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RNAClub 2024

RNA4T Kick-off Meeting

19–20 September
CEITEC, Brno, Czech Republic

ABSTRACTS BOOK

Organiser

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RNAClub 2024

Programme

19 SEPTEMBER

8:30–10:00 Registration and welcome coffee

10:00–10:10 Welcome

10:10–11:40 **Session 1: Translation (Chair: Leoš Valášek)**

- **10:10** Michal H. Kolář – Structure and dynamics of the narrowest part of the ribosome exit tunnel
- **10:25** Julie Kovářová – Alterations of the translation termination machinery are linked with stop codon reassignment
- **10:40** Bankatesh Kumar – Control of gene expression by queuosine tRNA modification and its impact on the virulence of *Leishmania mexicana* Ⓟ
- **10:55** Klára Pospíšilová – Uncovering the roles of individual eIF3 subunits Ⓟ
- **11:10** Khushboo Sharma – Polysome profiling of cell cycle Ⓟ
- **11:25** Jakub Dušek – PentaGen s.r.o., sponsor's presentation

11:40–13:15 Lunch break and poster viewing

13:15–13:55 **Igor Ulitsky – Noncoding RNAs as Therapeutic RNAs and Targets**

13:55–15:10 **Session 2: Transcription (Chair: Libor Krásný)**

- **13:55** Michal Hocek – Enzymatic Synthesis of Base-Modified RNA with Engineered DNA Polymerases
- **14:10** Nabajyoti Borah – Molecular insights of RNA polymerase recycling by mycobacterial HelD Ⓟ
- **14:25** Jarmila Hnilicová – RIP-seq reveals novel RNAs that interact with RNA polymerase in bacteria
- **14:40** Maria-Bianca Mititelu – Discovery of Dinucleoside Diphosphates acting as 5’ RNA Caps in Mammalian Cells Ⓟ
- **14:55** I.T.A.-Intertact s.r.o., sponsor's presentation

15:10–16:25 Poster session with coffee and refreshments

16:25–17:05 **Michaela Frye – Targeting RNA modifications in cancer**

17:05–18:20 **Session 3: Non-coding RNA (Chair: Petr Svoboda)**

- **17:05** Katarína Juríková – Telomeric lncRNA TERRA localizes to stress granules in human ALT cells Ⓟ
- **17:20** Aleš Obrdlík – Deciphering the Multifunctional Roles of m6A and m6Am Demethylases in Human Cells

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17:35	• Nenad Radivojević – The role of coilin in snRNP biogenesis Ⓟ
17:50	• Dragana Vukić – Exploring interactomes of ADAR1 isoforms and their response to Interferon induction Ⓟ
18:05	• Edward Curtis – Exploring the functional potential of nucleic acids using artificial evolution
18:20	Student's Prize Voting, Concluding Remarks
19:00	Young Scientists' Prizes announcement
19:00–21:00	Buffet dinner, networking

Ⓟ indicates eligibility for the Young Scientists' Prizes

RNA4T Kick-off Meeting

Programme

20 SEPTEMBER

8:30–9:00 Welcome coffee

9:00–9:30 **Introduction of RNA4T (Michal Hocek)**

9:30–10:15 **Transcription and its regulation (WP1)**

- **9:30** Michal Hocek
- **9:45** Libor Krásný
- **10:00** Richard Štefl

10:15–11:30 **RNA modifications and mechanisms of post-transcriptional regulation (WP2)**

- **10:15** Hana Macíčková Cahová
- **10:30** Edward Curtis
- **10:45** David Staněk
- **11:00** Petr Svoboda
- **11:15** Branislav Večerek

11:30–11:45 Coffee break

11:45–12:45 **Translational control and protein synthesis by rational design (WP3)**

- **11:45** Štěpánka Vaňáčová
- **12:00** Zdeněk Paris
- **12:15** Leoš Valášek

12:30–13:15 ISAB working lunch

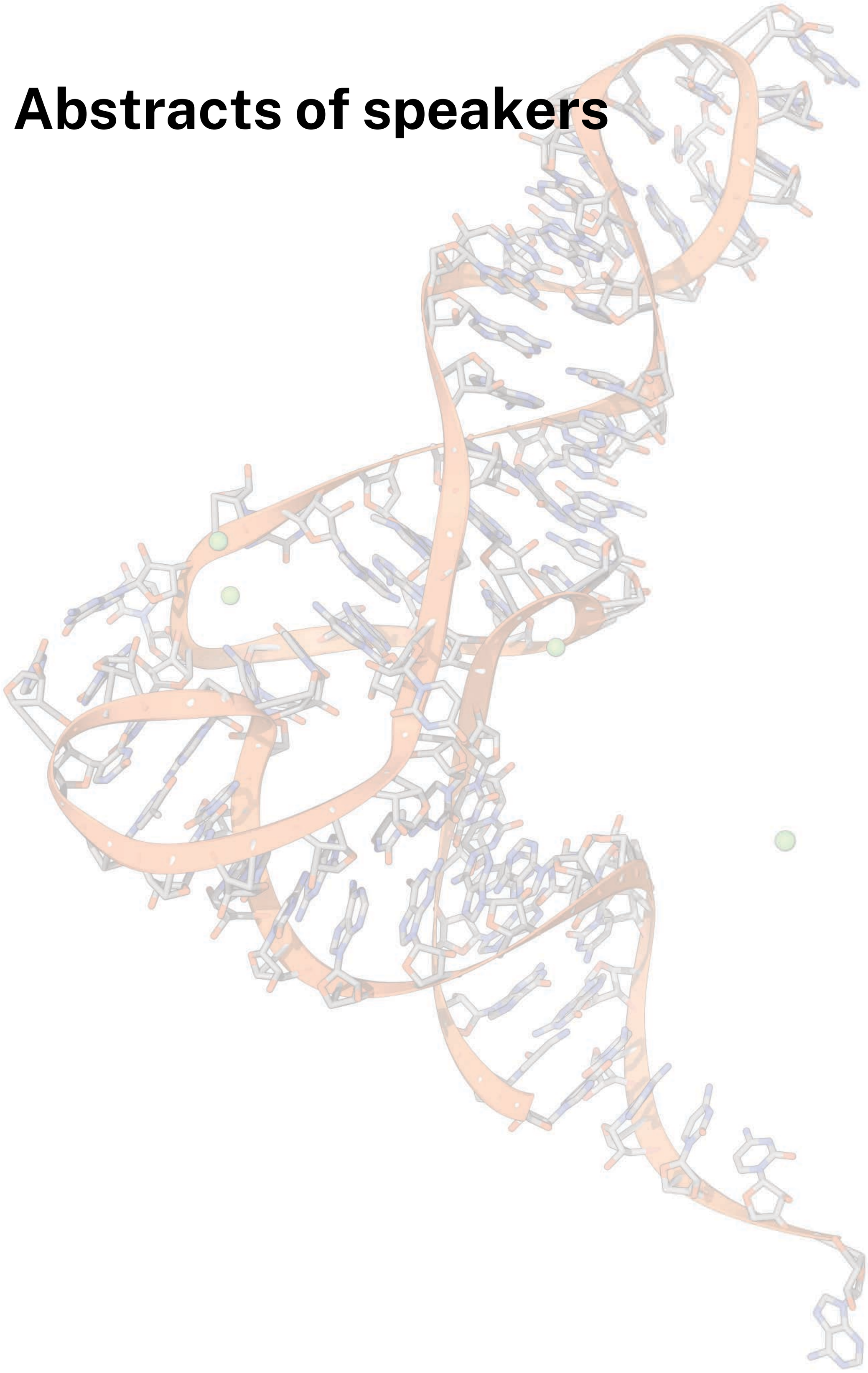
13:15–14:15 ISAB discussion with PIs

14:15–16:30 Networking with project partners

ISAB Members:

Witold Filipowicz, Michaela Frye, Matthias Hentze, Claudia Kutter, Martin Jacko

Abstracts of speakers



01 Structure and dynamics of the narrowest part of the ribosome exit tunnel

Michal H. Kolář

University of Chemistry and Technology, Prague, Czech Republic

During translation, nascent polypeptides (NPs) emerge from the ribosome through a 10-nm long tunnel. This tunnel is primarily formed by ribosomal RNA (rRNA), with contributions from a few ribosomal proteins. As NPs pass through this tunnel, they interact with rRNA and ribosomal proteins for the first time. These interactions that have significant physiological implications across all domains of life. Notably, ribosomal proteins uL4 and uL22 create the narrowest part of the ribosomal tunnel, though the evolutionary reason for this constriction remains unclear. To understand how this constriction influences NP translocation through the tunnel, we investigated its dynamics by analyzing over 200 experimental structural models of bacterial ribosomes available in the Protein Data Bank (PDB). We also conducted atomistic molecular dynamics simulations of the entire ribosome with NPs of various lengths. Our findings indicate that the constriction is conformationally flexible. The PDB survey identified Y60 and R67 on uL4, and R92 and R95 on uL22 as the most flexible residues. Simulations revealed that the width of the constriction varies, depending on the length of the NP. These results enhance our understanding of the initial stages of protein synthesis, shedding light on the structural dynamics of the ribosomal tunnel constriction.

