan1 is also sensitive to 5-FU which can be suppressed by overexpression of tRNASerCGA or the correction of base pair mismatches in the body of the tRNA. Inhibition of tRNA methylation therefore represents a new way to increase fluoropyrimidine toxicity.

23 Mariachiara Grieco (University of Milan; Italian Institute for Genomic Medicine, Milan, Italy)

Characterizing MYC-driven interplay of co- and post-transcriptional alterations in prostate cancer using direct RNA long-read sequencing

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MYC is a master transcription factor and a known driver of prostate cancer (PCa) progression. Aberrant alternative splicing (AS) contributes to heterogeneous phenotypes of PCa, whereby many AS events are associated with oncogenic signals promoted by transcription factors such as MYC. Similarly, post-transcriptional alterations in RNA methylation have also been associated with the onset of PCa. The development of long-read (LR) sequencing technologies has greatly enhanced our ability to map the transcriptome at full-length isoform resolution, also enabling the identification of RNA modifications. In this light, we assessed MYC-driven co- and post-transcriptional alterations in PCa using LR direct-RNA sequencing data. In particular, we analyzed LR direct-RNA sequencing data of three biological MYC siRNA-treated and control replicates from human prostate cancer cell line PC3. By a de novo isoform reconstruction we identified 19,543 isoforms across 12,502 genes, shedding light on 5,218 novel isoforms. By performing differential expression analysis at gene- and isoform-level we observed the contribution of MYC in regulating expression levels of factors related to the spliceosome machinery and post-transcriptional processes, such as RNA methylation and RNA localization. Moreover, we characterized MYC-driven AS events in genes implicated in spliceosome activity. By mapping N6-methyladenosine (m6A) sites along the full-length isoforms, we identified 29,744 m6A sites, including 1,391 sites significantly differentially methylated upon MYC depletion. Among the most significant sites, we detected changes in m6A methylation of a productive isoform of SF3B1. This gene is the most frequently mutated spliceosomal gene across cancers driving tumor progression. Differentially methylated SF3B1 isoforms were also characterized by the inclusion of AS events, differential isoform expression level and usage, suggesting the interplay between m6A modification and other RNA processing mechanisms in PCa.

Overall, our results revealed a multifaceted landscape regulated by MYC that simultaneously modulates isoform expression, AS and m6A modification in PCa. In particular, the splicing-related SF3B1 gene emerged for being differentially methylated and expressed, and presenting distinct isoform usage upon MYC depletion. This crosstalk highlights the strict link between MYC and AS in PCa, modulated by m6A as a cis-acting mark. Moreover, this study

underpinned LR sequencing as a highly valuable technology for providing a comprehensive portrayal of the transcriptome at higher resolution, as suggested by m6A differentially methylated sites. Understanding the role of MYC in corrupting the transcriptional landscape through post-transcriptional modifications such as m6A is fundamental to retrieving novel candidates for therapeutic purposes for MYC-driven cancers.

24 Valerie Griesche (DKFZ, German Cancer Research Center, Heidelberg, Germany)

Impact of adenosine mRNA modifications on the immune function of macrophages

Valerie Griesche

DKFZ - German Cancer Research Center, Heidelberg, Germany

Macrophages are unique in their high level of plasticity which allows their rapid adaptation to a changing environment. While the proinflammatory phenotype of macrophages is well characterized, the role of different RNA modifications in macrophage adaptability has only recently attracted scientific interest. It was found that mRNA modifications play a crucial role in modulating macrophage responses to proinflammatory stimuli, yet, a defining link between RNA modification sites and phenotype remains elusive. In this study we aim to catalogue and connect specific sites of N6-methyladenosine (m6A) modification and Adenosine to Inosine (A-to-I) RNA editing to the fate of individual transcripts and macrophages' proinflammatory response. While the activation of macrophages remained mostly unchanged in the absence of ADAR1 mediated A-to-I RNA editing, depletion of m6A by METTL3 knockout or inhibition resulted in deficient upregulation of inflammation-related transcripts and cell surface markers upon stimulation. Simultaneously, m6A promoted phagocytosis, possibly in a receptor-dependent manner. The ongoing mapping of the RNA modification sites will shed light on the connection between mRNA modification and macrophages' immunological response. Overall, our study stresses the significance of mRNA modifications in modulating macrophage function. A deeper understanding of this process holds great potential for therapeutic intervention in chronic inflammation and cancer.

25 Marco Guarnacci (The Australian National University, Canberra, Australia)

Substrate diversity of NSUN enzymes and links of 5-methylcytosine to mRNA translation and turnover

Marco Guarnacci[1]; Dr. Pei-Hong Zhang; Madhu Kanchi; Hung Yu-Ting; Hanrong Lin; Hanrong Lin; Nikolay Shirokikh; Li Yang; Thomas Preiss

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Maps of the RNA modification 5-methylcytosine (m5C) often diverge markedly not only because of differences in detection methods, data depth and analysis pipelines but also biological factors. We re-analysed bisulfite RNA sequencing datasets from five human cell lines and seven tissues using a coherent m5C site calling pipeline. With the resulting union list of 6,393 m5C sites, we studied site distribution, enzymology, interaction with RNA-binding proteins and molecular function. We confirmed tRNA:m5C methyltransferases NSUN2 and NSUN6 as the main mRNA m5C “writers,” but further showed that the rRNA:m5C methyltransferase NSUN5 can also modify mRNA. Each enzyme recognises mRNA features that strongly resemble their canonical substrates. By analysing proximity between mRNA m5C sites and footprints of RNA-binding proteins, we identified new candidates for functional interactions, including the RNA helicases DDX3X, involved in mRNA translation, and UPF1, an mRNA decay factor. We found that lack of NSUN2 in HeLa cells affected both steady-state levels of, and UPF1-binding to, target mRNAs. Our studies emphasise the emerging diversity of m5C writers and readers and their effect on mRNA function.

26 Wei Guo (Division of Biochemistry, Mannheim Institute for Innate Immunoscience (MI3), Heidelberg University, Mannheim, Germany)

tRNA splicing and tRNA modifications across various species

Wei Guo; Stefano Russo; Francesca Tuorto

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tRNA, harboring more than 90 different types of chemical structures, is the most heavily modified RNA molecule. Among these modifications, Q (queuosine) and m5C (5-methylcytosine), in the anticodon loop region, prove to be important for the translation process. Q is a hypermodified nucleoside at position 34 of specific tRNAs (Tyr, Asp, Asn, His) catalyzed the TGT complex. Eukaryotes obtain this modified nucleobase from bacteria, either with the diet or from their microbiota. m5C, present at position 34 of tRNA Leu and positions 38, 72, and in the variable loop of various tRNAs, is orchestrated by NSun2, NSun6, and Dnmt2 enzymes. Brain functions are greatly affected by the absence of these modifications. During maturation, tRNA precursors go through several processes such as the cleavage of the leader and trailer sequences, the modification of discrete positions by various modification enzymes and a subset of tRNAs go through splicing. In eukaryotes, precursors of tRNAs with introns (Tyr, Leu, Ile, Arg) are cleaved by the TSEN complex and the tRNA exon ends are ligated by RtcB coupled with other factors. tRNA splicing is an essential step, since introns enlarge the anticodon stem and only mature tRNAs have the structured anticodon loop necessary for the decoding. My work focuses on the interplay between tRNA modifications and tRNA splicing, looking evolutionarily at worms, fruit flies, mice, and human cell lines as models. Using Northern blotting, mass spectrometry, cryo-EM, and other techniques, we are currently identifying the modification status on individual tRNA and tRNA precursors at isodecoder level.

27 Alexander Hammermeister (Jagiellonian University Krakow, Poland)

Structural characterization of tRNA binding by the human FTSJ1-THADA methyltransferase complex

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tRNAs are extensively post-transcriptionally modified at various positions and the anticodon stem loop (ASL) is a modification hotspot. Modifications in the ASL are predominantly involved in the translational decoding process and their lack is often associated with various human diseases. Human FTSJ1 is a tRNA 2'-O-methyltransferase (MTase) that modifies the ribose 2'-OH moiety of residues 32 (Nm32) and 34 (Nm34) in the ASL of 21 reported tRNAs. FTSJ1 forms a complex with either THADA to methylate residue 32 or with WDR6 to methylate residue 34. Mutations in human FTSJ1 are linked to X-linked intellectual disability and cancer. 17 tRNAs, out of the 21 described FTSJ1 targets, carry only the THADA dependent Nm32 modification, two tRNAs only have the WDR6 dependent Nm34 methylation, whereas tRNAPhe and tRNATrp are modified at both positions. The exact molecular mechanisms that lead to tRNA selection and site specificity by the THADA-FTSJ1 complex remain unknown. To address this issue, we purified the heterologous expressed human FTSJ1-THADA complex to homogeneity. We use single particle cryogenic electron microscopy (cryo-EM) to determine the high-resolution structure of FTSJ1-THADA in different states and with different tRNA species. Furthermore, we use complementary biochemical and biophysical approaches to characterize the binding specificity and methylation activity towards different tRNA substrates in vitro. Based on our structure of the complex, we mapped and analysed residues on THADA and FTSJ1 that are involved in tRNA binding and protein-protein interactions.

28 Shanice Jessica Hermon (Max Planck Institute of Molecular Physiology, Dortmund, Germany)

Labelling of pseudouridine in RNA and its mass spectrometry-based sequence read-out

Shanice Jessica Hermon

Max Planck Institute of Molecular Physiology, Dortmund, Germany

Genetic modifications are crucial for human development, and their dysregulation is connected to pathology. Pseudouridine (Y) is one of the most prevalent and dynamic modification in RNA, and was shown to suppress immune responses in mRNA vaccines. However, the biological role of Y remains poorly understood as certain key limitations and challenges in the detection of Y are yet to be overcome. In account of this, we report the usage of bisulphite labelling strategy for the efficient, and quantitative mass spectrometry-based detection of Y. We show a labelling efficiency greater than 99%, in isolated yeast tRNAs hosting multiple Ys in parallel, which is currently not achieved in existing LC-MS based labelling detection methods. Our study thus provides a highly quantitative method which could be used for Y profiling across different cell states, to identify novel biomarkers and drug targets.

29 Hung Xuan Ho (Goethe University Frankfurt am Main, Germany)

Autophagy regulates YTHDF protein degradation during contact inhibition

Hung Xuan Ho PhD[1]; Alexandra Stolz[1]; Gunter Meister[2]

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The YTHDF protein family has been linked to cancer development and progression, while mechanistically recognizing N6-methyladenosine (m6A) on cytosolic mRNA. This most abundant mRNA modification plays crucial roles in diverse cellular and (patho)physiological processes. Although the level of YTHDF1, YTHDF2, and YTHDF3 is crucial for mRNA's fate, the homeostasis of the YTHDF family at the protein level is poorly understood. Here we report that YTHDF protein levels rapidly decrease upon the onset of contact-inhibition in cells. We further confirm that YTHDF family members are functionally redundant in supporting cell cycle progression/proliferation. However, while depletion of YTHDF proteins causes cell death in HCT116 cancer cells that lost contact-inhibition, contact-inhibited MRC5 cells remain unaffected. Increased mRNA levels in various cancer cells hint towards transcriptional regulation of YTHDF levels. Instead, we found YTHDF to be regulated on the protein level, involving both autophagy and the ubiquitin-proteasome system. Along this line, we found autophagy flux to significantly increase after contact-inhibited cells reach full confluence, a regulation that was absent in cells that lost contact-inhibition. Taken together our study sheds light onto the molecular mechanisms regulating YTHDF protein homeostasis and indicates a therapeutic window for specific cancer treatment.

30 Marko Jörg (Johannes Gutenberg University Mainz, Institute of Pharmaceutical and Biomedical Sciences, Germany)

Analysis of specific tRNA modification patterns and RNA methyltransferases leads to new insights into pathological aging and Alzheimer's disease.

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Alzheimer's disease (AD) is the most common neurodegenerative disease, characterized by gradual cognitive decline and later dementia. About 15% of the over-65s and over 50% of the over-80s are affected by AD worldwide. Despite intensive basic research, the pathogenesis of AD is only partially understood, and no high-efficacy treatment options are available. New approaches focus on the ribonuclease angiogenin (ANG), tRNA fragments (tRF) and RNA modifications in pathological aging and AD development. Especially tRNA modifications play a crucial role in RNA function and stability, but their role in pathological aging and AD remains unknown. To incorporate RNA modifications, specific proteins are required. This group of proteins is collectively known as RNA-modifying proteins (RMPs) and is further subdivided into three distinct groups: "writers", "readers", and "erasers". "Writers" chemically label RNA; "erasers" remove them again; and "readers" selectively recognize and bind to certain specific chemical RNA modifications leading to producing a cellular response. Therefore, we used an Liquid Chromatography with tandem coupled mass spectrometry (LC-MS/MS) based approach in different aging and AD cell, animal, and human postmortem brain tissue models to determine whether specific tRNA modifications contribute to mitochondrial defects following the dynamic changes in modification patterns along the pathological process of aging and AD. The tRNA modification 5-methylcytosine (m5C) located in the anticodon and variable loop region at positions 34/38/48/49/50 plays a crucial role in the stress-induced ANG-mediated tRNA cleavage. First results of LC-MS/MS and Western Blotting revealed various changes in tRNA modifications in pathological aging and AD, especially for m5C and the writer NOP2/Sun RNA Methyltransferase 2 (NSUN2) in an age- and sex-specific manner. Our group also determined an age-, sex- and AD-dependent dysregulation of ANG expression. These results suggest that ANG and tRNA modifications act as critical factors in the further development of AD or other neurodegenerative diseases. This line of research could be a new road to defining early biomarkers for AD and represent an important step toward developing new therapeutic strategies to improve the symptoms of AD patients.