02 Alterations of the translation termination machinery are linked with stop codon reassignment

Julie Kovářová, Nathalia Ballesteros Chitiva, Zdeněk Paris

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During translation, a stop codon is recognized by the protein release factor 1 (eRF1), which binds into the A-site of a translating ribosome. Next, it is accompanied by a GTPase called release factor 3 (eRF3) facilitating release of the newly synthesized peptide. However, the principles of translation termination remain unknown in the newly described trypanosomatid *Blastocrithidia nonstop*, which contains a non-canonical genetic code with all three stop codons reassigned for amino acids. We use *Trypanosoma brucei* to study the closely related protist *B. nonstop*, since trypanosome is a well-established model organism amenable to genetic manipulations. Interestingly, comparison of the protein sequences from these two trypanosomatids reveals numerous substitutions specific for *B. nonstop*, thus implying to play a role in stop codon read-through. We have generated *T. brucei* cell lines with eRF1 and eRF3 depleted by RNAi, which showed very strong growth defects after depletion of each of the proteins, indicating their essentiality. Analysis by polysome profiling exhibited global defects in translation in each of these cell lines, consistent with the expected phenotypes. These *T. brucei* cell lines provide a great tool to test several specific single point substitutions or large extensions in these proteins, which we have identified in *B. nonstop*. For instance, we have already shown that Ser67 for Ala in eRF1 leads to increased stop codon read-through. Our system will allow to characterize the individual variants of eRF1 and eRF3 and their contributions to translation termination efficiency and fidelity.

03 Control of gene expression by queuosine tRNA modification and its impact on the virulence of *Leishmania mexicana*

Bankatesh Kumar,[1,2] Michalea Boudova,[1,2] Julie Kovářová,[1] Thalia Pacheco Fernandez,[3] Sneha Kulkarni,[1] Abhay Satoskar,[3] Zdeněk Paris [1]

1 Institute of Parasitology, Biology Centre CAS, České Budějovice, Czech Republic
2 Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic
3 Departments of Pathology and Microbiology, Ohio State University, Columbus, USA

The complex life cycle of the human parasite *Leishmania mexicana* requires rapid translational adaptation to enable survival between two distinct environments, the insect and the mammalian host. In these medically important protists, transcriptional control is lacking due to the unusual organisation of the genome. tRNA modifications provide an option to regulate gene expression post-transcriptionally. One such a case is the queuosine (Q) modification of the wobble position 34. The location of Q in the anticodon of several tRNAs implicates its strong influence on translation. Interestingly, the levels of Q-tRNAs change significantly during parasite differentiation. To further investigate the role of Q-tRNA modification, we used CRISPR/Cas9 technology to target and knock out the TGT2 subunit of the enzyme that is responsible for the formation of Q-tRNAs in *L. mexicana*. Our results show that while the Q modification is not critical for parasite differentiation, it is indispensable for *L. mexicana* infectivity and survival within macrophages, as shown by fluorescent microscopy. Moreover, to understand how Q-tRNA can affect translation through codon bias, we performed a dual luciferase assay. The obtained results suggest that queuosine modification is required for global translation, with a slight preference for U-rich codons. Furthermore, proteomic analysis of TGT2 knockout parasites revealed significant downregulation of leishmanolysin (GP63), a key virulence factor on the parasite surface. This correlates with reduced infectivity of Q-tRNA depleted parasites, highlighting the critical role of this tRNA modification in translation and maintenance of parasite virulence.

04 Uncovering the roles of individual eIF3 subunits

Klára Pospíšilová[1], Anna Herrmannová,[1] Jan Jelínek,[2] Leoš Valášek[1]

1 Laboratory of Regulation of Gene Expression, Institute of Microbiology of the Czech Academy of Sciences, Prague, Czech Republic
2 Laboratory of Bioinformatics, Institute of Microbiology of the Czech Academy of Sciences, Prague, Czech Republic

The 12-subunit mammalian eukaryotic translation initiation factor 3 (eIF3) is the largest and most complex of translation initiation factors and has been implicated in numerous steps of translation initiation, termination and ribosomal recycling. Expression of all 12 eIF3 subunits is interconnected, as downregulation of a single subunit by siRNA often leads to a concomitant and specific decrease in protein levels of other eIF3 subunits resuting in the existence of several relatively stable eIF3 subcomplexes. Recent studies suggest that the unbalanced expression of eIFs is not only an indirect consequence of neoplasia but itself contributes significantly to cell transformation, tumor development, cancer cell survival and metastasis. To comprehensively study the effects of eIF3 remodeling, we took advantage of well-established knock-downs of subunits a, b, f, g, h, i, l, and m of human eIF3 in HeLa cells and investigated their impact on differential gene expression translatome-wide by Ribo-Seq. We observed that alterations in eIF3 subunit stoichiometry affect different transcripts characterized by different 5'UTR or 3'UTR length and complexity differently. The effects that single subunit downregulations have on gene expression clearly reflect the modular nature of eIF3. Together this work provides a summarization of the effects of individual eIF3 subunits downregulation on gene expression and helps understand the individual roles of each eIF3 subunit studied.

05 Polysome profiling of cell cycle

Khushboo Sharma, Kristina Roucova, Marharyta Ramanava, Martin Pospisek, and Tomas Masek

Laboratory of RNA Biochemistry, Department of Genetics and Microbiology, Faculty of Science, Charles University, Viničná 5, 128 44, Prague 2, Czech Republic

mRNA Translation is a highly regulated process and even more so during cell cycle transition where and the activation or degradation of proteins mediate progression through the various phases of the cell cycle. In numerous efforts have been made to study this regulation using genome-wide studies which include proteomic and ribo-seq approaches. These studies have shown that translation is not only globally regulated but is also gene specific for each phase of the cell cycle. However, the major drawback of these studies is the use of synchronisation to obtain the certain phase. It is unknown how the synchronisation might affect the gene regulation itself, despite certain studies showing changes in posttranslational modifications due to synchronisation. The research data also lacks any information about the influence of mRNA features like UTR length, structure, and composition on it is translatability.

Here, we coupled the well-established, high-sensitive polysome profiling method (Scarce sample polysome profiling; SSP-Profiling) with flow cytometry to obtain unperturbed cells from different phases of cell cycle and to evaluate their transcriptome and translatome. To achieve this, we used non- leukemic lymphoblastoid cell line, NCNC, with diploid karyotype. The cells were mildly fixed to be able to sort them before performing SSP- profiling. Our data demonstrates translational profile of G1, S and G2M, for 50K cells. As expected, light and heavy polysomal fractions cluster together with transcriptome but distinct from non polysomal fraction. Using differential gene expression, we identify translationally regulated genes specific to each phase of the cell cycle which are further characterised by gene ontology. Presently, we are working with the functional characterisation of the same. The establishment of this method broadens the scope of translation study in biologically limited samples with the possibility of coupled flow assisted sorting.

This research was supported by the project OP JAC CZ.02.01.01/00/22_008/0004575 RNA for therapy, Co-Funded by the European Union and National Institute of virology and bacteriology (Programme EXCELES, ID Project No. LX22NPO5103) – Funded by the European Union - Next Generation EU and by The Czech Science Foundation (GACR, no. 22-27301S).

06 How to save money and worries with PentaGen

Jakub Dušek

PentaGen, s.r.o.

The lecture will present the portfolio of Singleron and Lexogen, which are represented on the Czech market by PentaGen.

Singleron offers pioneering solutions in the domain of single cell transcriptomics, supported by over a hundred patents. The core of the technology is the SCOPE chip, which separates individual cells for subsequent preparation of the NGS library. This allows manual library preparation without the necessity for a specialized instrument.

Lexogen, also referred to as "RNA experts", is a company that offers a diverse range of products with an excellent price/performance ratio. This is made possible by a strong focus on in-house R&D. Their solutions cover the transcriptomics workflow from A to Z, i.e. from RNA preparation to NGS library preparation to data analysis.

07 Noncoding RNAs as Therapeutic RNAs and Targets

Igor Ulitsky

Weizmann Institute of Science, Rehovot, Israel

The development and rapid deployment of mRNA vaccines for SARS-CoV-2 has substantially boosted the interest in mRNA therapeutics, which joined the decade of successes of RNAi and antisense-oligonucleotide (ASO) therapeutics, several of which are now used in the clinic for both rare and common conditions. These build upon decades of research in RNA biology, but the realization of their full potential requires additional leaps forward in both understanding the biology of potential RNA targets and in the development of RNA molecules with desired features. My lab studies the functions and modes of action of long noncoding RNAs (lncRNAs), with a particular focus on lncRNAs that are conserved in evolution. I'll describe our discovery of the *CHASERR* long noncoding RNA as a conserved repressor of the *CHD2* gene, whose disruption leads to severe phenotypic consequences in both mice and humans and which can serve as a therapeutic target for ASO-based treatment of CHD2 haploinsufficiency. I'll also describe our studies into understanding how the sequences and structures of longer RNAs can be used to manipulate gene regulatory networks within cells.

08 Enzymatic Synthesis of Base-Modified RNA with Engineered DNA Polymerases

Michal Hocek[1,2], Mária Brunderová[1], Matouš Krömer[1]

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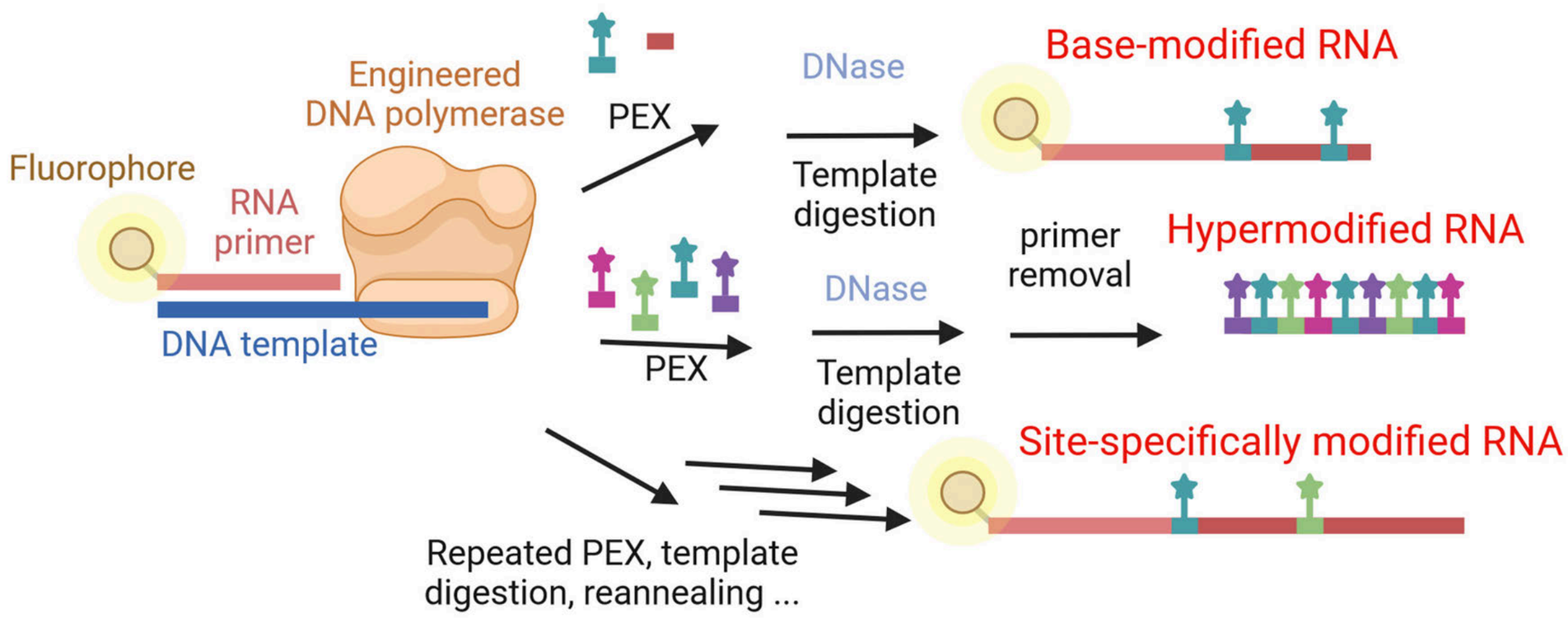
A new method for enzymatic synthesis of base-modified RNA was developed using engineered thermophilic TGK DNA polymerase [1] in primer extension of an RNA primer to afford RNA strand containing one or several different base-modified nucleotides (Scheme 1). [2] The method can be used for the synthesis of hypermodified RNA containing all four modified nucleotides using a set of four base-modified NTPs. We also developed site-specific or segmented introduction of one or two modifications at defined positions in diverse RNA molecules, including mRNA. This methodology can be used for expedient synthesis of diverse types of base-modified RNA for applications in chemical biology, therapy and diagnostics.



Scheme 1. Enzymatic synthesis of modified RNA with engineered DNA polymerase.

1. Cozens, C.; Pinheiro, V. B.; Vaisman, A.; Woodgate, R.; Holliger, P. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, 109, 8067–8072.
2. Brunderová, M.; Havlíček, V.; Matyašovský, J.; Pohl, R.; Poštová Slavětínská, L.; Krömer, M.; Hocek, M. *Nat. Commun.* **2024**, 15, 3054.

Funding by Czech Science Foundation (20-00885X) and by the Ministry of Education, Youth and Sports of the Czech Republic grant RNA for therapy (CZ.02.01.01/00/22_008/0004575) is gratefully acknowledged.

09 Molecular insights of RNA polymerase recycling by mycobacterial HelD

Nabajyoti Borah[1,4], Tomáš Koval[3], Barbora Brezovská[1], Petra Sudzinová[1], Tomáš Kouba[2], Tomáš Koval[3], Jan Dohnálek[3], Hana Šanderová[1], Martin Hubálek[2] and Libor Krásný[1]

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2 Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Flemingovo náměstí 542/2, 166 00 Prague, Czech Republic
3 Institute of Biotechnology of the Czech Academy of Sciences, v.v.i., Průmyslová 595, 252 50, Vestec, Czech Republic
4 Department of Genetics and Microbiology, Faculty of Science, Charles University, Prague, Czech Republic

Mycobacterial HelD is a class II HelD protein that releases RNA polymerase (RNAP) from non-functional transcriptional complexes. Structural studies have shown that HelD can occupy both the primary and secondary channels of RNAP by dedicated domains, thereby removing nucleic acids from the enzyme. Subsequently, for RNAP to be fully recycled, HelD must be released as the RNAP-HelD complex is transcriptionally inactive. However, the mechanism of this release is unknown. To address this process in the model organism *Mycobacterium smegmatis*, we first pulled down and identified proteins associating with HelD to identify its binding partners that might affect the release. The experiments revealed that HelD may co-exist in a complex with σ^A, the primary sigma factor, and RbpA, an essential transcription factor found in mycobacteria. We then reconstituted this complex *in vitro*, including also model DNA promoters, and used Cryo-EM to solve a series of its structures. These structures capture HelD in different positions relative to RNAP, suggesting a mechanism of its dissociation from RNAP. This model was validated biochemically, demonstrating that the release of HelD is facilitated by the promoter open complex. Furthermore, the biochemical analysis showed that this process is ATP-stimulated when both ATP binding and hydrolysis facilitate HelD release. Collectively, this study provides mechanistic insights into HelD release from RNAP, revealing gradual dissociation of HelD from RNAP during transcription initiation.

This work was supported by the National Institute of Virology and Bacteriology (Programme EXCELES, ID Project No. LX22NPO5103)-Funded by the European Union- Next Generation EU; institutional support of IBT CAS, v.v.i. (RVO: 86652036), Grant Agency of Charles University in Prague: GAUK 236823, Biocev-CMS of CIISB,Instruct-CZ Centre,supported by MEYS CR (LM2023042) and European Regional Development Fund-Project„UP CIISB" (No. CZ.02.1.01/0.0/0.0/18_046/0015974).

10 RIP-seq reveals novel RNAs that interact with RNA polymerase in bacteria

Viola Vaňková Hausnerová,[1,2] Dilip Kumar,[2] Mahmoud Shoman,[1,2] Marek Schwarz,[3] Martin Modrák,[3,4] Jitka Jirát Matějčková,[1] Martin Převorovský,[5] Libor Krásný [2] and **Jarmila Hnilicová**[1]

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2 Laboratory of Microbial Genetics and Gene Expression, Institute of Microbiology of the Czech Academy of Sciences, Czech Republic
3 Laboratory of Bioinformatics, Institute of Microbiology of the Czech Academy of Sciences, Czech Republic
4 Department of Bioinformatics, Second Faculty of Medicine, Charles University, Czech Republic
5 Department of Cell Biology, Faculty of Science, Charles University, Czech Republic

Bacterial transcription is an important target of antibiotics. Rifampicin, which inhibits bacterial RNA polymerase (RNAP), is the first-line drug to treat tuberculosis. We propose that in nature, bacteria have evolved their own mechanism to reversibly sequester and potentially inhibit RNAP by 200 - 400 nt long, structured RNAs. These RNAP-associated regulatory RNAs are highly abundant in specific growth conditions and show a high variability between different species. We have established a new protocol, a combination of RNA immunoprecipitation and next generation sequencing (RIP-seq), and identified RNAs interacting with RNAP in model gram-positive bacteria *Bacillus subtilis* and also in two mycobacterial model organisms, *Mycobacterium smegmatis* and the pathogenic *Mycobacterium tuberculosis*, and in the producers of amino acids and antibiotics – *Corynebacterium glutamicum* and *Streptomyces coelicolor*. We showed that in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*, the majority of RNAP molecules are bound to Ms1 or MTS2823 RNAs, respectively, in stationary phase of growth. In *Corynebacterium glutamicum*, we found structurally different CoRP RNA (Corynebacterium RNA Polymerase binding RNAs) to sequester RNAP. In addition, we have identified other species-specific RNAs that associate with RNAP, revealing a previously unknown landscape of RNAs interacting with RNAP. Future experiments will define mechanistic functioning of these RNAs and address whether molecules based on these RNAs (or their partial sequences) could be used as antibacterial compounds to target RNAP. The advantage is that these compounds could be species-specific, target only bacterial pathogens and not the other bacteria in the human microbiome.