31 Igor Kaczmarczyk (Jagiellonian University Krakow, Poland)

Molecular mechanisms of queuosinylation in humans

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Transfer RNA (tRNA) plays a central role in decoding genetic information into proteins. Recent studies have revealed that modifications in the tRNA anticodon regulate the speed and fidelity of ribosomal translation, supporting proper co-translational folding dynamics of the nascent polypeptide chain. Queuosine (Q) is a modified nucleoside present at position 34 of four tRNAs tRNA(Asp)GUC, tRNA(Asn)GUU, tRNA(His)GUG, and tRNA(Tyr)GUA in both prokaryotes and eukaryotes. However, only bacteria possess the full enzymatic pathway capable of synthesizing the required precursors from GTP. Eukaryotes harbor only the enzyme that allows to conduct the last step of the modification pathway – the human tRNA guanine transglycosylase (TGT) that is called queuine tRNA-ribosyltransferase (QTRT). Thus, eukaryotic organisms rely on an exogenous supply of queuine (modified guanine), which they can incorporate into their tRNAs post-transcriptionally. Bacterial TGT acts as a homodimer, whereas human QTRT is a heterodimer, composed of the catalytic QTRT1 and non-catalytic QTRT2 subunits. Numerous studies have shown the important role of Q in regulating protein synthesis and the activity of other tRNA modification enzymes. Moreover, queuosinylated tRNA(Asp) and tRNA(Tyr) are further decorated with mannose and galactose, respectively. Although many structural studies have aimed to provide mechanistic insights into QTRT1 and QTRT2, we still lack the full molecular understanding of how queuosinylation of human tRNAs is performed by the QTRT1/2 complex.

The main goal of this project is to provide a comprehensive understanding of how four tRNAs are specifically recognized by the human QTRT1/2 complex and what the exact mechanism of the enzymatic reaction looks like. We aim to obtain structural snapshots of the QTRT1/2-tRNA complex during the accommodation of target tRNAs in the pre-reaction (non-inhibited) state and to structurally resolve how queuine is accommodated by the protein-tRNA complex. Ultimately, we plan to fully characterize the dynamic transitions within the reaction center over the course of the enzymatic reaction. To achieve this goal, we use single-particle cryogenic electron microscopy (cryo-EM), which we complement with functional analyses of structure-guided mutants using a variety of biochemical assays.

32 Chih-Yuan Kao (Mannheim Institute for Innate Immunoscience, Medical Faculty Mannheim, Heidelberg University, Germany)

Role of m6A mRNA modification during macrophage activation

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Macrophages are cells of high plasticity in response to different environmental cues. In many cancer types, tumor-associated macrophages (TAMs) represent a heterogeneous cell population within the tumor microenvironment, and their activity is related to cancer progression and invasion. Likewise, the dysregulation of macrophages contributes to chronic inflammation. Our goal is to understand how macrophage plasticity is regulated by mechanisms that control gene expression at the posttranscriptional level. Recent evidence indicates that editing and modification of mRNA contribute to macrophage activation and reprogramming. However, we currently lack a systematic understanding of how different posttranscriptional modifications are coordinated and influence each other to regulate the fate of mRNAs within macrophages. To address whether dynamic changes in mRNA modifications control macrophage plasticity and activation, we focus on the most abundant mRNA modification, N6-adenosine methylation (m6A), and used CRISPR/Cas9 to generate knockout (KO) mouse RAW264.7 macrophage lines lacking METTL3, the writer of m6A. In the absence of METTL3, m6A levels were reduced as expected, and global translation was reduced when macrophages were activated by interferon-gamma and lipopolysaccharide. As in the KO cells, the response to these inflammatory cues was impaired when macrophages were treated with a METTL3 inhibitor. We are now in the process of measuring translation at a transcript-specific level using Ribo-Seq, and mapping changes in m6A modification using TadA-assisted m6A sequencing as well as Nanopore sequencing. This study will add to our mechanistic understanding of how mRNA modifications shape macrophage responses.

33 Liam Keegan (Central European Institute for Technology at Masaryk University (CEITEC MU), Brno, Czech Republic)

An RNA editing-independent function of ADAR1 inhibits PKR activation in mice.

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The deamination of the adenosine base to inosine in dsRNA is catalyzed by the ADAR RNA editing enzymes. In mammals, there are two active ADAR enzymes; ADAR1 and ADAR2. The editing activity of ADAR1 is essential for the discrimination of self and non-self RNA by the innate immune cytoplasmic dsRNA sensors MDA5 and RIGI that signal downstream to MAVS. In humans, mutations in ADAR1 that decrease RNA editing activity cause Aicardi Goutières Syndrome (AGS). Adar null mutant mice are embryonic lethal and die by E12.5. Both conditions are characterized by high aberrant interferon induction. We hypothesized that since Adar, Mavs double mutant mice survive till birth but die as pups, usually within 14 days, other innate immune dsRNA-driven pathways must be in play. Adar null mouse embryos have an increased expression of Pkr (Eif2ak2), a protein kinase that is activated by unedited dsRNA. We show that the early death of the Adar, Mavs double mutant pups and severe gut defects arising from death of proliferating gut stem cells stem and their aberrant differentiation are rescued in Adar, Mavs, Eif2ak2 triple mutant mice. We also report a direct regulatory interaction between PKR and ADAR1 that inhibits the phosphorylation of the PKR kinase domain and activation of PKR. Using ADAR1 mutants expressed in cells, we show that ADAR1 interacts through its third dsRNA binding domain (RBDIII) with PKR, in an editing-independent manner. Our results indicate that in addition to dsRNA editing, ADAR1 protein-protein interactions regulate the innate immune response.

34 Kira Kerkhoff (Goethe University Frankfurt am Main, Germany)

Shelf life study of aqueous nucleoside solutions during storage at different temperatures

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The epitranscriptome is an important area of research in many aspects. Dynamic changes in RNA modifications are crucial to stress adaptation and are associated with several human diseases. In addition, the current knowledge of chemical RNA modifications is already being used for the development of new drugs. Recently, the exchange of uridine to N1-methyl-pseudouridine has played a key role in overcoming unwanted immune reactions for COVID-19 vaccines. One method of analysing RNA is based on the digestion into nucleosides, which are then measured by LC-MS/MS. To use this method for quantification, a multi-point calibration with an external standard is required. This standard contains the nucleosides in dissolved form, usually in water, and is often stored for long periods, sometimes years. Although the quality of the standard is a limiting factor for the accuracy of this method, no data are available on the stability of individual nucleosides in solution. Here, we used UV-MS-coupled Liquid Chromatography to determine the shelf life of a total of 50 modified nucleosides. We present the results of the first months of nucleosides stored at various temperatures (20°, 4°, -20°, -80°C) and an elevated temperature of 40 °C for accelerated stability testing. Our laboratory has already shown pitfalls that need to be considered when quantifying RNA modifications using nucleoside MS and now this shelf-life study will help us to uncover further drawbacks related to the quality of the external standards.

35 Lukasz Koziej (Jagiellonian University, Krakow, Poland)

Atomic resolution profiling of elongating ribosomes

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Transfer RNAs (tRNAs) are the most densely and diversely modified nucleic acids in our cells. During elongation modifications in the tRNA anticodon stem-loop can influence the efficiency of translation, affecting tRNA binding to the ribosome, mRNA decoding, proofreading, suppressing/enhancing codon frameshifting, and translocation. The effect of specific modification is typically determined by comparing a wild-type strain with strains that lack the responsible modification enzyme or enzymes. Recent advances in RNA sequencing allow us to obtain global ribosomal profiles of cells and identify those specific mRNA codons that are most affected by the loss of a given RNA modification. To determine the underlying molecular cause for the ribosomal stalling, in vitro reconstituted translation systems can be used in combination with single particle cryo-EM to obtain a detailed atomic picture of the selected modification. However, these systems are technically challenging, and the translation rates of a custom transcript may not reflect the endogenous setting. The establishment of a reconstituted translation system requires substantial quantities of material and rigorous testing. For instance, each component (i.e. initiation and elongation factors, small and large ribosomal subunits) needs to be purified or synthesized (i.e. mRNA) individually. Finally, purifications of specific, fully modified eukaryotic tRNAs and tRNAs lacking a chosen modification are particularly challenging. To circumvent these technical limitations, we have developed a complementary method, which allows us to generate a quantitative atomic insight into ribosomal elongation directly from yeast and human cells by performing ribosomal profiling and single particle cryo-EM analyses from the same sample. Our scheme can provide a global picture of translation elongation at codon resolution and at the same time informs about the distribution of ribosome states at up to 1.9 Å resolution. Finally, we used our approach to show that the lack of a conserved modification delays the binding of tRNA to cognate codons in polysomes engaged in translation elongation.

36 Anna Kuszczynska (Lodz University of Technology, Lodz, Poland)

Two-step conversions of uridine and cytidine to their variously C5-C functionalized derivatives

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C5-substituted pyrimidine ribonucleosides constitute an important class of compounds, that have found practical use as biological standards and pharmaceutical agents with antibacterial, antiviral and antitumor properties.[1] In this context, there is a high demand for simple and effective methods for obtaining C5-functionalized uridines and cytidines, particularly those with a C5-C bond, which is challenging to form.

The most commonly utilized strategies for the creation of carbon-carbon bond at C5-position involve: hydroxymethylation,[2] aminomethylation by the Mannich reaction[3] and Pd-catalyzed reaction of 5-iodo-nucleosides to introduce alkyl, alkenyl, alkynyl or aryl groups.[4,5] All these methods require, however, additional ribose protection and a final deprotection step, making them costly and time-consuming.

Herein we report a two-step approach to introduce several chemically diverse

C5-C substituents: carboxyl, nitrile, ester, amid and amidine into uridine and cytidine.[6,7] The method utilizes the CF3-functionalized pyrimidine nucleosides, which turned out to be easy-convertible upon incubation with nitrogen or oxygen nucleophiles. Employed strategy does not require incorporation of additional protective groups for ribose hydroxyl functions.

Research funded by National Science Centre: OPUS 22, UMO-2021/43/B/ST4/01570, 2022-2026

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37 Chengkang Li (Goethe University Frankfurt am Main, Germany)

Nano-flow HILIC-MS-based site-specific assessment of RNA modifications

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Being one of the most abundant and important macromolecules in all organisms, RNAs undergo multiple modifications (epitranscriptome) post-transcriptionally, which could affect their structures and/or functions, codon usage preference, and protein translation efficiency accordingly. To date, the constantly growing number of RNA modifications has reached 170 already, while many RNA modifications are found to be involved in many neurological diseases and cancers. Surprisingly, recent studies have revealed potential adverse effects of certain popular pharmaceutical drugs on RNA modification landscape, making the RNA modification an unavoidable topic in nowadays' life science, especially after the big success of the RNA based vaccination in tackling the recent global pandemic (COVID-19). Therefore, a better understanding of RNA modifications (e.g. of drug or stress treated cells), in terms of modification type, quantity, and exact location, will be beneficial to the mechanism study of diseases and the development of more targeted and healthier therapeutic drugs. After decades of efforts, researchers have now been able to accurately quantify multiple and specific types of RNA modifications using advanced mass spectrometric approaches, e.g. the nucleic acid isotope labeling mass spectrometry (NAIL-MS), which has never been possible before by using in vitro transcription (IVT)-based RNA sequencing techniques. However, a robust approach for site-specific localization of RNA modifications is still an unsolved but promising challenge in epitranscriptomic study. Although ion-pairing reagent-assisted reverse phase chromatography is by far the most popular strategy for oligonucleotide separation based on ionic strengthen hydrophobicity prior to the pinpointing of RNA modifications using mass spectrometry (MS), the use of ion-pairing reagents of these approaches potentially suffers from certain practical limitations, e.g. impaired MS sensitivity, instrument contamination, poor robustness. Therefore, a cleaner – ion-pairing reagent-free separation technique, e.g. hydrophilic interaction chromatography (HILIC), is in high demand for the accurate investigation of MS-based site-specific RNA modifications. To the best of our knowledge, we are the first lab that are able to nicely separate, ionize, and sequence different lengths of synthetic oligonucleotides (up to 40 bases) using, particularly nano-flow, HILIC chromatography coupled with mass spectrometry. According to our MS result, nearly infinite signal-to-noise ratio is recorded in the (MS1) extracted ion chromatogram (XIC) by only injecting samples in "ng" magnitude, while high MS2 fragmentation coverage rates (≥ 97%) are obtained under data-dependent acquisition (DDA) mode. The recent implementation of the method on RNase digested tRNA isoacceptors unveils immeasurable potential of our new method in the upcoming tides of epitranscriptomics.

38 Christine Lietz (Johannes Gutenberg-University Mainz, Institute of Pharmaceutical and Biomedical Sciences, Germany)

Impact of Plant Extracts on Mitochondrial Function via mRNA-Mediated Mechanisms in Alzheimer's Disease

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Mitochondrial dysfunction is one of the major pathologic hallmarks in late onset Alzheimer's disease (LOAD). Several alterations in mitochondrial function are described such as reduced mitochondrial membrane potential (MMP), lower ATP levels and enhanced reactive oxygen species (ROS), mainly induced by Amyloid b. These alterations are assumed to be strongly associated with a decreased effectivity of the respiratory chain (OXPHOS) especially complex I. Previous data from our group demonstrated that enhanced N1-methylation of adenosine (m1A) in mRNA encoding ND5 subunit of complex I interfered with its translation. This might lead to complex I and mitochondrial dysfunction.

Therefore, we aimed to investigate whether plant extracts such as Ginkgo biloba or other potent antioxidant extracts affect mitochondrial dysfunction.

To identify the protective effects of a selection of 48 defined plant extracts on mitochondrial function, a screening was performed in SH-SY5Y Mock cells by measuring ATP levels under physiological conditions and mitochondrial membrane potential (MMP) against rotenone-induced complex I damage. Glycolytic involvement was excluded by analyzing lactate production.