11 Discovery of Dinucleoside Diphosphates acting as 5’ RNA Caps in Mammalian Cells

Maria-Bianca Mititelu[1,2], Ondřej Nešuta[1], Hana Cahova[1]

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Currently, more than 170 RNA modifications are known in both prokaryotes and eukaryotes, but the role of majority of them is not well understood. 5’ termini of eukaryotic mRNAs and certain viral RNAs are distinguished by a specific structure known as the 7-methylguanosine (m7G) cap. This cap plays crucial roles in transcription, stability and translation of mRNAs. The recent discovery of various noncanonical RNA caps, such as diadenosine tetraphosphate (Ap_4A)[1], coenzyme A (CoA),[2] and nicotinamide adenine dinucleotide (NAD)[3,4], in mammalian cells has prompted a reassessment of our understanding of RNA cap function and metabolism. In this study, we present the discovery of new dinucleoside diphosphate molecules acting as 5’ RNA caps in mammalian cells using liquid chromatography–mass spectrometry (LC–MS) technique. We observed that the intracellular concentration of these molecules increases after oxidative stress. Furthermore, our screening of known RNA decapping enzymes revealed that two of them can cleave this modification from RNA. We also explored possible biosynthetic pathways. Since the free forms of these dinucleoside diphosphates were not detected in cellular extract, we focus on putative RNA capping enzymes. We are currently establishing a sequencing protocol employing specific enzymes that will facilitate the identification of RNAs harboring dinucleoside diphosphate structures at the 5’ end.

1. Frantisek Potuznik, J. et al. Diadenosine Tetraphosphate (Ap(4) A) Serves as a 5' RNA Cap in Mammalian Cells. *Angew Chem Int Ed Engl* **63**, e202314951, doi:10.1002/anie.202314951 (2024).
2. Kowtoniuk, W. E., Shen, Y., Heemstra, J. M., Agarwal, I. & Liu, D. R. A chemical screen for biological small molecule-RNA conjugates reveals CoA-linked RNA. *Proc Natl Acad Sci U S A* **106**, 7768-7773, doi:10.1073/pnas.0900528106 (2009).
3. Cahova, H., Winz, M. L., Hofer, K., Nubel, G. & Jaschke, A. NAD captureSeq indicates NAD as a bacterial cap for a subset of regulatory RNAs. *Nature* **519**, 374-377, doi:10.1038/nature14020 (2015).
4. Chen, Y. G., Kowtoniuk, W. E., Agarwal, I., Shen, Y. H. & Liu, D. R. LC/MS analysis of cellular RNA reveals NAD-linked RNA. *Nat Chem Biol* **5**, 879-881, doi:10.1038/nchembio.235 (2009).

12 Targeting RNA modifications in cancer

Michaela Frye

German Cancer Research Center, Heidelberg, Germany

13 Telomeric lncRNA TERRA localizes to stress granules in human ALT cells

Luca Larini,[1,2] Elena Goretti,[1,3,4] Eleonora Zulian,[1] Emma Busarello,[1] Stefano Maria Marino,[1] Mona Hajikazemi,[2] Katrin Paeschke,[2] Toma Tebaldi,[1,5] Emilio Cusanelli,[1]

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TERRA is a long non-coding RNA derived from the ends of chromosomes. It has a number of well-described nuclear roles including telomere maintenance and homeostasis. A growing body of evidence now points at its role in human cells outside of nucleus—it has been found to be a component of extracellular vesicles, a player in inflammation signalling and its capacity for translation has been shown. We set out to study whether TERRA is directly present in the cytoplasm of human cancer cells and what may be its eventual role. We used telomerase-negative osteosarcoma cells as a model, as they harbor elevated levels of TERRA and accumulate increased levels of telomere DNA damage. We demonstrate, using a combination of single-molecule RNA FISH, dCas13-based visualization, cellular fractionation, proteomics and transcriptome analysis that TERRA is present in the cytoplasm of human cells, especially upon various stress stimuli, and that it associates with stress granules. We will discuss how cytoplasmic TERRA may be involved in nucleo-cytoplasmic stress communication and speculate on the role of RNA secondary structures in the process.

14 Deciphering the Multifunctional Roles of m6A and m6Am Demethylases in Human Cells

Aleš Obrdlík[1], Shwetha Krishna[1], Anton Zuev[2], Helena Peschelová[1], Veronika Kozlová[1], Praveenkumar Rengaraj[1], Veronika Rajecká[1], Michal Šmída[1], David Potěšil[1], Jana Dobrovolná[2], Štěpánka Vaňáčová[1]

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2 Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czech Republic

N6-methyladenosine (m6A) and N6,2'-O-dimethyladenosine (m6Am) are crucial RNA modifications found across various RNA classes in eukaryotic cells. These modifications significantly impact numerous aspects of RNA metabolism, such as transcription, pre-mRNA processing, localization, stability, and translation. Consequently, methyladenosines in mRNA are essential for development and are implicated in a range of pathologies, including obesity, cancer, and viral infections. While the establishment of m6A(m)s in pre-mRNA, including their writer and reader proteins, has been extensively studied, our current knowledge about the regulation of these two modifications, is limited. In mammals, the removal of m6A and m6Am is facilitated by at least two adenosine demethylases, namely ALKBH5 and FTO, respectively. To comprehend the influence of these demethylases on RNA methylation and elucidate their regulatory patterns within cellular contexts, our laboratory has employed an integrated approach encompassing omics-wide and biochemical techniques over recent years. In this presentation, we will delineate our latest findings, offering insights into the potential functions of m6A(m) eraser proteins in maintaining cellular homeostasis, their potential regulation, and unprecedented mechanistic insights into their functions in maintaining cellular homeostasis and genome stability.

15 The role of coilin in snRNP biogenesis

Nenad Radivojević[1], Martina Groušlová[1], Hana Petržílková[2], David Staněk[1]

1 Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic
2 European Molecular Biology Laboratory, Grenoble, France

Cajal bodies (CBs) are membraneless nuclear organelles involved in the metabolism of spliceosomal small nuclear ribonucleoprotein particles (snRNPs). During their biogenesis, snRNPs have been observed to transiently localise to CBs, whereas disruption of their maturation leads to their accumulation. While the importance of CB in snRNP maturation is clear, the precise mechanism and function remain elusive. Coilin, a CB marker protein that has been shown to provide scaffolding and induce CB formation, may serve as a targeting signal and quality control factor in the final steps of snRNP biogenesis. Previous studies have shown that coilin's Tudor-like and RG-box domains interact with Sm proteins and the SMN complex, respectively, while iCLIP experiments indicate its association with different classes of RNA. In this study, we aim to elucidate the nature of the interaction between coilin and snRNPs and determine whether coilin can discriminate between mature and immature snRNP particles. Here, we identify the RG-box as an RNA-binding domain capable of pulling down snRNA in vitro. In particular, arginines within the RG-box provide the interaction interface, possibly mediated by electrostatic interactions. Using the C214 coilin fragment and several of its mutant variants, we have shown that both the RG box and the Tudor-like domain are required for efficient association with U2 snRNP. In the future, we plan to solve the structure of the C214 fragment in complex with the maturing snRNP and to elucidate the role of coilin as a quality control factor in snRNP assembly.

16 Exploring interactomes of ADAR1 isoforms and their response to Interferon induction

Dragana Vukić[1], Anna Cherian[1], Salla Keskitalo[2], Rita Bong[2], Martin Marônek[1], Leena Yadav[2], Liam Keegan[1], Markku Varjosalo[2], Mary O'Connell[1]

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Adenosine deaminase acting on RNA 1 (ADAR1) is an RNA editing enzyme that catalyzes the hydrolysis of adenosine to inosine in double-stranded RNA (dsRNA). ADAR1 plays a multifaceted role in maintaining homeostasis and responding to various stress conditions, through both editing-dependent and editing-independent mechanisms. ADAR1 exists in two isoforms: the constitutively expressed ADAR1p110 isoform, which is primarily localized in the nucleus, and the interferon (IFN)-inducible ADAR1p150 isoform, which is predominantly cytoplasmic. The ADAR1p150 isoform plays a crucial role in preventing aberrant activation of antiviral dsRNA sensors. We mapped the stable interaction network of endogenous ADAR1 in A549 cells and interactome of individual isoform in Flp-In 293 T-Rex cells. In order to capture a more transient protein network, ADAR1 isoforms were tagged with a biotin ligase, thereby labeling proteins in close proximity. Furthermore, we employed RNase A digestion and a mutant ADAR1 variant that is unable to bind to dsRNA to investigate the interactions facilitated by RNA molecules. Given that the expression of the ADAR1 p150 isoform is regulated by IFN, we also examined ADAR1's interactome under IFN stimulation with IFNα or HMW poly(I:C). We obtained a comprehensive dataset of the ADAR1 interactome under both homeostatic and IFN-stimulated conditions. By employing these different approaches, we were able to identify both known and novel ADAR1 interactors and proximal protein networks, as well as isoform-specific and dsRNA-dependent interactions. Upon IFN induction, we observed the formation of novel protein interactions of both ADAR1 isoforms, enriched for IFN response regulators. Finally, employing BioID we observed a dynamic of the ADAR1 proximal network upon the introduction of HMW poly(I:C). This included the association of ADAR1p110 with components of the nuclear export machinery and dsRBPs, and the association of ADAR1p150 with NF-κB signaling regulators and components of the cellular condensates.