Our group identified 9 most promising plant extracts with protective effects on these cellular powerhouses at certain concentrations tested (0.01, 0.1, 1, 10 µg/mL) after 24 h of treatment.

To characterize mitochondrial function more specifically, confocal microscopy was used to investigate mitochondrial dynamics. Furthermore, we selectively analyzed mitochondrial complex activities, especially complex I, after extract treatments by measuring the oxygen consumption rate (OCR) with an Agilent Seahorse XF Analyzer.

To elucidate the molecular mechanism of these highly interesting plant extracts, we will examine alterations in protein expression, followed by determining the methylation levels of ND5 mRNA after extract treatment using Illumina Sequencing analysis.

Our findings could highlight a new potential therapeutic approach for Alzheimer's disease treatment, specifically targeting mitochondrial health.

39 Lorena Martin-Morales (Laboratory of Cancer Stemness, GIGA-Stem Cells, University of Liege, Belgium)

tRNA optimality shapes colorectal cancer-initiating Lgr5+ identity through the regulation of mRNA translation

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Lgr5-expressing colorectal cancer stem cells (CSCs) display extensive plasticity and are responsible for cancer initiation and metastasis outgrowth. Recent studies have demonstrated that the establishment of Lgr5+ CSCs occurs in a niche-independent manner during liver metastasis, suggesting that cancer-initiating Lgr5+ stem cells possess the intrinsic capability to sustain stem cell-like multipotency and self-renewal programs. Nevertheless, the key molecular players of this intrinsic plasticity are yet unknown.

tRNAs are heterogeneous and highly modified molecules necessary for the accurate translation of mRNAs into proteins, and whose active role regulating translation has been well-documented both in health and disease. A growing amount of data has demonstrated that optimal tRNA pools are essential to sustain cancer initiation, invasion and resistance to therapy, uncovering the unexpected role of these molecules in cancer biology.

Here, we postulated that tRNA optimality is essential to sustain the intrinsic cancer-initiating Lgr5+ identity. To this end, we combined multi-omic approaches to identify the specific translational dynamics promoted by Wnt-driven transformation in healthy stem cells. Our results demonstrate the presence of distinct tRNA pools, tRNA modifications and tRNAmodifying enzymes (TMEs) in cancer-initiating Lgr5+ CSCs compared to their healthy counterpart. Functionally, a CRISPR-Cas9 drop-out screen performed on intestinal healthy and cancer stem cells, identified tRNA aspartic acid methyltransferase 1 (Trdmt1) as an essential TME for Lgr5+ CSC survival, yet dispensable in healthy stem cells. Genetic depletion and chemical inhibition of this enzyme prevent the formation of CSC-derived intestinal organoids by impairing the mRNA translation of key regulators that support the multipotency and self-

renewal programs of Lgr5+ stem cell-like cancer-initiating cells. Together, these findings demonstrate that tRNA biology plays a key role in sustaining the intrinsic plasticity of Lgr5+ CSCs and pave the way for the development of targeted therapies against colorectal cancer stem cells.

40 Nassim Meziane (Sorbonne Université - Institut de Biologie Paris-Seine, France)

The RNA methyltransferase FTSJ1: roles in neural morphology and translation efficiency

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Transfer RNAs (tRNAs) are essential components of the translation machinery, relying on modified ribonucleotides for their structure and decoding capacities. 2'-O-methylation (Nm) is one of the most widespread RNA modification. Nm is catalyzed by FTSJ1, a stand-alone methyltransferase (MTase) in humans. The loss of FTSJ1 has been linked to intellectual disability (ID), although the underlying mechanisms of this pathology remain unclear.

Our research on human neural progenitor cells (NPC) revealed that inhibiting FTSJ1 leads to an increase in the number of dendritic spines, a characteristic frequently observed in neurodevelopmental disorders. Notably, this phenotype is conserved in a subset of neurons from Drosophila larvae carrying mutations in FTSJ1 orthologs. The Drosophila mutants exhibited similar neuronal morphological defects, as well as a significant reduction in long-term memory. Transcriptome analysis further indicated the deregulation of specific mRNAs and miRNAs involved in brain development in human cells. This disruption in gene expression may contribute to the morphological defects observed in both human cells and Drosophila.

Given the central role that tRNAs play in translation, we conducted a transcriptome-wide profiling of ribosome footprints in human patient cell lines, together with the Drosophila model lacking FTSJ1 activity. These analyses are underway.

Overall, our results indicate significant regulation of brain-specific genes and highlight morphological defects affecting neuronal cells in both humans and Drosophila lacking FTSJ1 RNA MTase. Our aim is to identify the genes involved in the defective morphology of neuronal tissues devoid of Nm, explore a potential regulation of their translation, and ultimately elucidate the mechanisms underlying FTSJ1-related intellectual disability.

41 Isabel Naarmann-de Vries (University Hospital Heidelberg, Germany)

Strategies for targeted RNA sequencing and RNA modification detection on the Oxford Nanopore platform

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The direct sequencing of RNA (direct RNA-seq) on the Oxford Nanopore Technologies (ONT) platform is currently the only technology to sequence RNA directly. The platform uses full length RNA without the need for cDNA synthesis and PCR amplification. This allows the direct assessment of RNA isoforms and also RNA modifications. Recently, a new generation of direct RNA-seq chemistry (RNA004) was introduced by ONT.

The current ONT protocols support the sequencing of polyadenylated RNA as well as calling of m6A and pseudouridine modification sites. Here, the RNA is targeted by an oligo(dT) adapter that binds to the poly(A) tail and RNA is sequenced from the 3'end.

However, this strategy covers only a small fraction of RNA species and modifications. Consequently, custom sequencing strategies are required to make also other RNA species accessible to direct RNA-seq. In the past, targeted sequencing of ribosomal RNAs (rRNA) [1] and transfer RNAs (tRNAs) [2,3] was established and is now transferred to RNA004 chemistry.

In other cases, one might be interested in sequencing of only particular polyadenylated RNAs (like reporter genes) and not the whole polyadenylated transcriptome. Furthermore, for very long RNAs, the 5'end might not be accessible by the standard sequencing approach. In order to specifically enable direct RNA-seq in these cases, we hypothesized that sequence-specific RNA cleavage would generate new, unique 3'ends that can be targeted by custom sequencing adapters. For this, it is imperative that the 3'end is exactly defined, as the T4 DNA ligase (used to ligate the sequencing adapter) does not tolerate gaps or flaps. We show that the widely used RNase H is not suitable for this approach. Instead, we identified a specific DNA endonuclease [4] that can be adapted to cleave RNA in an RNA-DNA hybrid at the specific recognition sequence. We show that this approach can be applied to either remove a poly(A) tail of a reporter gene or cleave rRNA and generates new 3'ends, which can be targeted by custom adapters and used in direct RNA-seq experiments.

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42 Ivanéia Nunes (University Hospital Heidelberg, Germany)

2'O-Methylation prevents release of uridines for TLR8 activation by Ribonuclease 6

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Toll-like receptor 8 (TLR8) has been identified as a sensor for foreign single-stranded RNA (ssRNA) in innate immune cells. RNA modifications affect TLR8 stimulation. Upstream of TLR8 ribonuclease (RNase) T2 and members of the RNase A family process RNA releasing monouridines and purine 2'3'-cyclophosphate-terminated fragments which engage in the binding sites 1 and 2 on TLR8, respectively. RNase 6, an RNase A family member, may also contribute to RNA processing as it is expressed highly in classical monocytes and dendritic cells where TLR8 expression overlaps. Therefore, this study aimed to characterize the features of RNase 6 cleavage and investigate whether RNA modifications might affect this process.

Comparative analysis of RNase 6 expression in monocytic cell lines showed that transdifferentiated BLaER1 cells express RNase 6 and T2. Thus, we generated BLaER1 cells lacking TLR8 or RNASE6 by CRISPR-Cas9. By similar means, RNase 6 deficient primary CD14+ monocytes were also generated. Upon live whole bacterial infection, and stimulation with total bacterial RNA (bRNA) supernatant was analyzed for cytokine detection by ELISA. Stimulations with bRNA predigested by recombinant RNase 6 were also performed and breakdown products were transfected into the cells. Similarly, chimeric short oligoribonucleotides modified by the addition of a single 2'-O-methyl (2'-O-Me) modification were ex cellulo digested and fragments were analyzed by TBE Urea PAGE.

Infection with live whole bacteria revealed decreased IL-6 and TNF production in cells lacking RNase 6. Likewise, stimulation with total bRNA isolated from those strains induced significantly less IL-6 in RNASE6-/- proving that RNase 6 is required for bacteria recognition. Additionally, the breakdown fragments from bRNA digestion with recombinant RNase 6 showed TLR8 stimulation in cells lacking endogenous RNase 6. Similar findings were observed when un- or predigested bRNA was delivered to CD14+ monocytes that had been edited to lack RNASE6 whereas pre-digestion induced higher TLR8 stimulation in non-targeted cells. These findings argue that this ribonuclease can generate fragments with immunostimulatory potential. Regarding the effect of 2'-O-Me RNA modification - that had been shown to impede TLR8 stimulation – when positioned in between uridine and adenosine (RNase 6 preferential cleavage site) – showed impairment of cleavage and also hampered TLR8 stimulation.

Taken together, our findings reveal the role of RNase 6 in processing bRNA for TLR8 activation by the generation of uridine-terminated fragments.

43 Bhargesh Indravadan Patel (Humboldt-University Berlin, Germany)

Queuosine salvage in fission yeast by Qng1-mediated hydrolysis to queuine

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Queuosine (Q for nucleoside or nucleotide and queuine (q) for the nucleobase) is a complex modification that replaces guanosine at the wobble position of tRNAs that display the G34-U35-N36 anticodon (i.e., tRNAAsn, tRNAAsp, tRNAHis, and tRNATyr). This modification plays an important role in regulating codon-anticodon fidelity, translational efficiency and tRNA stability. This altogether maintains the protein homeostasis within the cell, and alleviates ER stress and unfolded protein response. As a result, dysregulation of the Q-modification has been associated with various pathological conditions like inflammatory bowel disease, neurodegenerative disorders and cancer. In the biosphere, the Q modification of tRNAs is present in most eukaryotes, but only eubacteria can synthesize Q de novo. In this process, guanosine triphosphate (GTP) is first converted to a precursor preQ1, and then replaces G34 on the tRNAs, which is catalyzed by the bacterial tRNA guanine transglycosylase (bTGT) enzyme. The preQ1 is finally converted to Q on the tRNA. In contrast, the eukaryotes salvage Q/q from the external environment, and the eukaryotic TGT (eTGT) can directly exchange guanine for q on tRNAs. In this context, when only Q is available for uptake, an enzyme that cleaves the N-glycosidic bond of Q to release the q nucleobase becomes essential for Q-modification. In this study, we characterised the eukaryotic queuosine nucleoside glycosylase (Qng1, also known as SPAC589.05c, Qtr3 and DUF2419) from Schizosaccharomyces pombe. Our study revealed that qng1Δ cells carry Q-modified tRNAs only when grown in a medium containing the q nucleobase, but not the Q nucleoside, indicating that they are unable to release the q nucleobase from the Q nucleoside. In agreement with this, recombinant Qng1 exhibited Q glycosylase activity in vitro. Interestingly, there was notable immediate Q hydrolysis upon incubation with Qng1, followed by reduced activity. When Q hydrolysis was coupled with eTGT-dependent tRNA queuosinylation, the activity of Qng1 increased, indicating that it is inhibited by its own product, the nucleobase q. Overall, our findings demonstrate that Qng1 functions as a Q nucleoside glycosylase in S. pombe, a function conserved in its homologs in higher eukaryotes.

44 Riccardo Pecori (German Cancer Research Center (DKFZ))

Optimization of ADAR1-recruiting guide RNAs (gRNAs) toward the generation of RNA-derived neoepitopes

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Increasing neoepitope formation is a promising strategy to sensitize tumors to immunotherapy. RNA editing by adenosine deaminase acting on RNA 1 (ADAR1) is the most abundant RNA modification, with more than 16 million RNA editing events cataloged in humans. ADAR1 deaminates adenosine to inosine (A-to-I) within dsRNA, is overexpressed in most cancers, and was found to be essential in certain subsets of tumor cells. RNA editing can also lead to non-synonymous substitutions in mRNA, generating epitopes that can be recognized by T cells and induce their activation.

ADAR1-recruiting gRNAs re-target endogenous ADAR1 to induce specific amino acid recoding. Here, we present the contribution of RNA editing to the ligandome of tumor cell lines and the thorough optimization of gRNAs toward the generation of functional and immunogenic neoepitopes leveraging ADAR1 RNA editing activity.

45 Michael Piechotta (University of Heidelberg, Germany)

QutRNA 2.0 – Queuosine (and other) RNA modification discovery from Nanopore direct tRNA

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Transfer RNAs (tRNAs) are a central constituent of mRNA translation by providing the link between the genetic code and amino acids in proteins. In eukaryotes, tRNAs are typically 76 – 90 nt long. Each tRNA carries an amino acid and has a characteristic three-base long segment (anticodon) that forms complementary bonds with the codon sequence in mRNA. Individual nucleotides of a tRNA can be chemically modified, changing the secondary structure and codon specificity or otherwise influencing the regulation and fate of tRNAs.

Precise and unambiguous identification of modified tRNA residues is imperative to completely understand their role in protein synthesis. Recently, protocols for sequencing of tRNAs using Oxford Nanopore Technology (ONT) have been established. ONT features direct sequencing, where modified residues can be identified by characteristic base calling error profiles or raw signal analysis.

Specific properties of tRNAs, such as their short length, the large number of identical copies in the genome, and the elevated modification density and frequency, challenge the bioinformatic analysis. Traditional heuristic read mapping strategies fail to achieve satisfying precision and recall.