17 Exploring the functional potential of nucleic acids using artificial evolution

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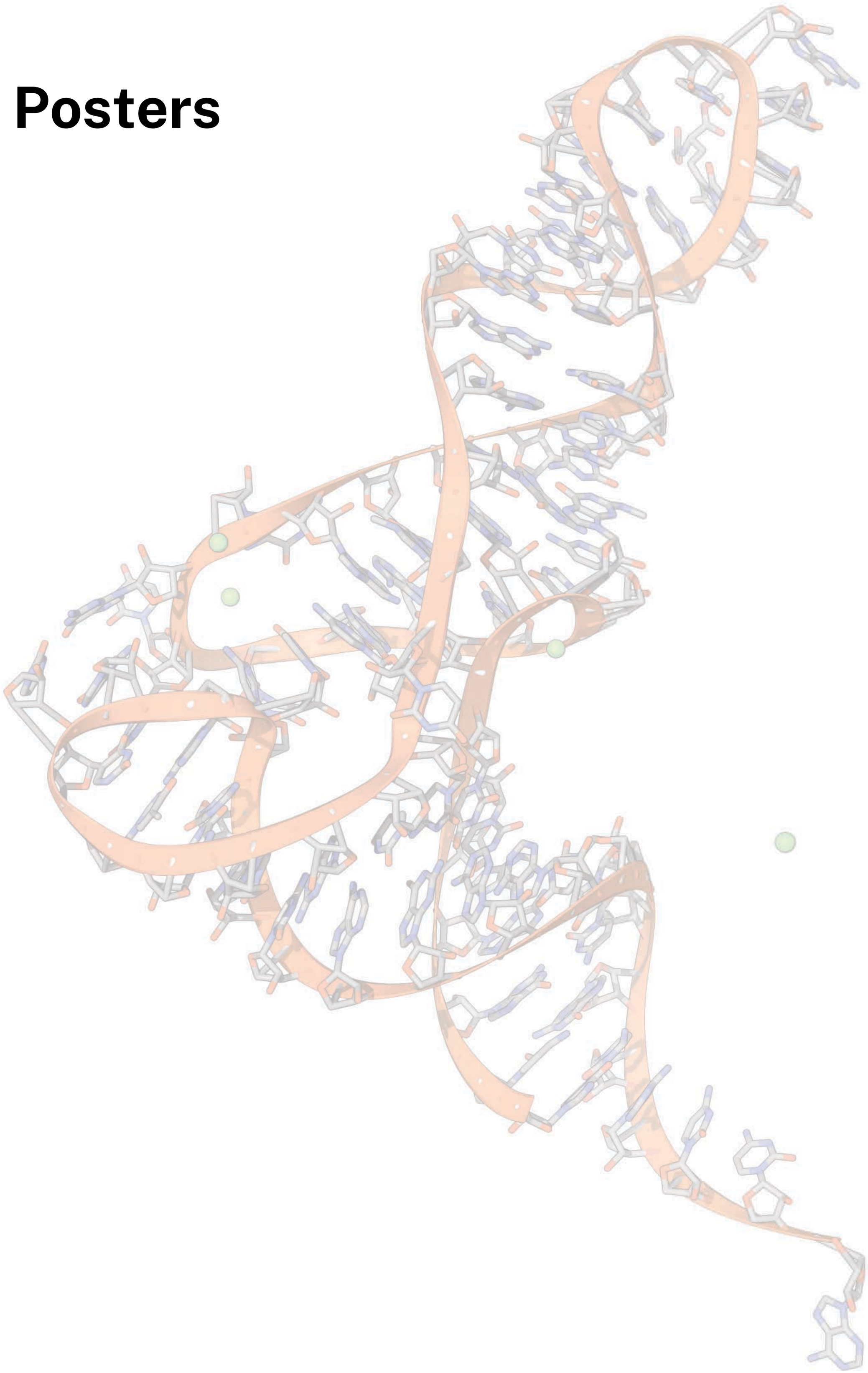
The double helix is well-suited for the storage and transfer of genetic information: the two strands are held together by base pairing, which allows each to act as a template for the other. However, the functional potential of nucleic acids is far greater than this canonical role might suggest. Our group uses artificial evolution to learn more about the interesting and useful things that DNA and RNA can do. This typically involves purifying rare molecules with a desired property (such as the ability to bind a ligand or catalyze a reaction) from large random sequence libraries. In my presentation I will highlight some of our recent progress. This includes the discovery of DNA enzymes that generate chemiluminescent, fluorescent, and colorimetric products and the development of new methods to explore sequence space using structured libraries and single-step selections.

Posters



P01 HelD: fighter against rifampicin in *Bacillus subtilis*

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At present, antibiotic resistance is a serious worldwide problem. Rifampicin is a clinically important antibiotic, an essential component of the anti-tuberculosis therapy. It binds to bacterial RNA polymerase (RNAP) on its β subunit and stops the synthesis of RNA at the stage of only several nucleotides transcribed. The HelD protein is an interaction partner of RNAP that releases stalled complexes of RNAP from DNA, thereby helping to restart transcription. Recently, there Class II HelD proteins from Actinobacteria were shown to provide resistance against rifampicin. In contrast, Class I HelD proteins from Firmicutes were suggested to lack this property. Here, we re-investigated the possibility of whether *Bacillus subtilis* HelD (phylum Firmicutes) plays a role in rifampicin resistance. First, contrary to the published results, we showed that the presence of the *helD* gene increases the minimal inhibitory concentration (MIC) to rifampicin. Second, we demonstrated that rifampicin induces expression of the *helD* gene and the regulation of *helD* expression is at the transcriptional and not translational level. Next, we identified a region in the 5' untranslated region of the *helD* gene, which is responsible for this induction. Results of experiments addressing the mechanism of regulation of *helD* gene expression and resistance of B. subtilis to rifampicin will be presented and discussed.

P02 Ribosome profiling: Tool to characterize the translation of the alternative genetic code in *Blastocrithidia nonstop*

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Blastocrithidia nonstop is a trypanosomatid in which all three stop codons are reassigned to encode amino acids. Previous findings from our group have provided insights into the molecular mechanisms behind this stop codon readthrough. Specifically, we identified tRNAs that decode UAG and UAA as glutamate, a shortened anticodon stem in the canonical tRNATrp that decodes the UGA stop codon as tryptophan, and a specific mutation (Ser74Gly) in a conserved region of release factor 1 that enhances the efficiency of UGA stop codon readthrough (Kachale et al., Nature 2023). Additionally, the overrepresentation of the UAA stop codon in 3' UTRs has led to the hypothesis that it has a dual function, either coding for glutamate or serving as a genuine stop codon.

Despite these discoveries, several questions regarding translation mechanisms remain unanswered, such as the impact of these modifications on translation efficiency and termination. Ribosome profiling (RiboSeq), a technique based on deep sequencing of ribosome-protected fragments (RPFs), offers significant advantages for studying translation dynamics, efficiency, and related processes. Standardizing and implementing this approach will allow us to investigate translational mechanisms directly in the model organism *B. nonstop*.

We have optimized a key step in the protocol for obtaining polysome profiles in *B. nonstop* to assess active translation processes. This optimization allows us to visualize mRNA fragments translated by a single ribosome (monosome) or multiple ribosomes (polysomes) that have been further ribonuclease-processed to obtain single ribosome-protected fragments, necessary to proceed with the RiboSeq methodology. However, the detection of highly ribonuclease-resistant disomes and their association with collision mechanisms, changes in translation speed and proximity to stop codons could provide a new approach to studying the translation mechanism in *B. nonstop*. Adapting ribosome profiling to this non-canonical protozoon for both monosome and disome populations will contribute to a deeper understanding of fundamental translation processes and stop codon readthrough, with high potential for the development of innovative RNA therapies.

P03 Correcting splicing of Prpf31 in retinitis pigmentosa

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Retinitis pigmentosa (RP) is a genetic disorder causing the loss of photoreceptors and consequently central and peripheral vision. Pre-mRNA processing factor 31 (PRPF31) is a splicing factor associated with ~10% of cases of the autosomal dominant form of RP. We have described a novel point mutation in the intron 10 of the Prpf31 gene which caused RP in one of the siblings in a family, while the parents were phenotypically unaffected. The splicing efficiency test of intron 10 using human cell lines and blood samples showed that intron 10 is not efficiently removed even in wild type samples. Next, we established a splicing reporter based on Prpf31 intron 10 and inserted the intronic mutation. This mutation not only eliminated the initial spliced product but also revealed a cryptic splice site, resulting in an alternative spliced product that was extended by 10 base pairs. Protein expression from the RP patient's blood sample also revealed decreased expression of PRPF31 and other splicing proteins. Furthermore, immunoprecipitation experiments with the mutated PRPF31 reporter revealed a diminished interaction with PRP6, which collaborates with PRPF31 in the assembly of the tri-snRNP (U4/U6.U5).Then, we designed antisense oligonucleotides (ASOs) targeting the potential binding sites of splicing regulators in exons 10 and 11 and intron 10, in order to correct the splicing in the mutated Prpf31. We introduced ASOs individually or in combination into cells and evaluated their effect on splicing of wild type and mutated Prpf31. We also have established induced pluripotent stem cell lines derived from patient mononuclear blood cells (PBMC) and would like to administer our ASOs for potential splicing improvements. ASOs are becoming a powerful tool to manipulate RNA processing and hold a therapeutic potential to treat various genetic disorders. So far in this study, we have identified a novel point mutation in Prpf31, one of the most mutated genes causing RP, characterized the mutation and identified ASO to improve its splicing, which may open a way to treat this rare disease.

P04 Identification and characterization of novel small regulatory RNA of *Bordetella pertussis* CT_521

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Recently we have applied the RNA-seq technology to discover hundreds of putative regulatory RNAs in B. pertussis, though the function of most of these riboregulators remains uncharacterized. Furthermore, we have shown that RNA chaperone Hfq, a key player in small RNA (sRNA) transactions, is required for virulence. RIL-seq (RNA interaction by ligation and sequencing) is a method combining experimental and computational approaches. It is based on *in vivo* ligation of Hfq-bound sRNAs-target mRNAs pairs, immunoprecipitation of Hfq-RNA complexes, sequencing of ligated chimeric RNAs and computational analysis. Thus, this method allows for global capture and mapping of *in vivo*-formed duplexes between sRNAs and target mRNAs. Our RIL-seq analysis revealed putative targets for several already identified sRNA including Candidate_Transcript_521 (CT_521). The expression of CT_521 sRNA is highly dependent on Hfq and can be induced by heat shock treatment. Furthermore, the expression of *CT_521* is moderately reduced in the absence of BvgA regulator but not in the presence of 50 mM sulfate. Thus, it appears that this sRNA requires unphosphorylated BvgA factor. We have deleted the CT_521 sRNA and analyzed the mutant by omics techniques (RNA-seq, LC-MS/MS). These data suggest that CT_521 is involved in regulation of sulfur transport and metabolism.

P05 Terrestrial Slugs as Prospective Animal Models for Studying RNA Silencing Pathways

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Mollusca, the second largest animal phylum, has been rather neglected in molecular biology research. Our aim is to shed light on this phylum and understand how RNA silencing pathways evolved in molluscs. To achieve this, we propose two terrestrial slugs Deroceras laeve and Deroceras invadens as new model organisms for studying RNA silencing. The selected slugs are perfect for their small size (2-3 cm), relatively short generation cycle (3-5 generations a year), and established lab cultures. Additionally, we included the Spanish slug *Arion vulgaris* into our analysis, for it is an important pest and invasive species.

To study RNA silencing, we sequenced small RNAs from all three slugs. We identified 154 high confidence miRNA precursors in *A. vulgaris* genome. However, only about half of them exist in *Deroceras spp.*, suggesting dynamic evolution of the miRNA pathway among slugs. Interestingly, piRNAs in *A. vulgaris* are ubiquitously expressed, targeting various types of repetitive elements (LINEs, LTRs, DNA transposons). Since genomes for *D. laeve* and *D. invadens* were unavailable, we sequenced them and so far completed an initial assembly for *D. laeve*: its genome is around 1.1 Gb and shows low heterozygosity rate. Genome of *D. invadens* is approximatelly 1 Gb. Furthermore, we sequenced transcriptomes of all three slugs, identifying genes involved in the RNA silencing pathway, including Dicer, two Argonaute, and two PIWI proteins. We identified the conserved DEDH tetrad motif in both Argonaute and PIWI proteins in the *Deroceras species*, confirming the presence of a functional RNA silencing machinery.

Presently, we are working on knocking-out Pax6 as a proof-of-concept experiment. Additional knock-outs will target genes involved in RNA silencing to extensively determine the role of small RNAs in all three slug species. Establishing genetic modifications will be crucial in developing terrestrial slugs into an experimental model system in molecular biology.

P06 The roles of the phosphotransferase system (PTS) in regulation of rifampicin resistance in *Bacillus subtilis*

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The bacterial phosphoenolpyruvate phosphotransferase system (PTS) is conserved in almost all types of eubacteria. It functions as a phosphorylation cascade and enables the transport and simultaneous phosphorylation of various carbohydrates. In *Bacillus subtilis*, the central protein of this cascade is histidine-containing phosphocarrier protein (HPr), which can be phosphorylated on His-15 or Ser-46. The two phosphorylated forms play roles in regulation of carbon metabolism and sugar transport across the cytoplasmatic membrane. Surprisingly, our proteomic data revealed that HPr was upregulated after treatment with subinhibitory concentration of the antibiotic rifampicin, which targets RNA polymerase. Subsequent experiments validated the result, showing that a strain with the HPr-encoding gene deleted was more sensitive to rifampicin. We then demonstrated that the resistance is dependent on the Ser46-phosphorylated form of HPr and the functional carbon catabolite repression (CCR) pathway involving catabolite control protein A (CcpA). Moreover, biochemical experiments with purified components of the PTS system analyzed by mass spectrometry indicated that the PTS system has a potential to phosphorylate and thereby inactivate rifampicin itself, suggesting a complex defence mechanism against this antibiotic. The results will be discussed and a model describing the involvement of the PTS system in rifampicin resistance will be presented.

This work is supported by grant No. 24-10700S from the Czech Science Foundation.

P07 A non-coding Ms1 RNA modulates activity of RNA polymerase in *M. smegmatis*

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A single RNA polymerase is responsible for transcription of all genes in bacteria and therefore this process is heavily regulated. First major control point is at transcription initiation stage, where various factors can be recruited to RNA polymerase and determine the outcome. One type of such regulators are non-coding RNAs (sRNAs), which can be highly abundant and take up to 60% of all trancripts.[1]

A newly discovered Ms1 RNA in mycobacteria has been shown to bind RNAP core, in contrast to 6S RNA which associates only RNAP holoenzyme in *B. subtilis*.[2]

The mechanisms by which Ms1 controls transcription initiation in *M. smegmatis* are not well understood. In order to address this interaction, we are using both - a recombinant and natively pull-down Ms1-RNAP complexes on cryo-EM. Initial findings indicate a strong presence of MS1 in the electron density map and that the Ms1 densities correspond well to the predicted secondary RNA structure. The outcomes from this study could potentially be utilized in the development of drugs against the human pathogen *M. tuberculosis*.

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P08 MoaB2, a newly identified transcription factor, binds to σ^A in *Mycobacterium smegmatis*

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In mycobacteria, SigA is the primary sigma factor that is essential for transcription initiation by allowing RNA polymerase (RNAP) to bind specifically to promoters of housekeeping genes. Despite its importance, our knowledge of SigA in mycobacteria remains limited. In this study, we performed an unbiased search for *Mycobacterium smegmatis* SigA interaction partners and identified several proteins, the most prominent of which was MoaB2. We verified and characterized the SigA-MoaB2 interaction by several methods and demonstrated that the interaction is direct and does not require RNAP. Alternative sigma factors did not interact with MoaB2. Using analytical methods, we determined the structure of MoaB2 from *M. smegmatis*, identified a unique, unstructured N-terminal SigA domain as key to this interaction and defined the protein complex stoichiometry. Functional experiments demonstrated that MoaB2 inhibits SigA-dependent transcription and increases SigA stability in the cell. We hypothesize that MoaB2 sequesters SigA to affect the transcriptional apparatus in a dual manner, potentially modulating gene expression. Overall, this study reveals a novel binding partner of mycobacterial SigA and paves the way for future investigation of this biochemical interaction.

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P09 Study of dinucleoside polyphosphates incorporation into RNA by *Escherichia coli* RNA polymerase and its regulation

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The discovery of a new class of 5' RNA caps, known as dinucleoside polyphosphates (Np_nN), in bacteria has opened new ways for studying RNA and its various functions. These molecules, also referred to as alarmones, play a significant role in the cell's response to metabolic changes. Our research demonstrates that Np_nNs are incorporated into RNA during transcription and that it is influenced by the metabolic status of the cell.

In our study, we observed the *in vitro* incorporation of Np_nNs into RNA by T7 RNA polymerase. Building on these findings, we directed our focus towards understanding the role of *Escherichia coli* RNA polymerase in the capping mechanism both *in vitro* and *in vivo*. A particular emphasis was placed on the role of sigma factors in this process. Sigma factors are proteins that bind to RNA polymerase core forming the holoenzyme, directing it to specific initiation sites for transcription. In our *in vitro* experiments, we observed that both sigma 70 (σ70), the housekeeping sigma factor, and sigma S (σS), the stationary phase sigma factor, are capable of producing Np_nNs- RNA. We observed that the in vitro production of capped RNA by *E. coli* RNA polymerase associated either with sigma 70, sigma S, or without any sigma subunit, differs. The difference in the amount of produced capped RNA indicates that the presence of sigma factors could influence the efficiency and perhaps the specificity of the RNA capping process. To investigate deeper the role of sigma factor S (σS) in the production of capped RNA, we extracted RNA from an *E. coli* strain with a mutated *rpos* gene (which encodes σS) and from a wild-type (Wt) strain at various stages of growth. This approach will allow us to assess the impact of σS on the capping process under different physiological conditions.

Overall, this work aims to enhance our understanding of the role of *E. coli* RNA polymerase during transcription initiated by Np_nN-RNA caps and the cellular response to stress. By unrevealing how sigma factors, particularly σS, affect RNA capping, we can gain insights into the complex regulatory mechanisms that govern bacterial RNA synthesis and response to environmental changes.