Here, we present QutRNA: https://github.com/dieterich-lab/QutRNA, a one-stop shop for aligning and detecting RNA modifications in tRNAs from ONT sequencing. The software consists of read mapping, modification detection, and visualization. We calculate optimal local alignments with parasail and assess their statistical significance to keep the proportion of false alignments below 5%. We use JACUSA2 to detect RNA modifications by calculating and comparing error profiles derived from mismatches, insertions, and deletions in paired sample groups. Finally, the tRNA profiles are visualized as heatmaps using the cloverleaf (Sprinzl) coordinates system, enabling comparison of tRNAs and identification of potential RNA modification sites.

We support the visualization of RNA modifications in the cloverleaf coordinates system for eukaryotic nuclear-encoded tRNAs and mt-tRNAs out of the box. Custom mappings from raw

sequences to cloverleaf coordinates can be performed by supplying custom files or employing secondary structure alignments against user-provided covariate models.

We showcase QutRNA 2.0 on tRNA sequencing from HCT-116 cells comprising pairwise comparisons of wild-type vs. known mutants of tRNA modification enzymes DNMT2, NSUN2, and QTRT1.

Sun Y, Piechotta M, Naarmann-de Vries I, Dieterich C, Ehrenhofer-Murray AE. Detection of queuosine and queuosine precursors in tRNAs by direct RNA sequencing. Nucleic Acids Res. 2023 Nov 10;51(20):11197-11212. doi: 10.1093/nar/gkad826. PMID: 37811872; PMCID: PMC10639084. "

46 Maria Polycarpou-Schwarz (Medical Faculty Mannheim, Heidelberg University, Germany)

m6A RNA methylation controls RNA stability

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Background

N6-methyladenosine (m6A) RNA methylation is the most prevalent, abundant and conserved internal cotranscriptional modification in eukaryotic RNAs. m6A RNA modification is added by the m6A methyltransferase core complex, METTL3/14 and WTAP. How m6A RNA methylation controls gene expression.

Methods

Primary human vascular endothelial cells (EC) monolayers were used to evaluate the EC-specific m6A effects. Transcriptome-wide single nucleotide m6A RNA methylation profiles were generated by array star nanopore direct long RNA sequencing. Molecular studies involving RNA methylation-specific RNA immunoprecipitation, transcriptomics, stability assays, RNAi and gain- and loss of function assays in primary ECs provided mechanistic insights.

Results

WTAP is the determinant of m6A RNA methylation in vascular ECs. Vascular endothelial cadherin (CDH5) is among the most important genes involved in the transition of endothelial cell to mesenchymal cell (EndMT). CDH5 transcript exhibited decreased methylation levels in WTAP-deficient cells compared to control ECs. Silencing of WTAP in ECs reduced VE-cadherin protein levels. We identified by iCLIP and DiCLIP experiments an interaction between the stabilizing RNA-binding protein Human Antigen R (HuR) and the 3'-untranslated Region (3'UTR) of CDH5 mRNA. Silencing of HuR, phenocopied the reduced VE-cadherin expression levels initially observed in WTAP-deficient ECs. Conversely, overexpression of HuR increases VE-cadherin expression levels in ECs. WTAP and m6A RNA methylation levels were both induced in response to TNF-alpha pro-inflammatory stimuli. Silencing of WTAP resulted in hyper-inflammatory response of ECs suggesting that m6A RNA methylation inhibits both autoinflammation and over-response to pro-inflammatory cytokines. We then determined HuR interactome in absence or presence of WTAP as well as in homeostatic (unstimulated) or TNF-alpha induced-pro-inflammatory conditions. HuR iCLiP-seq and RIP-seq experiments

revealed HuR binding sites in the 3'UTR of several pro-inflammatory genes regulating local adhesion assembly, TGFBR-signaling, cell migration and differentiation suggesting that the RBP HuR is critically involved in the regulation of EndMT. Co-silencing of WTAP and HuR rescued the autoinflammatory phenotype observed in WTAP-deficient ECs suggesting the notion that m6A RNA methylation is a master upstream regulator of HuR-mediated RNA stability or processing of several pro-inflammatory genes.

Conclusion

Collectively, these findings suggest that m6A RNA methylation controls HuR interactome, and, thus endothelial cell plasticity."

47 Jiri Frantisek Potuznik (IOCB Prague, Czech Republic)

Diadenosine Tetraphosphate (Ap4A) Serves as a 5' RNA Cap in Mammalian Cells

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RNA modifications have long been connected to foreign nucleic acid detection and the innate immune response. In particular, the canonical eukaryotic N7-methylguanosine (m7G) RNA cap and its modifications are a key component of this recognition process. While the m7G cap was long thought to be the only eukaryotic RNA cap, the recent discovery of non-canonical RNA caps such as nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD), and others has changed our understanding of post-transcriptional gene regulation. Here, we present the discovery of a new type of RNA cap in eukaryotes – diadenosine tetraphosphate (Ap4A). Free Ap4A is the most abundant dinucleoside polyphosphate in eukaryotic cells and can be incorporated into RNA by RNA polymerases as a non-canonical initiating nucleotide (NCIN). We use liquid chromatography-mass spectrometry (LC-MS) to show that Ap4A-RNA capping is not dependent on the intracellular concentration of free Ap4A. We further identify two enzymes that are capable of cleaving the Ap4A-RNA cap in vitro, NUDT2 and DXO and we show that Ap4A-RNA is not translated. Interestingly, we also show that even though free Ap4A has been linked to the innate immune response and its dysregulation, Ap4A-RNA does not induce the expression of genes responsible for nucleic acid sensing such as RIG-I, MDA-5, IFNB1 or members of the IFIT family. As such, Ap4A-RNA is recognized as self by the cell and exists as a natural part of the cellular transcriptome. This discovery of a new cap opens a previously unexplored area of eukaryotic RNA regulation.

48 Stefan Prodic (The Australian National University, Canberra, Australia)

SWARM: Single-molecule Workflow for Analysing RNA Modifications

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Over 170 chemical modifications encompass the epitranscriptome and play a pivotal role in regulating RNA properties and function across various RNA classes. However, conventional methods for RNA modification detection are hindered by extensive protocols that lack isoform-level resolution and restrict studies to a single modifications per experiment, limiting comprehensive exploration of the dynamic and diverse epitranscriptome. Here we describe SWARM, a robust approach for the detection of m6A, m5C, pseudouridine, and ac4C from the same sample in individual RNA isoforms. SWARM exploits nanopore direct RNA sequencing signals, which capture continuous native individual RNA molecules. SWARM attains exceptional accuracy in single-molecule modification detection for multiple RNA modifications through a state-of-the-art neural network approach trained on a broad array of diverse nanopore signals. We implemented a versatile workflow which can be easily adapted for detecting and analysing any RNA modification with sufficient training and testing data, through which we produced models for both RNA002 and RNA004 nanopore sequencing kits. Moreover, we put strong emphasis on achieving high modification specificity which is crucial for simultaneous analysis of different modification types. Applying SWARM to numerous independent datasets illustrated consistent and reliable detection of mRNA modified sites and their modification rates which align well with experimentally validated data. Our rigorous analysis shows that SWARM delivers confident detection of multiple RNA modifications from a single sample and provides a robust framework for comparing RNA modification landscapes between samples. Our work opens a wealth of possibilities towards uncovering diverse RNA modification landscapes in countless contexts, which is a significant leap towards deciphering the dynamics and functional relevance of the epitranscriptome.

49 Yuyang Qi (Goethe University Frankfurt am Main, Germany)

The modification landscape of human cytosolic tRNAs

Yuyang Qi; Stefanie Kaiser

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Transfer RNAs (tRNAs) function as adapter molecules, providing the amino acids required for protein biosynthesis. There are 47 tRNA isoacceptors annotated in the human genome that decode 20 amino acids. Despite their small size, tRNAs are the most extensively modified RNAs in human cells, with an average of 13 modifications per molecule. These modifications are crucial for tRNAs in many ways, such as stabilizing their structure, promoting correct codon-anticodon recognition and increasing translation efficiency. Recently, the complete chemical structures of human mitochondrial tRNAs have been unveiled. However, the complete landscape of human cytosolic tRNAs still remains unknown. Although several studies and databases contain information about human cytosolic tRNA modifications, there is no systematic demonstration of the modification profiles of all human cytosolic tRNAs. To fill this gap, we performed a systematic mass spectrometric analysis of human cytosolic tRNAs, purified using an oligonucleotide hybridization assay. After enzymatic hydrolysis the tRNAs are analyzed either qualitatively or quantitatively using LC-MS/MS. By the combination of nucleoside analysis and the already known canonical sequences, we determined the identity and position of the modifications on various human cytosolic tRNAs. Our results contribute to a better understanding of the function of tRNA modifications and provide a solid foundation for subsequent research in this field.

50 Stefano Russo (Mannheim Institute for Innate Immunoscience, Medical Faculty Mannheim, Heidelberg University, Germany)

Q-tRNA modification in the protein homeostasis and fine-tuning of the translation machinery

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tRNA works as a key adaptor molecule which translates the transcriptome to the proteome. The flexibility of codon-anticodon interactions and the codon degeneracy enable many tRNAs to decode multiple codons thanks to the contribution of post-transcriptional modifications located at or adjacent to the tRNA anticodon loop. Among these modifications, queuosine (Q) is a naturally occurring modified ribonucleoside found in the first (wobble) position of the anticodon of tRNAs for Aspartate, Asparagine, Histidine and Tyrosine. Eukaryotes do not possess biosynthetic pathways for queuine (q), the nucleobase precursor to Q, and must obtain it from diet or gut microbiota. The base q is incorporated in tRNAs by a tRNA-guanine-transglycosylase (TGT) composed of the QTRT1-QTRT2 enzymes complex. Our group has developed human cell lines and mouse knockout models, in addition to queuine-deprived cell lines, to study the biological function of this tRNA modification. In particular, we are using human HeLa and HCT-116 and primary mouse neuronal cell cultures to study how the lack of Q affects translational processes. By the means of Northern and Western blotting, FACS, immunohistochemistry, polysome profiling and other methods, we aim to unravel the mechanisms and cellular pathways that link the altered decoding of the transcriptome to the changes in proteostasis and the neuronal phenotypes observed in absence of Q. We are further highlighting the role of tRNA modifications in surveillance checkpoints, like Ribosome Quality Control (RQC), No-Go mRNA Decay (NGD) and the Unfolded Protein Response / Integrated Stress Response (UPR/ISR).

51 Marco Sachse (Heidelberg University, Mannheim, Germany)

Intronic Alu RNA editing couples mRNA stability with pre-mRNA processing

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Background: Adenosine deaminase acting on RNA-1 (ADAR1) binds to double-stranded RNAs (e.g Alu elements) and deaminates adenosine to inosine (A-to-I), a process called RNA-editing. Although most of the RNA-editing events are located in introns, the role of intronic RNA editing in gene expression remains elusive.

Methods: RNA-sequencing, RNA-editing studies, gain/loss-of-function assays, gene expression analysis, western blot, individual cross-linking immunoprecipitation (iCLIP) and RNA immunoprecipitation (RIP) studies in human primary endothelial cells were employed.

Results: RNA-sequencing and RNA-editing studies revealed editing events in CTSK, an inflammation-stimulated extracellular matrix degradation enzyme with an established role in inflammatory diseases including atherosclerosis. CTSK is extensively edited within the Alu regions of intron 5, which is also enriched of HuR binding sites. Silencing of ADAR1 resulted in a 2-fold downregulation of CTSK mature mRNA and a 2-fold upregulation of pre-mRNA while ADAR1 overexpression exerted the opposite. Accordingly, silencing of HuR reduced CTSK expression by >2-fold. Subsequently, CTSK protein expression was siginificantly reduced upon ADAR and HuR silencing, respectively. Importantly, iCLIP and RIP experiments confirmed that HuR interacts with intronic edited regions of CTSK. In the absence of RNA editing, HuR did not bind to CTSK. Under proinflammatory and hypoxic conditions, CTSK mRNA is 10-fold and >2-fold upregulated, whereas silencing of ADAR1 and HuR reduced CTSK mRNA expression, respectively.

Conclusion: Intronic Alu RNA-editing enables proper pre-mRNA processing by HuR, a primate-specific mechanism that plays a decisive part in inflammatory gene expression.

52 Raffael Schaffrath (University Kassel, Institute for Biology, Germany)

Autophosphorylation of conserved yeast and human casein kinase 1 isozymes regulates Elongator dependent tRNA modifications

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Casein kinase 1 (CK1) family members are crucial for ER-Golgi trafficking, calcium signalling, DNA repair, tRNA modifications and circadian rhythmicity. Whether and how substrate interactions and kinase autophosphorylation contribute to CK1 plasticity remains largely unknown. Here, we undertake a comprehensive phylogenetic, cellular and molecular characterization of budding yeast CK1 Hrr25 and identify human CK1 epsilon (CK1ε) as its ortholog. We analyse the effect of Hrr25 depletion and catalytically inactive mutants in vivo and show that perturbations in CK1 activity lead to stress-induced growth defects, morphological abnormalities and loss of Elongator-dependent tRNA modification. We use purified Hrr25 protein to identify distinct autophosphorylation patterns and phospho-sites on several physiological substrates in vitro and find only human isozyme CK1ε can replace yeast Hrr25 functions essential for tRNA modification and cell proliferation in vivo. Furthermore, we demonstrate that human and yeast CK1 orthologs share conserved autophosphorylation sites, which regulate their activities and mutually exclusive interactions with Elongator subunit Elp1 and Sit4, a phosphatase antagonist of Hrr25. Thus, autophosphorylation controls CK1 activity and regulates the tRNA modification pathway. Our data offer mechanistic insights into regulatory roles of CK1 that are conserved from yeast to human cells and reveal a complex phosphorylation network behind CK1 plasticity.