Oldřich, Hudeček., Roberto, Benoni., Paul, E., Reyes-Gutiérrez., Martin, Culka., Hana, Šanderová., Martin, Hubálek., Lubomír, Rulíšek., Josef, Cvačka., Libor, Krásný., Hana, Cahová. (2020). Dinucleoside polyphosphates act as 5'-RNA caps in bacteria. Nature Communications, 11(1):1052-1052. doi: 10.1038/S41467-020-14896-8.

P10 Retinitis pigmentosa-linked mutations impair the snRNA unwinding activity of SNRNP200

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Retinitis pigmentosa (RP) is a hereditary disorder caused by mutations in more than 70 different genes including those that encode proteins important for pre-mRNA splicing. Most RP-associated mutations in splicing factors reduce either their expression, stability or incorporation into functional splicing complexes. However, we have previously shown that two RP mutations in SNRNP200 (S1087L and R1090L) behaved differently, and it was still unclear how these mutations affect the function of the protein. To investigate this in the context of functional spliceosomes, we used iCLIP in HeLa cells. We found that both mutations in the RNA helicase SNRNP200 change its interaction with U4 and U6 snRNAs. The significantly broader binding profile of mutated SNRNP200 within the U4 region upstream of the U4/U6 stem I strongly suggests that its activity to unwind snRNAs is impaired. This was confirmed by helicase activity assays comparing mutant and WT protein.

P11 Understanding the structural basis of genome replication in Rift Valley Fever Virus

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Rift Valley Fever Virus (RVFV) possesses a segmented negative sense single-stranded RNA as its genetic material. RVFV poses a significant public health risk due to its ability to infect humans and the absence of specific medical treatments.[1] RNA replication and transcription in RVFV are catalysed by the multi-functional and monomeric viral RNA-dependent RNA polymerase, referred to as L-protein (LP). LP initiates replication of viral RNA (vRNA) into complementary RNA through *de novo* replication initiation. It utilizes a capped mRNA primer obtained from the host to generate viral mRNA during transcription. The cap snatching process is executed by the endonuclease domain and the cap-binding domain of LP.[2] However, existing structural data on RVFV L-protein lack visualization of the endonuclease domain, cap-binding domain, and multiple intermediate steps essential for a comprehensive understanding of replication and transcription.

Our aim is to generate high-resolution cryo-EM structures of LP's replication and transcription intermediates, allowing us to elucidate these essential mechanisms at the near-atomic scale. This comprehensive structural understanding of RNA replication and transcription mechanisms in RVFV can provide promising opportunities for drug development.

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P12 Assembly of the Xrn2/Rat1–Rai1–Rtt103 termination complexes in mesophilic and thermophilic organisms

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Transcription termination is essential for delineating the genetic information stored in DNA, as it establishes the boundaries of transcriptional units.[1] In yeast, there are two model pathways how the termination of mRNA coding genes is organized: allosteric and torpedo models. The torpedo model considers that the unprotected free 5'-end of the mRNA transcript is digested by nuclease until it collides with RNA polymerase II (RNAPII), leading to dissociation from the template. Studies in the yeasts showed that exposed free 5'-end of RNA serves as an entry point for Xrn2/Rat1, a 5'-3' exonuclease. Stimulated by its cofactor Rai1, the Xrn2/Rat1-Rai1 complex greatly stimulates spontaneous termination.[2] However, the exact mechanism of how the Xrn2/Rat1-Rai1 complex is recruited to the site of transcription and how RNAPII is released from the DNA is unknown. Findings also suggest that the torpedo complex is recruited by Rtt103, recognizing Ser2[3] and or Thr4 phosphorylation marks of RNAPII. This allows us to hypothesize that Rtt103 helps to recruit the 5'-3' RNA termination machinery to the site of transcription.
Due to the variations in Xrn2/Rat1-Rai1-Rtt103 complexes in mesophiles and thermophiles, we set out to investigate whether and how these complexes assemble in yeast Saccharomyces cerevisiae and fungi Chaetomium thermophilum. Using a combination of structure biology techniques including cryo-EM, size exclusion chromatography, crosslinking mass spectrometry, and NMR we reveal differences in how the two torpedo complexes assemble in mesophiles and thermophiles. Our observations suggest that thermophilic organisms have adapted protein-protein interfaces to favor the presence of highly structured elements, whereas mesophilic organisms prefer the utilization of unstructured elements that fold upon binding to their interaction partners.

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P13 Role of R-loops in oncogene-induced replication stress

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Oncogene-induced replication stress plays a critical role in cancer initiation and progression. Activation of oncogenes, by various mechanisms, affects the speed and timing of DNA replication and generates obstacles for the replication fork, which can result in DNA damage, including DNA double-strand breaks, and consequent chromosomal rearrangements characteristic for cancer cells. A significant source of replication stress is head-on collisions between transcription and replication complexes (TRCs). Although the role of TRCs in oncogene-induced transformation is increasingly recognized, the factors contributing to increased frequency of TRCs and their genotoxic effects as well as mechanisms underlying resolution of TRCs remain elusive.

In this study, we have focused on replication stress induced by retrovirus-mediated overexpression of cyclin E and hRAS-V12 oncogenes. Although these oncogenes have different mechanisms of action, we confirmed that activation of either of them results in increased numbers of potentially genotoxic R-loops, three-stranded structures composed of RNA:DNA hybrid and ssDNA loop. We used RNaseH1-based approaches to visualize the oncogene-induced formation of R-loops (catalytically inactive RNaseH1) and to study the role of R-loops (wild-type RNaseH1) in observed fork slowing/stalling and in consequences of this replication stress. We showed that replication forks stalled in response to oncogene overexpression can be restarted via the previously described MUS81-ELL-LIG4 pathway that resolves R-loop-associated TRCs. Understanding how cells with activated oncogenes overcome the replication stress and restart stalled replication forks might reveal new targets for anti-cancer therapies.

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P14 A novel mutation in *ADARB1* effect its activity

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One of the most abundant and extensively studied RNA modifications within this field is the deamination of adenosine to inosine by the family of enzyme; adenosine deaminase acting on RNA (ADAR). Three members of the ADAR family, ADAR1, ADAR2, and ADAR3, have been identified in humans, but only ADAR1 and ADAR2 are enzymatically active. ADAR2 plays a key role in regulating neuronal excitability and inhibition. It is involved in editing of transcripts encoding ion channels and neurotransmitter receptors, proteins critical for neuronal electrical signalling.[1] ADAR2's editing activity can modulate the function of these proteins, thereby impacting on the excitability levels of neurons.

Previous research has shown that some ADAR2 mutations found in children with seizures can reduce RNA editing efficiency, which may be related to severe symptoms.[2] Therefore, understanding ADAR2's functions and the consequences of its dysregulation is of significant interest for both basic neuroscience research and the development of potential therapies for related diseases. Here we report a novel mutation in *ADARB1*, the gene encoding ADAR2. In addition, we perform proteomic analysis of the brains of a mouse model lacking Adar2 protein.

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P15 Requirements for both Dcr-2 and cGlr1/Sting dsRNA-activated signaling for aberrant innate immune induction in Adar ^{5G1} null mutant flies lacking adenosine to inosine editing in dsRNA

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Drosophila Adar is mainly expressed in the nervous system (Jepson et al. 2011) and carries out A-to-I RNA editing in dsRNA hairpins in pre-mRNAs. Edited mRNAs are numerous in CNS and enriched in ion channels and neurotransmitter receptor subunits which produce new edited proteoforms (Duan et al. 2017). Loss of Adar RNA editing activity in Adar ^{5G1} null mutant flies leads to a severe locomotion defect, consistent with loss of edited CNS proteoforms, and also to aberrant innate immune AMP induction (Deng et al. 2020). The AMP induction is suppressed by silencing of Dicer-2 in cholinergic neurons (Deng et al. 2020) and may be similar to aberrant activation of Dicer-related vertebrate cytoplasmic antiviral dsRNA sensors by unedited dsRNA. We sought to determine whether knocking down the antiviral cGas-Like Receptor1 (cGlr1), recently shown to be activated by dsRNA in Drosophila, or Sting receptor which acts downstream of cGLR1, can rescue aberrant AMP induction and other defects in Adar5G1 flies. We found that ubiquitous RNAi knockdown of cGlr1 in Adar ^{5G1}, arm> cGlr1 RNAi flies rescues the aberrant immune induction in heads; however, it does not rescue the locomotion defect or reduced survival to eclosion. Similar RNAi knockdown of Sting improves survival and rescues aberrant immune induction but not locomotion defects. Furthermore, the double null mutant Adar ^{5G1}; cGlr1 KO prevents the immune induction and significantly improves the locomotion. It is important to note that cGLR1 KO alone has no effect on wildtype locomotion. These data suggest that the innate immunity and neuronal defects in Adar ^{5G1} null mutant involve both Dcr-2 and cGlr1/Sting pathways, perhaps working together.

P16 High-throughput screening for enhancing PRPF31 expression in retinitis pigmentosa model

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Retinitis pigmentosa (RP) is the most common inherited retinal disease, characterized by the progressive degeneration of photoreceptors. RP patients suffer from night blindness and the disease can eventually lead to complete loss of vision. Among the proteins linked to RP, splicing factors such as *PRPF31* are notable. Mutations in *PRPF31* resulting in functional loss of one allele and haploinsufficiency account for nearly 10% of autosomal dominant RP. However, PRPF31-linked RP demonstrates incomplete penetrance, as carriers of these mutations can be prevented from developing the disease if the remaining wild-type allele produces sufficient amounts of functional protein. Currently, there is no cure for PRPF31-dependent RP, though vitamins and nutritional supplements can slow its progression.