53 David Scherf (University of Kassel, Germany)

Phosphorylation by casein kinase 1 Hrr25 regulates Elongator and wobble uridine modifications

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Kinases regulate a multitude of cellular processes, often by the reversible attachment of phosphate groups to substrate proteins. In yeast, phosphorylation by casein kinase 1 (CK1) Hrr25 was shown to affect Elongator, a multi-subunit (Elp1-Elp6) complex that modifies uridine bases in the wobble position of tRNA anticodons. To further study Hrr25 functioning, we established an ATP analogue sensitive hrr25-I82G mutation in vivo that allowed us to characterize Elongator activity upon chemical inhibition of the kinase. The so called Shokat mutation enabled dose-dependent inhibition of Hrr25 activity that correlated with a progressive decline of Elongator function and wobble uridine modification. We next asked whether this negative effect is conserved among eukaryotes and identified CK1ε as the human homolog of Hrr25 able to support Elongator function and tRNA modification in yeast. The analogue sensitive kinase mutant together with the conservation of Elongator phosphorylation between yeast und human cells can open up future opportunities in the research of tRNA anticodon modifications that are clinically relevant for the formation of human diseases including cancer and neuropathies.

54 Sabine Schneider (LMU Munich, Germany)

Demethylation of m3C in mitochondrial tRNA by AlkBH1

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Translation efficiency is fine-tuned through the post-transcriptional modification of tRNAs. Recently it was shown that the methyltransferase METTL8 isoform 1 is located in the mitochondria, where it methylated C32 in the anticodon-arm of human serine and threonine tRNAs to 3-methyl-cytidine (m3C) and hence impacts on the translation of components of the respiratory chain. Here we show that the human AlkBH1, a member of the Fe2+ and α-ketoglutarate dependent-family of dioxygenases, is demethylating m3C in mitochondrial tRNA in vitro and human cell lines and thus the likely natural antagonist of METTL8.

55 Merlin Schwan (Heidelberg University Biochemistry Center (BZH))

Processing and degradation of ncRNA by MTREC – molecular insights into modular organization and poly(A)-binding

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Heidelberg University Biochemistry Center (BZH)

Eukaryotic cells produce a large variety of RNA molecules including non-coding RNAs (ncRNAs). Although these ncRNAs arising from pervasive transcription are capped and polyadenylated like mRNAs, they are rapidly recognized and selectively degraded in the nucleus by the RNA exosome. In fission yeast, studies on the Mtr4-like protein 1 (Mtl1) and the zinc-finger protein Red1 recently led to the identification of a novel exosome targeting complex called the MTREC (Mtl1-Red1 core), which is required for targeting meiotic or mis-spliced mRNAs and cryptic unstable transcripts (CUTs) to the exosome. Our data obtained from the model organism Chaetomium thermophilum suggest that Red1 is a scaffolding protein, acting as a docking platform for the submodules that recognize their specific RNA targets. Notably, structural information on how the different exosome targeting complexes specifically recruit their RNA targets is still missing to date.
The RMN submodule, comprising Rmn1, Pab2 and Red5, plays an important role in recruiting CUTs for degradation. Here we show that Pab2 is a poly(A)-binding protein which forms oligomers upon RNA-binding. Using biophysical techniques and electron microscopy, we gained valuable insights in the molecular organisation of this submodule. Additionally, the comparison between Pab2 and its orthologue Pab1 revealed a potential new mode of poly(A)-binding. These findings improve the understanding of how MTREC selectively recruits targets for degradation by the nuclear exosome.

56 Maialen Sebastian-delaCruz (University of the Basque Country (UPV/EHU), Leioa, Spain)

Linking viral infections and autoimmunity: Ac4C as a novel epitranscriptomic regulator

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Celiac disease (CeD) is a complex, chronic inflammatory disease mainly affecting the intestine, in which genetic and environmental factors are implicated. Viral infections have been proposed as environmental triggering factors for some intestinal inflammatory disorders. In the case of CeD, dsRNA viruses such as rotavirus and reovirus are the main triggering viral candidates. Reoviral infections have been reported to induce the characteristic loss of tolerance (LOT) to dietary gluten that occurs in CeD patients, through the upregulation of Interferon Regulatory Factor-1 (IRF1). In addition, viral infections are also known to alter the epitranscriptomic landscape of the host cell.

On this basis, we hypothesized that epitranscriptomic modifications might be involved in the relationship between viral infections and CeD. In this line, we have recently described an m6A-mediated mechanism that regulates IRF7 upon the combination of viral infection and gluten ingestion. However, IRF1 does not seem to be under m6A control in this setup and whether is regulated by epitranscriptomic mechanisms in the context of CeD remains unclear. N4-acetylcytidine (Ac4C) is a recently identified epitranscriptomic modification on mRNA molecules. It is catalyzed by the enzyme N-acetyltransferase 10 (NAT10), the only human enzyme to has both acetyltransferase and RNA binding activities. Besides, IRF1 mRNA has been reported to harbor Ac4C sites.

Thus, to decipher whether Ac4C could be involved in IRF1 regulation in the intestine, we analyzed NAT10 and IRF1 expression in biopsy samples from 15 celiac pediatric individuals and 15 controls. We observed that both NAT10 and IRF1 are upregulated in CeD patients. Furthermore, their expression showed significant correlation. In addition, quantification of total RNA acetylation revealed higher Ac4C levels in CeD patients, in accordance the with increased NAT10 expression. Next, we assessed if viral infections affect total RNA acetylation. Transfection of Poly(I:C), the synthetic analog of dsRNA molecules, in HCT-15 intestinal cells, resulted in alteration of NAT10 expression, and in an increase of total Ac4C levels. As expected,

IRF1 was also augmented after mimicking viral infection. Manipulated NAT10 expression by an overexpression plasmid did not affect IRF1 expression. However, silencing of NAT10 resulted in a reduction of IRF1 protein levels. Lastly, RNA immunoprecipitation of Ac4C modifications followed by RT-qPCR, confirmed the presence of this modification in IRF1 mRNA in intestinal cells.

Altogether these results suggest that IRF1 could be regulated by NAT10 and Ac4C-mediated mechanisms in the intestine contributing to the LOT observed in autoimmune CeD.

57 Aditya Sethi (The Australian National University, Canberra, Australia)

Co-transcriptional m6A deposition precedes global splicing catalysis

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Splicing-mediated exon-junction complex (EJC) deposition has been implicated as a central determinant of the m6A epitranscriptomic landscape. However, recent studies suggest that m6A deposition begins co-transcriptionally, potentially preceding much of splicing catalysis. How splicing and m6A cooperate to direct m6A topology remains an open question.

In this study, we resolve the kinetics of co-transcriptional m6A methylation and splicing catalysis via direct RNA sequencing of RNA polymerase II-bound pre-mRNA. We use nanopore signals to distinguish and separate co-transcriptional and post-transcriptional reads, accurately mapping thousands of m6A sites to the mammalian nascent epitranscriptome. We leverage nanopore long-read to simultaneously measure transcriptional elongation, splicing catalysis, and m6A deposition at single molecule resolution, uncovering widespread co-transcriptional m6A deposition across pre-mRNA molecules. Using our epitranscriptome maps, we identify m6A hypermethylation domains on fully-unspliced molecules, demonstrating that m6A is deposited on pre-mRNA prior to any splicing catalysis. Surprisingly, we observe a global depletion of m6A methylation in the vicinity of unspliced exon-intron junctions, implicating EJC-independent mechanisms which must supress m6A at these regions.

In summary, we reveal rapid co-transcriptional m6A deposition kinetics that precede most splicing catalysis. We demonstrate that the selectivity of m6A deposition is established early during transcription, independently of the EJC.

58 Hamrithaa Shanmuganathan (Philipps University Marburg, Germany)

NAD-RNA mediated control of gene expression during macrophage infection by Klebsiella pneumoniae

Hamrithaa Shanmuganathan

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Pathogens often target the nicotinamide adenine dinucleotide (NAD+) metabolism during infection, and defects in this metabolism have been associated with various human disorders. Thus, the NAD metabolism has emerged as a potential therapeutic target. NAD can be incorporated at the 5'-end of RNA and NAD caps have been found to (i) protect bacterial RNA from rapid decay and (ii) promote RNA degradation in mammalian cells. Cellular processes that involve the addition, removal, or modification of RNA caps are of particular interest. By targeting NAD capped RNAs with NAD consuming enzymes like NudC, a mechanism might be provided where alterations of the NAD metabolism can be linked to alterations in gene expression especially during Klebsiella pneumoniae infection of human macrophages. Here, we provide first insights into the presence of NAD-RNAs in the human pathogen K. pneumoniae by liquid chromatography-mass spectrometry (LC-MS) and NAD capture-seq. Fifty-three specific NAD-RNAs were identified in K. pneumoniae. Sequencing analysis showed that all enriched transcripts contain an A at the transcription start site and upstream promoter motifs were identified, suggesting that the addition of NAD-caps occurs co-transcriptionally. We are investigating the modulation of bacterial NAD and NAD-RNA levels by knocking out the the Nudix hydrolase NudC. Initial infection studies showed a lower phagocytosis rate and infectivity of the knockout strain. Recombinant K. pneumoniae NudC was purified and its NAD-RNA decapping activity was verified. We aim to investigate the impact of NAD-RNA integrity on gene expression during macrophage infection by comparing the wildtype K. pneumoniae with the NudC knockout strain.

59 Ameya Sinha (Goethe University Frankfurt am Main, Germany)

A functional genomics approach to dissect the role of the epitranscriptome in influenza biology

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RNA viruses, as its eponymous name suggests, embed their genetic information in the form of RNA. Of these, Influenza A virus (IAV) is an obligate intracellular parasite that depends heavily on host machinery for its replication and propagation. While it encodes a polymerase for RNA synthesis, it must make effective use of host RNA-binding proteins to coordinate splicing, nuclear import and export, and translation of each viral RNA segment. Central to the biology of every RNA molecule, including viral RNAs, are chemical modifications that it undergoes collectively labelled as the 'Epitranscriptome'. More than the 170 known modifications determine the fate of all RNAs within the cell, impacting their location, processing, and interaction with other biomolecules within the cell. In the context of an infection, these modifications can either be protective or lethal when considering the host immune response. A catalogue of 214 different RNA-modifying proteins (RMPs) catalyze the addition or removal of these chemical moieties in multi-step processes or are known to interact with modified RNA. Here, we sought to study these host RNA modifying proteins en masse in the context of an influenza infection by carrying out a single-cell CRISPR screen. Specifically, we carried out a CRISPR interference (CRISPRi) experiment with a guide pool of RMPs in human lung carcinoma cells (A549) infected with the Influenza A H1N1 PR8 virus isolate and subjected the cells to single-cell RNA sequencing and guide capture. First, we validated that the perturbation of these RMPs by CRISPRi led to a downregulation of their cognate target. Next, we tagged each RMP knockdown with the host transcriptional response and infection status of the cell concurrently. We identified RMPs that upon knockdown are refractory to IAV and conversely others that make the cells more susceptible to infection. Of interest were also perturbed cells that show high viral load, but do not produce productive progeny due to the loss of one or more IAV segments. We also identified a transcriptionally distinct cluster of perturbed cells where interferon signaling was strongly upregulated in contrast to the fact that IAV generally suppresses such an immune response, implicating the importance of these RMPs in immune evasion. This work will further be complemented with validating and dissecting the mechanistic role of several of these RMPs in controlling viral infections in a systematic way.

60 Anton Škríba (Institute of Organic Chemistry and Biochemistry AS CR, Prague, Czech Republic)

HILIC as an alternative separation method for RNA-caps and short oligonucleotides analysis

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Recent expansion of RNA chemical modifications field opened new questions regarding post-transcriptional gene regulation. Apart from internal modifications, in our group we mostly focus on 5'-RNA caps. These caps are typically derived from various metabolites such as cofactors [1,2] and dinucleotide polyphosphates [3]. Although their presence is believed to influence RNA stability, cellular metabolism, and mRNA translation, their exact role remains poorly understood. The physicochemical properties of RNA caps, which include high hydrophilicity, acidic phosphate functional groups, and nucleobases, make their liquid chromatography mass spectrometry (LC-MS) detection challenging. Currently, these molecules are analyzed by reversed-phase chromatography with ion-pairing agents, such as alkylammonium salts. This technique is well established in the oligonucleotides field, however the presence of high salt concentration suppresses the ionization and lowers the sensitivity of mass spectrometry detection. We have employed an alternative method - HILIC (hydrophilic interaction chromatography), which does not need such strong ion-pairing agents and can be used even for analysis of longer oligonucleotides [4].

This work presents an overview of protocols used in qualitative and quantitative analysis of canonical and non-canonical 5'-RNA caps in bacteria and mammalian tissue cell cultures. The method consists of RNA isolation, digestion by various enzymes (Nuclease P1, NudC, alkaline phosphatase), purification by solid phase extraction and subsequent analysis by LC-MS. As examples, we present application of this protocol in detection of NAD cap in RNA from HIV-infected MT4 cells and in Bordetella pertussis bacteria, dephospho-coenzyme A RNA cap in Escherichia coli, Ap4A RNA cap in human embryonic kidney and rat basophilic leukemia cells and hypermethylation of canonical cap in small nuclear RNA. The structural identification of these caps was validated based on retention time, m/z ratio and compared to commercial standards. In some cases, fragmentation spectra were used to confirm the identity of the caps.

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61 Claudia Sudol (Sorbonne Université - Institut de Biologie Paris-Seine, France)

Functional redundancy in tRNA dihydrouridylation

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Transfer RNA (tRNA) undergoes chemical modifications during its maturation process, catalyzed by modification enzymes. These post-transcriptional modifications can serve as quality control markers for the RNA structure or enhance the fidelity and efficiency of translation. Among these modifications is dihydrouridine (D), which is highly abundant and conserved across all domains of life. Dihydrouridine is found predominantly in tRNA and, more recently, in messenger RNA (mRNA). D is the product of the C5=C6 double bond reduction in uridine. The formation of dihydrouridine in tRNAs is catalyzed by a large family of flavoproteins called dihydrouridine synthases (Dus), which belong to the COG0042 (Cluster of Orthologous Group) family of flavoproteins. These enzymes have been shown to use flavin mononucleotide (FMN) as a redox coenzyme and NADPH as a hydride source necessary for reducing flavin. Dihydrouridine is commonly found at multiple canonical sites in tRNAs (D16-D17-D20-D20a-D20b-D47). This distribution varies depending on the considered organism and/or the tRNA. Despite its prevalence, mechanisms of dihydrouridine biosynthesis, particularly in prokaryotes, remain ill-characterized.

To overcome this gap, we examined D-biosynthesis in a model Gram-positive bacterium, Bacillus subtilis. By combining genetic, biochemical, and deep-sequencing methods, we discovered that B. subtilis uses two FMN-dependent Dus-like flavoproteins, BsDusB1 and BsDusB2, to introduce D residues into tRNAs. DusB1 is unique in its ability to oxidize both NADH and NADPH, while DusB2 prefers NADPH, catalyzing D formation at specific tRNA positions. This study revealed functional redundancy in these enzymes, targeting only a subset of tRNAs and suggesting an evolutionary process that may lead to more specialized enzymes. These findings provide new insights into the biosynthesis of dihydrouridine in prokaryotes and underscore the role of enzyme redundancy in this process.

62 Yu Sun (Humboldt University Berlin, Germany)

Detection of queuosine and queuosine precursors in tRNAs by direct RNA sequencing

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tRNAs carry diverse chemical modifications that play crucial roles in tRNA stability, folding, and fine-tuning mRNA decoding. Queuosine (Q) modification is a hypermodified 7-deaza-guanosine analogue found at the Wobble position 34 in NAC/ U-decoding tRNAs, and its presence equilibrates translational speed between the C- and U-ending codons. Our previous study has shown that in the fission yeast Schizosaccharomyces pombe, Q modification enhances the translational speed of the C-ending codons and decreases the speed of the U-ending codons. Furthermore, the presence of Q is also involved in the regulation of translation accuracy. A deeper understanding of Q has been challenging due to the limited availability of high-throughput methods for its detection.

In this study, we have harnessed direct RNA sequencing (Nanopore sequencing, Oxford Nanopore Technology, ONT) to detect Q modification on in vitro-transcribed tRNAs as well as on tRNAs ex cellulo from S. pombe and Escherichia coli. To obtain end-to-end base calling, adapters are ligated to the 5' and 3' end of tRNAs. Nanopore sequencing of in vitro-transcribed Q-tRNAs revealed increased base-calling errors at and around Q34 positions. Q34 could be distinguished from G34 by comparing read signatures from Q-modified and non-modified tRNAs using the software JACUSA2. Next, this approach was employed for the detection of Q modification in Q-tRNAs ex cellulo from S. pombe, which showed that Q modification on ex cellulo Q-tRNAs is also detectable. The miscalling profiles caused by Q modification varied depending on the sequence context in the tRNA, and the bases UQU at Q34 often were miscalled as C. Furthermore, the analysis of tRNAs from E. coli strains with defects in queuosine biosynthesis allowed us to distinguish between Q and its precursors preQ1 and preQ0. Specifically, misincorporation and insertion rates for Q and preQ1 were similar, while the deletion rates were lower for preQ1. preQ0 generated less stark base-calling errors compared to that of Q and preQ1 modification, which is in line with the fact that it is chemically most similar to G. Taken together, direct RNA sequencing is a powerful tool for the detection of Q and its precursors at single-base resolution.

63 Carla Ricarda Till (University Regensburg, Germany)

Investigation of molecular mechanisms shaping the m6A landscape

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Methylation of Adenosine (m6A) is one of the most prominent mRNA modifications regulating gene expression by affecting fundamental processes like mRNA degradation and translation. m6A is installed within a consensus sequence referred to as the DRACH motif (D=G/A/U; R=A/G; H=A/C/U) by the m6A-writer complex, consisting of METTL3, METTL14, WTAP, VIRMA, ZC3H13 and HAKAI. While distribution of DRACH motifs in a transcript does not display a distinct pattern, most of the m6A sites are located around the stop codon, in the 3' UTR and in long exons while a small percentage of modified sites can be found in the 5' UTR. One of the known mechanisms that shape this m6A landscape is the physical exclusion of the m6A-writer complex from splice-site proximal regions by the Exon Junction Complex (EJC). However, this model does not fully account for the discrepancy between predicted and modified m6A sites.

The investigation of further aspects affecting the m6A methylation pattern is the aim of this study. For this purpose, in vitro methylation assays will be performed on non-methylated mRNA isolated from human METTL3 knockout cells using recombinant proteins of the m6A-writer complex. Via m6A-sequencing, the in vitro methylation pattern will be mapped against its in vivo methylated counterpart. This will enable us to identify sites that are methylated exclusively in vitro but not in vivo, sites that are neither methylated in vitro nor in vivo and sites that are methylated in vivo but not in vitro. Based on this data set, we will investigate molecular mechanisms that prohibit or mediate methylation at the identified sites. The knowledge gained by this research could enhance our understanding of how the m6A landscape is shaped.

64 Sabrine Toubdji (Sorbonne Université - Institut de Biologie, Paris-Seine, France)

Biological and biochemical characterization of dihydrouridylation in bacterial ribosomal

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Dihydrouridine (D) is a prevalent and evolutionarily conserved modification found mainly in tRNAs and, to a lesser extent, in mRNAs. In E. coli, it extends to position 2449 of the 23S rRNA, strategically located near the ribosome's peptidyl transferase site. Despite the existence of known dihydrouridine synthases (DUS), which utilize NADPH and FMN, the enzyme responsible for biosynthesizing D2449 has remained elusive.

This study introduces a rapid method for detecting D in rRNA, involving reverse transcriptase blockage at the rhodamine-labeled D2449 site followed by PCR amplification (RhoRT-PCR). Through analysis of rRNA from diverse E. coli strains, including those with chromosomal deletions and point mutations, the yhiN gene was pinpointed as the ribosomal dihydrouridine synthase, now designated as RdsA.

Biochemical characterizations revealed RdsA as a novel class of flavoenzymes dependent on FAD and NADH, exhibiting a complex structural topology. In vitro assays demonstrated that RdsA dihydrouridylates an rRNA transcript, mimicking a segment of the peptidyl transferase site, suggesting an early introduction of this modification before ribosome assembly. Phylogenetic studies unveiled the widespread distribution of the rdsA gene in the bacterial kingdom, emphasizing the conservation of rRNA dihydrouridylation.

These findings underscore nature's preference for utilizing reduced flavin in the reduction of uridines and their derivatives, highlighting the importance of this modification in RNA biology and bacterial physiology.

Biological characterization using polysome profiling analysis and ribosome Cryo-EM studies to investigate how dihydrouridine modification influences ribosomal function and cellular processes. The results suggest that D-2449 play significant roles in the structural dynamics and architectural stability of rRNA, thereby impacting translation efficiency and accuracy. Overall, these results emphasize the importance of dihydrouridine in RNA folding dynamics and its broader implications for cellular processes and evolutionary adaptations.

65 Andrey Turchinovich (Heidelberg University, Mannheim, Germany)

High-throughput studies of the RNA-binding protein HuR targetome

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Background

Human antigen R (HuR), preferentially associates with AU-rich elements (AREs) within 3'-UTRs of mammalian mRNAs regulating their steady-state levels. HuR-regulated mRNAs encode proteins involved in multiple molecular pathways including those strongly related to progression of cardiovascular diseases (CVDs). RNA immunoprecipitation (RIP) and individual nucleotide resolution cross linking and immunoprecipitation (iCLIP) are being harnessed to characterize RNA binding sites of RBPs, including those of HuR protein in multiple previous reports. However, their sensitivity can be limited by standard RNA sequencing methods which rely on either 3’-adapter ligation or long RNA-seq. The main goal of this project was to investigate HuR-associated RNAs in human umbilical vein endothelial cells (HUVECs) using RIP and iCLIP methods coupled with ultrasensitive semi ligation-based RNA-seq library preparation method. In addition, we developed a novel approach “direct CLIP (DiCLIP)” and benchmarked it with standard iCLIP on HUVECs HuR IP samples.

Methods

Both RIP and DiCLIP pellets were preformed from paraformaldehyde fixed HUVECs lysates according to in-house developed workflows using anti-HuR as well as control IgG antibody. Upon total RNA isolation, stranded small RNA-seq libraries were prepared using 3’-polyadenylation and 5’-ligation of RNA adapters and sequenced on Illumina NextSeq 550.

Results

Multiple transcripts have been detected by RNA-seq in RIP pellets when using as little as 0.6-6 ng total RNA inputs. Furthermore, 67% RNA transcripts (8079 out of 12076 detected) were significantly (BH adj. pvalue < 0.05) enriched in anti-HuR vs. IgG RIP pellets. Downstream GO analysis showed that mRNAs strongly associated with HuR (> 3.5 LFC versus IgG background) encode proteins enriched in mTORC1, VEGF and TGF-beta signaling pathways which are related to development of atherosclerosis and other CVDs. Multiple transcripts have been detected by RNA-seq in DiCLIP pellets when using as little as 0.3-0.5 ng total RNA inputs. Furthermore, DiCLIP peaks were in close proximity/overlapping putative HuR binding motifs, while classical iCLIP peaks were 10-12 bases downstream of HuR binding sites. Interestingly, DiCLIP identified multiple HuR binding sites which were overlooked in classical iCLIP datasets.

Finally, we show that N6-methyladenosine (m6A) methylation significantly influences HuR binding of multiple transcripts confirming its crucial role in various cellular processes.

Conclusion

Classical RIP coupled with ultrasensitive semi-ligation based small RNA-seq revealed multiple previously unreported mRNAs associated with HuR protein. Newly developed protocol DiCLIP enables characterization of RNA binding sites of RBPs from low sample inputs and with higher resolution as compared to classic iCLIP methodology.

66 Folkert van Werven (Francis Crick Institute, London, United Kingdom)

Towards a molecular understanding of the m6A machinery in cell fate control in yeast

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N6-methyladenosine (m6A) is a highly abundant and evolutionarily conserved messenger RNA (mRNA) modification. This modification is installed on mRNAs by a hetero-multimeric enzyme known as the m6A methyltransferase complex (MTC) and read by a conserved class of proteins characterized by the YTH domain. Using Saccharomyces cerevisiae, also known as budding yeast, as a model, we aim to dissect the molecular functions of the MTC and a single m6A reader protein, which are both active and important for the early stages of the meiosis programme. We recently demonstrated that, unlike the mammalian bipartite MTC, the yeast MTC is unipartite yet multifunctional. The mRNA-interacting module within the MTC exerts m6A-independent functions, while the catalytic components of the MTC are responsible for m6A deposition. Both functions are critical for meiotic progression. In yeast, the m6A mark is read by the conserved YTH protein Pho92, which mediates m6A-mRNA decay contingent on active translation and the CCR4-NOT complex. We propose that yeast serves as an exceptional model for studying the fundamental molecular mechanisms related to the function and regulation of m6A-modified mRNAs. Our findings valuable insights into the conservation and diversification of m6A regulatory pathways across species.

67 Alexandre Magno Vicente (Goethe University Frankfurt am Main, Germany)

Comparative Epitranscriptome Unveils Stress-Specific tRNA Modification Reprogramming across Eukaryotes and Prokaryotes under Heat

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The control of gene expression in stress response is an essential mechanism of adaption. To date, many studies have unveiled genes that are crucial to stress response. Recently, studies focusing on the post-transcriptional level of gene regulation have shown that RNA modifications play a role in stress response in many ways, such as configuring the RNA stability, translation efficiency, RNA structure and alternative splicing. Although small in size, tRNAs are the most extensively modified RNA in the cells. These small RNAs are commonly associated with a stress-specific reprogramming of RNA modifications, to fine-tune the control of translation. Here, we performed a comparative epitranscriptome analysis using different organisms and a common stress condition. More specifically, Arabidopsis thaliana, Escherichia coli and Saccharomyces cerevisiae were grown under optimal and heat-stress conditions to find out specific heat-stress tRNA modifications that are exclusive or mutual among these organisms. For this purpose, total RNAs were extracted under heat-stress conditions. Subsequently, tRNAs were isolated using SEC (Size Exclusion Chromatography), digested into single nucleosides and submitted to LC-MS/MS for quantification. Overall, we observed a complex profile of RNA modifications under heat for both eukaryotes and prokaryotes. Interestingly, specific up- and down-regulated RNA modifications are shared among these organisms. acp3U, for example, is down-regulated in both E. coli and A. thaliana exclusively under heat treatment. Therefore, our results suggest that a heat stress tRNA reprogramming potentially plays important regulatory and translational roles in all domains of life.

68 Marta Walczak (Max Planck Research Group, Malopolska Centre of Biotechnology, Jagiellonian University, Krakow, Poland)

A Cascade of Questions: Unraveling the Complexities of tRNA Thiolation

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In all living organisms, tRNA modifications are important for the proper and efficient synthesis of cellular proteins. The exchange of oxygen with sulfur (thiolation) at the C2 position of wobble base uridines (U34) facilitates codon recognition and reading frame maintenance. In humans, the highly conserved CTU1-CTU2 complex carries out the final step of the reaction and thiolates four cytoplasmatic tRNAs, namely tRNA(Lys)UUU, tRNA(Glu)UUC, tRNA(Gln)UUG and tRNA(Arg)UCU. Lack of thiolation in U34 of tRNAs affects intracellular proteostasis and leads to occurrence of various neurodegenerative disorders. Patient-derived mutations in the CTU2 subunit are found to be associated with the DREAM-PL (dysmorphic facies, renal agenesis, ambiguous genitalia, microcephaly, polydactyly, and lissencephaly) syndrome. The exact molecular mechanisms behind the CTU1-CTU2-mediated sulfur transfer reaction and the tRNA selectivity remain elusive. Here, we aim to uncover and explain the mechanism of tRNA thiolation in humans and other eukaryotes at the molecular level. In detail, we aim to characterize the binding specificity of different tRNA substrates, to image different reaction intermediates at high-resolution by single particle cryo-EM and to identify the role of additional binding partners. To achieve those goals, we established an efficient expression and purification protocol for the human CTU1-CTU2 complex. The already obtained reconstructions enable us to identify key regions involved in tRNA recognition, as well as to define the reaction center. Moreover, we found that the CTU1-CTU2 complex can exist in various, previously undescribed oligomeric states, which seem to be highly relevant for its function and the regulation of the entire thiolation pathway. We examine the reaction kinetics of the purified complex using radioactive assays and complementary biochemical approaches that can provide direct insight into the sulfur transfer via the pathway components. Besides the focus on characterization of the CTU1-CTU2 complex, the project also aims to reconstitute the entire tRNA thiolation pathway in vitro. Our in vitro studies are complemented by analyses

of thiolation in human cell lines, which allows us to verify our structural findings and their clinical impact on the cellular level.

69 Lukas Walz (Institute of Pharmaceutical and Biomedical Sciences, Johannes Gutenberg-University Mainz, Germany)

N1-methylation of adenosine (m1A) in ND5 mRNA leads to complex I dysfunction in Alzheimer's disease

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With an increasingly older population, the number of patients with neurodegenerative disorders such as Alzheimer's disease (AD) grows continuously. AD, defined by neuronal loss accompanied by cognitive decline, will therefore pose a significant burden. Yet treatment options remain suboptimal, necessitating further research.

A disruption of energy metabolism and its harmful effects on neuronal health are early events in AD pathology. Malfunctioning mitochondria are a pathological feature linking aging and neurodegenerative disorders. Disease-specific characteristics enhance mitochondrial dysfunction occurring during aging, leading to a tipping point where compensatory mechanisms are exhausted, and neuronal deterioration ensues. This mitochondrial impairment in AD is attributed to defects in complexes I and IV of the respiratory chain.

The mitochondrial encoded subunit of complex I ND5 is essential for its function. ND5 transcripts are known to harbor N1-methyladenosine (m1A) at position 1374 within their coding sequence, incorporated by its writer enzyme TRMT10C. m1A interferes with protein production by disrupting Watson-Crick base pairing. We aimed to investigate the hypothesis that complex I dysfunction in AD is induced by hypermethylation of ND5, facilitated by a pathological increase of TRMT10C. Our research utilized a diverse range of techniques, such as western blot analysis, Illumina sequencing, RNA modification analysis, bioinformatics, and assays for mitochondrial respiration.

We identified that mediators of mitotoxicity in AD, Aβ and reactive oxygen species, increased the expression of TRMT10C in AD-specific cell models. This led to a rise in m1A levels on ND5 transcripts, which we detected via a misincorporation-based sequencing. Subsequently, we observed decreased ND5 protein quantities. Overexpression of TRMT10C increased m1a levels, disturbed mitochondrial respiration, and decreased mitochondrial membrane potential. Consequently, siRNA-mediated TRMT10C knockdown normalized m1A levels and rescued ND5 protein expression. Our group also identified elevated TRMT10C expression in post-mortem AD brain tissue and further substantiated these findings by analysis of published

RNA-Seq data. m1A sequencing of AD patient brain tissue revealed hypermethylation of ND5 mRNA, with consequently decreased ND5 protein levels.

We propose a new mechanism contributing to mitochondrial dysfunction in AD, where a pathological augmented level of m1A on ND5 mRNAs, mediated by increased expression of TRMT10C, leads to diminished protein levels of ND5 and thereby to a disturbance of complex I and mitochondrial respiration.

This newly proposed pathway of Complex I dysfunction in AD contains promising targets for early pharmacological intervention and exciting opportunities for diagnostic tools.

70 Annabelle C. Weldert (Institute of Pharmaceutical and Biomedical Sciences, Johannes Gutenberg-University, Mainz, Germany)

Development of heterobifunctional degraders for RNA and RNA-modifying enzymes

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Proteolysis targeting chimeras (PROTACs) are novel therapeutic modalities that have emerged in recent years. These heterobifunctional compounds recruit E3 ligases, such as cereblon (CRBN) or Von Hippel-Lindau factor (VHL), in close proximity to a protein of interest (POI), leading to targeted degradation of the POI. Since 2019, several PROTACs have entered clinical trials, mainly targeting proteins involved in different types of cancer and autoimmune diseases.1 We designed and synthesized PROTACs targeting the human METTL3/14 methyltransferase. METTL3/14 is involved in multifaceted biological activities, such as cell cycle, apoptosis, autophagy, and differentiation, and has an impact on numerous types of cancers including breast cancer, lung cancer, and acute myeloid leukemia.2 Additionally we are interested in, ribonuclease-targeting chimeras (RIBOTACs). Similar to PROTACs, RIBOTACs function as targeted degraders, however, targeting an RNA of interest (ROI) instead of a POI. RIBOTACs can selectively bind and degrade ROIs by activation of RNase L, part of the innate immune system, an otherwise latent ribonuclease.3 Choosing the optimal composition of heterobifunctional molecules includes deciding on optimal linker length, type of recruiter, and type of POI/ROI-binder. Therefore it is important to characterize the interaction between our molecules and their respective interaction partner. Hence, we employed various biophysical methods such as fluorescence polarization (FP), and Förster resonance energy transfer (FRET) based assays. Moreover, characterization of the ternary complexes is essential for successful degrader development. To achieve this, we developed a homogeneous time-resolved fluorescence (HTRF)-based method in our PROTAC studies. Additionally, we investigate the ternary complex using the FP assay.

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71 Hagen Wesseling (Goethe University Frankfurt am Main, Germany)

Artificial nucleases to deplete ribosomal RNAs

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Besides the abundant ribosomal and transfer RNA (rRNA and tRNA, respectively) transcripts, there are tens of thousands of long coding (mRNA) and non-coding transcripts (lncRNA) within each cell. To date, studies have only revealed the occurrence of RNA modifications in a small number of these long RNA transcripts. One reason for this is that most mRNAs and lncRNAs are low abundant and therefore difficult to isolate. In addition, they are equal in length to rRNA, which makes up 90 % of total RNA, preventing size-based purification. State of the art mRNA purification protocols are based on poly(A) enrichment with subsequent rRNA depletion. The inaccessibility of non-poly-adenylated RNAs, residual remnants of ribosomal and transfer RNAs in mRNA samples, as well as high costs, are only some of the limitations of these methods. Thus, a method to make (pre)mRNAs and lncRNAs accessible for LC-MS analysis is lacking.

Here, we show that the use of artificial nucleases to cleave ribosomal RNA into shorter fragments allows size-based purification of mRNAs and other long RNAs. Sequence-specific cleavage of rRNA is achieved by reverse-complementary DNA oligonucleotides linked to a tris(2-aminobenzimidazole) structure. After this cleavage, rRNA fragments as well as other short RNAs can be removed from long coding and non-coding RNAs, which makes the long RNAs accessible for subsequent LC-MS analysis. Comparison with RNase H-based rRNA depletion shows that artificial nucleases cleave with higher specificity and therefore lead to decreased off-target RNA degradation.

In summary, we present a novel tool to deplete interfering rRNA, which in turn enriches lncRNAs and (pre)mRNAs. Subsequently, the function of lncRNAs and the modification patterns of long RNAs can be investigated in future studies.

72 John Williams (University of East Anglia, School of Biological Sciences, Norwich, United Kingdom)

The parental contribution of modified snRNAs

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Cellular RNA modifications provide dynamic and diverse control mechanisms for gene expression, including pre-mRNA splicing. Pre-mRNA splicing requires the involvement of small nuclear RNAs (snRNAs) U1, U2, U4, U5 and U6, along with approximately 100 proteins, to assemble a functional spliceosome. These snRNAs carry various modifications, some with known and unknown roles in splicing. Our group studies the U6 snRNA m6A modification, which is deposited by C. elegans METT-10 and human METTL16 methyltransferases (Mendel et al., 2018; Pendleton et al., 2017). Our group recently showed the essential role of U6 m6A in splicing (Shen et al., 2024). When C. elegans lack METT-10, they exhibit specific temperature-sensitive phenotypes, including slow growth (Gro), protruding vulvas (Pvl) and sterility (Ste). However, mett-10 null animals derived from heterozygous mothers do not exhibit these phenotypes; instead, they display maternal effect lethality (Dorsett et al., 2009). In C. elegans, maternal effect lethality refers to the survival of animals carrying homozygous mutations when derived from heterozygous mothers. At the same time, their own offspring are non-viable (Kemphues et al., 1988).

This project aims to uncover the parentally inherited factor(s) rescuing mett-10 temperature-sensitive phenotypes while also causing maternal effect lethality in the subsequent generation. We generated two fluorescent strains to easily follow the wild type and mutant mett-10 alleles in genetic crosses. We will first investigate the pre-mRNA splicing profiles in zygotic vs maternal-zygotic mett-10 mutants. Next we will investigate the U6 snRNA m6A levels in zygotic mutants using the GLORI-seq approach (Shen et al., 2024).

73 Nur Yesiltac (Goethe University Frankfurt am Main, Germany)

Site-specific Detection of Pseudouridine by Destruction of Uridine using Mass Spectrometry

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As an essential mediator between the genetic code encoded in DNA and the cellular processes regulated by proteins, RNA plays a central role in the complex world of biological phenomena. Epitranscriptomics is concerned with the dynamic and reversible nature of RNA modifications, which have been shown to play a key role in various diseases, in particular neurological disorders and cancer. A comprehensive understanding of the dynamics of RNA modifications is crucial for the further development of disease diagnostics and possible therapeutic interventions.

In this scientific challenge, nucleoside mass spectrometry (MS) is proving to be a robust tool for the detection and quantification of RNA modifications. Although conventional enzymatic hydrolysis at the nucleoside level is powerful, it lacks important sequence information. To overcome this limitation, an innovative oligonucleotide MS technique is currently being investigated. By using dinucleotide-specific RNases, in particular colicin E5, this technique generates informative oligonucleotide fragments of about 15 to 35 nucleotides. Subsequent analysis using high-resolution Orbitrap-MS enables an understanding of the RNA sequences and overcomes the limitations of conventional MS approaches.

However, the identification of isomers, especially uridine (U) and pseudouridine (Ψ), remains a challenge. Hydrazine is used to discriminate between U and Ψ in sequencing studies, where reaction with U under combined hydrazine/aniline treatment results in RNA cleavage that allows discrimination of U and Ψ. In this study, we systematically vary the reaction conditions, including concentrations, incubation times and temperatures, to achieve near-complete conversion of U while minimising RNA cleavage and ensuring no depletion of pseudouridine. Absolute quantification of U by nucleoside MS confirms the successful hydrazine-mediated conversion of U without affecting Ψ. Using nucleoside MS, we have successfully quantified U in absolute terms to determine the number of uridines that have reacted with hydrazine, and we have shown that pseudouridine is not affected by hydrazine. In summary, these results demonstrate the robustness of hydrazine treatment in the detection of pseudouridine in RNA sequences and provide an alternative method for the site-specific identification of mass-silent pseudouridine.

74 Elena Zemlyanskaya (Institute of Experimental Botany, Czech Academy of Sciences, Prague, Czech Republic)

How does tRNA modification t6A affect plant development?

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Among all RNA types, tRNAs stand out as possessing the most extensive array of covalent modifications. However, a comprehensive understanding of the biological role of tRNA modifications, especially in multicellular organisms, remains elusive. The main focus of our project is threonylcarbamoyladenosine (t6A), one of the most ancient tRNA modifications that marks A37 position of ANN-decoding tRNA molecules. t6A is involved in maintaining translation fidelity in eukaryotes. We characterize the components required for the t6A formation in Arabidopsis thaliana and reveal that major t6A biosynthesis genes are indispensable for the earliest steps of plant morphogenesis. Our preliminary data suggest that conditional knockdowns of the key t6A biosynthesis genes show non-overlapping phenotypes, indicating auxiliary roles for these genes in other pathways. Furthermore, we show that t6A is highly abundant as a free compound in plants, in contrast to other adenosine modifications typical for tRNA. Ultimately, by examining the subcellular localization of t6A biosynthesis proteins, we present that the two sequential steps of t6A biosynthesis are likely compartmentalized in Arabidopsis, with the first step occurring in plastids.

75 Hannah Müller (Institute of Pharmaceutical and Biomedical Sciences, Johannes Gutenberg-University Mainz, Germany)

Structural dynamics of the E. coli Guanidine-II Riboswitch

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Riboswitches are cis-regulatory RNA elements in the 5' UTR of several bacteria. They are able to influence multiple steps during protein expression, including transcription, translation and RNA-decay. The Guanidine-II Riboswitch is a translational ON-Switch, meaning that binding of its ligand guanidinium to the aptamer leads to a conformational change and through that translation initiation. More specifically the two hairpins P1 and P2 each coordinate a guanidinium ion and form a kissing loop, which leaves the Shine-Dalgarno-sequence free for ribosome binding. This conformational change can then be detected via smFRET microscopy and can help to further clarify how precisely the switching works. Under closer investigation were especially changes in the primary structure of the RNA and whether they influence the structural dynamics of the riboswitch. In the future the analysis of longer smFRET recordings could furthermore lead to an improved understanding of time dependent conformational changes.

76 Vanessa Krause (Institute of Pharmaceutical and Biomedical Sciences, Johannes Gutenberg-University Mainz, Germany)

Structural dynamics of assembly and turnover in eukaryotic H/ACA RNPs

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H/ACA ribonucleoprotein complexes mediate the sequence-specific pseudouridylation of ribosomal and spliceosomal RNAs, thus playing an important role in ribosome and spliceosome biogenesis. The complex consists of a snoRNA with two hairpin structures with an internal loop where the enzymatic reaction takes place on a substrate RNA which is recruited via base pairing. Eukaryotic H/ACA complexes contain two sets of four core proteins Cbf5, Nop10, Gar1 and Nhp2, with Cbf5 being the catalytic subunit, which bind to each of the hairpins. Despite the basic mechanisms being characterized using archaeal model complexes, the role of several features of eukaryotic H/ACA ribonucleoprotein complexes remain elusive. In particular, the structural and functional roles of both the unique bipartite architecture and the presence of eukaryote-specific protein domains are poorly characterized. We investigate protein-protein as well as protein-RNA-interactions and structural dynamics using smFRET spectroscopy to gain a better understanding of their complex functions and their role in cellular RNA processing.

77 Clara Johanna Grampp (Institute of Pharmaceutical and Biomedical Sciences, Johannes Gutenberg-University Mainz, Germany)

Identification of substrate recognition specificities for 2'-O-methylation of guanosine by TrmH

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Posttranscriptional RNA modifications have been proposed as structural determinants that help the immune system discriminate self and non-self nucleic acids. The 2'-O methylation of guanosine at position 18 in tRNAs (Gm18), found in bacteria, archaea, and eukaryotes, is known to antagonize the human immune receptors TLR7 and TLR8. 1

Due to the immune-antagonizing effects of Gm18, we aim to investigate its biological and physiological roles. To do this, it is essential to identify specific sequence and structural features in RNA that are recognized by the 2'-O-methyltransferase for subsequent substrate methylation. For this purpose, various minimized tRNA constructs based on the TrmH substrate S.cerevisiae tRNAPhe are being tested. The constructs are generated by dividing the tRNA into 5' and 3' halves, which are then further truncated. In a next step, substrate recognition and methylation efficiency are determined using radioactive labeling.

78 Marc Lander (Institute of Pharmaceutical and Biomedical Sciences, Johannes Gutenberg-University Mainz, Germany)

Development of a novel approach for microbiome analysis based on tRNA abundance

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The human organism as well as the environment are vulnerable to various diseases and threads. However, many diseases are not necessarily caused by a specific pathogen, but by an imbalance in the gut microbiome of the host organism.(1,2) The same applies to the diversity of the soil microbiome and associated environmental dependencies.(3) For a better understanding of different microbiomes and their impact on their respective ecosystem, robust analytical methods are mandatory. Common microbiome analysis is based on the isolation of genomic DNA followed by the amplification of 16S or 18S rRNA sequences of interest and their analysis on a next generation sequencing platform. The included PCR amplification is a crucial step for library preparation but introduces bias to the results based on the used primer pairs. This includes unequal sequence amplification and complete omission of specific important taxa.(4,5)

The objective of this work is the development of a new approach for the analysis of different microbiomes, e.g. from soil or the intestine. Thereby we focus on microbial changes due to antibiotic exposure and disease related mouse models. Based on literature data(6,2) we identify highly diverging tRNA sequences for different microbiome members and design unique complementary oligomer pools according to Kristen et al.(7) For microbiome analysis total RNA content <200 nt of soil and intestine samples are isolated and analyzed via a modified version of the DORQ-Seq approach developed by Kristen et al.(7)

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Liposomal based screening approach for the delivery of new DNMT2 inhibitors

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Modifications of ribonucleic acids RNAs are gaining increasing attention due to their significant impact on various physiological and pathophysiological processes in organisms. One field is the development of small molecules to inhibit various methyltransferases such as DNA methyltransferase 2 (DNMT2), which catalyses the methylation of cytosine to 5-methylcytosine. To avoid the time-consuming synthesis of membrane-crossing prodrugs for a first test of the cellular inhibition potential, we developed a delivery system based on liposomes. Liposomal nanoparticles in a size range of 100-200 nm with encapsulated inhibitor are taken up by cells together with their cargo. The encapsulated inhibitor is subsequently released and begins to exhibit its biological activity.

For the identification of potentially suitable liposomal formulations, two established liposomes were modified and then tested by flow cytometry. In the next step, we encapsulated several model cargos and quantified the liposomal-mediated cellular transfer potential as well as the cytotoxicity.

As a result, we were able to identify a first liposomal formulation that showed a high delivery potential, which is now being used for further screening experiments.

80 Sophie N. Mulartschyk (Institute of Pharmaceutical and Biomedical Sciences, Johannes Gutenberg-University Mainz, Germany)

Identification of interaction sites between tRNA and its modifying enzymes using Chemical Crosslinking

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Post-transcriptional modifications significantly determine the structural and functional properties of tRNA.[1,2] Methylation of nucleobases catalyzed by methyltransferases (MTases) is the most most abundant and important type of modification.[3-5] Editing enzymes like MTases, responsible for the incorporation of modifications in the sequence of RNA, control the functionality of the tRNA.[1,3,6-,8] Dysregulation of the enzymatic control through expressional abnormalities leads to aberrant RNA modifications which are associated with the ability to cause human diseases.[6-10]The discovery of RNA-protein interactions contributes to the understanding of complex mechanisms involved in biological processes and pathogenesis. By unveiling these correlations, new therapeutic approaches can be developed. [8-10]

The aim of this work was the development of a suitable method which enables the analysis of interaction points between tRNA and its modifying enzymes DNMT2, Trm(A, D,E) and TGT based on chemical crosslinking in combination with LC-MS/MS.

By inducing covalent linkage in the presence of chemical agents the tRNA-protein complex is captured and stabilized for further analytical steps.[11] UV-crosslinking is considered as conventional method but has its limitations, whereby alternatives are demanded.[12] Therefore, we test the bifunctional alkylating agents chlorambucil and busulfan as potential crosslinkers, due to their observed ability to covalently link nucleic acids and protein in an individual manner.[13-15]

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Detection and investigation of oxidatively damaged RNA

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Oxidative stress plays a major role in biological systems occurring naturally in physiological processes.[1,2] In particular, significant amounts of reactive oxygen species (ROS) are produced in mitochondria as part of the respiratory chain. On the other hand, the immune system generates hypochlorous acid (HOCl), catalyzed by the enzyme myeloperoxidase, to fight pathogens as part of the immune response. HOCl induces chlorination and oxidation of various biomolecules such as lipids, peptides and nucleic acids, altering their native function and structure.[3] Known modifications include *N*-chloramines, 8-chloro- and 8-oxopurines as well as 5-chloro- and 5-oxopyrimidines. Especially, guanine as the most susceptible nucleobase towards oxidation has been the primary target of investigation, leading to the identification of several oxidized derivatives such as spiroiminodihydantoin (Sp), guanidinohydantoin (Gh), oxazolone (Z), imidazolone (Iz) and diiminoimidazole (Diz). Oxidation-induced damage has been well investigated on the level of DNA; however, the effect on RNA is poorly understood. Our goal is to investigate the effects of oxidative stress on RNA using methods such as UV absorbance, LC-MS/MS and Next Generation Sequencing (NGS). Furthermore, a novel NGS method is established named OxiAbSeq, a chemistry-based deep sequencing method to study RNA oxidation profiles, in particular G oxidation products and abasic sites.[4,5]

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82 Martina Krämer (Institute of Pharmaceutical and Biomedical Sciences, Johannes Gutenberg-University Mainz, Germany)

LC-MS analytics of eukaryotic and synthetic caps

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The canonical cap of most eukaryotic and viral mRNAs consists of N7-methylguanosine (m7G) which is linked to the first nucleotide of the RNA through a reverse 5' to 5'- triphosphate bridge. Recently, synthetic mRNA has attracted considerable public attention due to its capacity for antiviral vaccines, leading to an increased need for analytical methods to evaluate cap structures. Here, we describe two different LC-MS/MS methods for the investigation of the presence of cap structures in in vitrotranscribed (IVT) mRNAs. The first method is based on the digestion of RNA to nucleoside level. Cap-relevant nucleoside modifications such as m7G and 2'-Omethylated nucleosides are quantified and normalized to the amount of injected RNA molecules enabling the determination of capping efficiencies of IVT mRNAs. Alternatively, a different digestion mixture can be used thereby keeping the triphosphate bridge intact. The analysis is performed as previously mentioned, except that cap dinucleotides are related to the amount of injected RNA. Isotopically labelled cap structures can be used as internal standards for LC-MS analysis to improve the reliability of quantification of cap structure populations. Purines can exchange a hydrogen atom at ring position 8 upon treatment with deuterated water. To prepare isotopically labelled cap dinucleotides, unlabelled ones containing two (modified) purine nucleotides were treated with heavy water, resulting in LC-MS signals with a mass shift of +2. Improvement of reaction parameters such as temperature and incubation time led to an almost quantitative conversion into double-deuterated cap analogs.

83 Zeynep Özrendeci (Institute of Pharmaceutical and Biomedical Sciences, Johannes Gutenberg-University Mainz, Germany)

Dynamics of modifications and processing of RNAs in the SARS-CoV-2 replication cycle

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In mammals, 2'-O-methylation of RNA is a molecular signature by which the cellular innate immune system distinguishes endogenous from exogenous messenger RNA. However, the molecular functions of RNA 2'-O-methylation are not well understood. Using RiboMethSeq analysis, we identified possible 2'-O-methylations at specific residues on the SARS-CoV-2 genome. Here, two sites predicted to be 2'-O-methylated were selected for further validation with an orthogonal approach. Herefore, selected sites in SARS-CoV-2 genome were point modified *via* three-way-one-pot splint ligation method. Point modified mRNA is isolated by real-time gel elution from agarose gels using blue-light detection. Isolated point modified SARS-CoV-2 mRNAs will be sequenced *via* Oxford Nanopore and RiboMethSeq with their respective full length IVTs in parallel for comparison. Upon confirmation of predicted modification sites, in vitro translation of the modified mRNA and functional analysis of the protein will be performed to gather a more detailed understanding of 2´-O-methylation and its role in immune evasion and viral replication. The application of this strategy beyond 2´-O-methylation by investigating the dynamics of other modifications together with their respective enzymes in an immunological and virological context is a crucial subject of RMaP program and will be suitable for further cooperations within and beyond RMaP boundaries.

84 Tristan Dewald (Institute of Pharmaceutical and Biomedical Sciences, Johannes Gutenberg-University Mainz, Germany)

Development of RNA-based PROTACs as a strategy for targeting RNA-modifying enzymes

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Dysfunction and dysregulation of RNA-modifying enzymes are associated with severe human diseases. For example, overexpression of the m5C-writer NSUN6 is associated with the progression of osteosarcoma.[1] Targeting RNA-modifying enzymes using conventional drug design strategies has proven to be challenging. Therefore, the development of novel therapeutic approaches is necessary to specifically and efficiently target these enzymes.

PROTACs (Proteolysis Targeting Chimeras) are a novel class of therapeutics that target the proteasomal degradation pathway. Due to their heterobifunctional design, PROTACs are able to bind both the protein of interest (POI) and an E3-ligase, which is responsible for marking the POI for degradation. By forming a ternary complex, the POI will be subsequently degraded by the proteasomal pathway.[2]

Instead of small molecules, short RNA-sequences can be used to specifically target the RNA-binding pocket of RNA-modifying enzymes. NSUN6 is known to have very well-defined substrates. It can recognize a specific sequence motif of five nucleotides (CUCCA), which is often incorporated into hairpin loop structures.[3] Therefore, the binding affinity of short RNA-sequences containing this sequence motif was tested and evaluated as potential ligands for the POI. Various modifications of the nucleobases and the backbone will be tested to improve cell permeability and the pharmacokinetic properties of the ligand. One prominent modification widely used in RNA-based therapeutics is the incorporation of phosphorothioates. Due to their increased cell permeability and increased nuclease resistance, phosphorothioates are commonly found in several FDA-approved aptamers and antisense oligonucleotides, such as Mipomersen (Kynamro®).[4]

After optimizing the ligand's properties, the RNA-construct will be conjugated with a linker and an E3-ligase-recruiter for the development and testing of the first RNA-PROTAC-candidates for targeting NSUN6.

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A novel approach to chemical derivatization of pseudouridine to improve RNAseq-based detection

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Pseudouridine is the most abundant modified nucleoside in RNA and was first discovered in 1951. Pseudouridine displays similar characteristics to uridine, such as identical Watson-Crick base pairing with adenine and an identical molecular mass. In order to distinguish these two bases, liquid chromatography (LC) in combination with downstream mass spectrometry (MS) can be used, making use of differing elution times and fragmentation patterns. As most information about the sequence context are lost during LC-MS analysis, further sequence-specific analysis is necessary to retrieve site-specific pseudouridine quantities in a distinct RNA.[1] Hence, several methods for sequencing of pseudouridine have been developed, such as Pseudo-seq, Ψ-seq, PSI-seq and CeU-seq, all relying on selective chemical treatment with CMCT.[2] Chemical treatment can also be found in nanopore sequencing, like the labeling of inosine with acrylonitrile in Nano ICE-Seq.[3] While chemical treatments with CMCT, acrylonitrile and methyl vinyl sulfone are commonly used, these bear several limitations such as incomplete reactions with pseudouridine and side reactions with other nucleotides.[1] Therefore, in the context of 'RMaP' project C01, other Michael acceptors were tested at different reaction conditions in order to detect pseudouridine with a high specificity and a preferably high sensitivity to enable mapping of pseudouridine at single nucleotide resolution. Reaction efficiency and side product formation of different reagents were monitored via LC-MS of digested tRNA after chemical treatment and will be followed by extensive testing on several artificial and native RNAs containing pseudouridine. Propargyl acrylate (PA) appears to be a good candidate with high reactivity, that also allows further derivatization by click-chemistry and ester hydrolysis. Finally, biological samples will be treated with promising candidates and analyzed via Illumina and Oxford Nanopore sequencing to elaborate efficiency and applicability of our new approach.