In this study, we propose and validate a high-throughput screening method based on EGFP-fluorescence readout to determine PRPF31 expression levels following treatment with clinically relevant drug libraries. With this aim, we established an RPE cell line with PRPF31 protein tagged with EGFP in one allele (heterozygous, Hz) or both alleles (homozygous, Ho). Changes in EGFP fluorescence of PRPF31-Hz cells are evaluated 24 hours post-treatment, while PRPF31-Ho cells serve as positive controls. Promising hits are further analyzed by flow cytometry and western blotting to optimize drug concentrations and validate the high-throughput results. Selected drugs that increase PRPF31 expression without compromising cell viability will be tested for their ability to enhance PRPF31 expression in patient-derived iPS cells and reverse their deleterious phenotype.

We believe that our proposed system will facilitate the identification of new drugs capable of positively influencing PRPF31 expression, potentially leading to novel therapeutic strategies for RP.

P17 Where transcription meets translation

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Transcription and translation are key steps in gene expression. Elongation factor Tu (EF-Tu) belongs to the group of translational GTPases. It binds and transports aminoacyl-tRNA to the ribosome. Moreover, EF-Tu has additional functions. EF-Tu interacts with the MreB protein, helping maintain the cell shape. Phages Qβ and MS2 use EF-Tu as a subunit of their RNA replicases for their proper function. EF-Tu also functions as a molecular chaperone. Finally, EF-Tu was shown to increase resistance of the translational apparatus to antibiotics tetracycline, streptomycin, spectinomycin, and erythromicin.

RNA polymerase (RNAP) is the key enzyme of transcription. During studies of *Bacillus subtilis* transcription, we identified that EF-Tu possibly associates with RNAP.

Here, we will present results from co-immunoprecipitation and western blot showing the interaction between EF-Tu and RNAP. We monitored the interaction in different growth phases and in rich and defined/minimal media. Furthermore, we will present *in vitro* transcription results addressing the effects of EF-Tu on RNAP.

This novel interaction suggests a coordinated regulation of transcription and translation, providing new insights into the complexity of gene expression regulation in *B. subtilis*. Further research into this interaction could uncover additional regulatory mechanisms and contribute to a broader understanding of bacterial gene expression.

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P18 Study of the effect of the 5´poly(A) sequence of mRNAs on translation initiation rate

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Translation represents a relatively well-studied process in both prokaryotic and eukaryotic organisms. However, certain principles of translation remain insufficiently explored. In yeast, there are highly specific virus-like elements (VLEs) whose specific transcripts are translated by an unknown mechanism. These transcripts exhibit unconventional 5' and 3' structures. The 5' ends contain 2-20 non-templated added adenosine residues and lack an N7 methylguanosine cap. At the 3' end, transcription termination occurs via weak terminator hairpins, and polyadenylation is completely absent.

The objective of this project is to elucidate the translation mechanism of VLE-specific transcripts. For this purpose, two reporter systems have been developed, based on luciferase reporter genes, whose transcripts bear a defined number of adenosine residues at the 5' end. The first reporter system is an integral part of the linear cytoplasmic plasmids (VLEs elements) of the yeast *Kluyveromyces lactis*. This system utilizes promoters that are intrinsic to these plasmids. These promoters determine the structure of the resulting reporter transcripts, which is crucial for our analysis. In contrast, the second system is based on a dual-expression 2μ vector, where the reporter genes are inserted under the control of strong yeast promoters with a modified transcription start site. This modification ensures that the resulting reporter transcripts carry a defined number of adenosine residues at the 5' end.

Through the use of reporter enzyme activity and other approaches, such as mapping the structures of reporter transcripts (5' RACE PCR), qPCR (transcriptome mapping), and the newly published TREX method, which enables the study of protein structures binding to specific mRNA sequences, answers are being sought regarding the unknown translation mechanism.

This research was supported by the project National Institute of virology and bacteriology (Programme EXCELES, ID Project No. LX22NPO5103) – Funded by the European Union - Next Generation EU and by the Project P JAC CZ.02.01.01/00/22_008/0004575 RNA for therapy, Co-Funded by the European Union.

P19 Unveiling druggable Sites in c-MYC 3’UTR: a high-throughput approach for targeting 'undruggable' oncogenes

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Oncogenic proteins often lack accessible binding sites for small molecule inhibitors, complicating therapeutic intervention. Targeting the 3' untranslated regions (3'UTRs) of oncogene mRNAs, crucial for mRNA stability and translational regulation, presents a novel and promising strategy. However, drug design based on predictive RNA structures is challenged by complex 3'UTR tertiary and quaternary formations. We present a high-throughput screening method to identify druggable pockets within 3'UTRs of cancer-related mRNAs, bypassing biases from secondary structure predictions. Overlapping fragments of the c-MYC mRNA 3'UTR were screened against a specialized heterocycle library using a Fluorescein-based Anisotropy (FA) assay. This approach successfully identified compounds that modulate RNA structure, indicated by significant changes in fluorescence anisotropy. Key regions of the c-MYC 3'UTR demonstrated substantial small molecule interactions, suggesting potential binding sites. Functional validation via dual-luciferase assays revealed that specific compounds could either inhibit or enhance c-MYC expression in cellular models. Correlating FA data identified 3'UTR regions inducing structural changes upon ligand binding, critical for protein or miRNA interactions. This methodology underscores the potential to target 'undruggable' oncogenes with small molecules, facilitating the discovery of specific binders for distinct 3'UTR regions. It promises broad applications across diverse cancer-related genes, revolutionizing therapeutic strategies contingent on RNA tertiary structure variability.

P20 Dissecting recycling: differential effects of *cis*- and *trans*-acting factors on ribosome recycling in yeast

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Recycling of 40S ribosomal subunits following translation termination entails the release of deacylated tRNA and release of the small subunit from mRNA. In yeast, this involves the Tma20/Tma22 heterodimer and Tma64, counterparts of mammalian MCTS1/DENR and eIF2D. MCTS1/DENR enhance reinitiation (REI) at short upstream open reading frames (uORFs) harboring penultimate codons that confer heightened dependence on these factors in bulk 40S recycling. Tma factors, by contrast, inhibited REI at particular uORFs in extracts; however, their roles at regulatory uORFs in vivo were unknown. We examined effects of eliminating Tma proteins on REI at regulatory uORFs mediating translational control of GCN4. We found that the Tma proteins generally impede REI at native uORF4 (primed for ribosome recyclicing) and mutant variants equipped with various penultimate codons regardless of their Tma-dependence in bulk recycling. The Tma factors have no effect on REI at native uORF1 (primed for REI), and equipping uORF1 with Tma-hyperdependent penultimate codons generally did not confer Tma-dependent REI. Thus, effects of the Tma proteins vary depending on the REI potential of the uORF and the penultimate codon, but unlike in mammals, are not principally dictated by the Tma-dependence of the codon in bulk 40S recycling. In addition, we employed the *GCN4* reporter system to screen a yeast deletion library for unknown factors influencing REI and/or recycling and found several promising candidates, the functional roles of which will be discussed.

P21 The regulation of RNA polymerase expression in mycobacteria

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Mycobacterial RNA polymerase (RNAP) is the target of rifampicin, an important drug used in the treatment of tuberculosis caused by *Mycobacterium tuberculosis*. The resistance to rifampicin is influenced by the RNAP level in mycobacterial cell. Previously, we discovered a small RNA Ms1 that binds RNAP in *M. smegmatis*. We found that Ms1 increases the amount of RNAP during stationary phase of growth, when the conditions are unfavorable.

We identified new promoter of *rpoB-rpoC* genes encoding two major RNAP subunits. mRNA transcribed from this promoter has an unusually long 5´UTR. We identified proteins binding to different promoter regions and the sequences that are important for the regulation by Ms1 RNA.

P22 See life through colors: Single-cell fluorescent-based RNAi sensor system for assessment of increased RNAi efficiency

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Current methods to measure RNAi activity involve luciferase-based reporter systems, which require the co-transfection of multiple plasmids. The limitations of these systems include the restriction to a single time-point measurement and the inability to determine the heterogeneity of reporter activity in individual cells. Here, we have developed a fluorescence-based RNAi reporter system in the form of a stable cell line, which expresses three knocked-in fluorescent proteins into highly expressed endogenous genes. This system enables us to monitor RNAi efficiency and addresses the aforementioned limitations, while offering flexibility in data collection, including single-cell resolution and sampling at multiple time points.

P23 eIF3 at the Tip of the AUG Recognition Process in Humans

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Translation initiation site (TIS) selection is a crucial step in translation as it determines the reading frame for decoding, ensuring that the correct protein is produced. If initiation occurs at an incorrect codon, it can result in the synthesis of a miscoded protein, wasting the cell's valuable resources and potentially creating toxic peptides. In eukaryotes, the 40S ribosome must scan the mRNA's 5' UTR in search of the TIS and respond to a complex set of signals that arise from interactions among the mRNA, the 40S ribosome, and specific initiation factors (eIFs) that coordinate TIS recognition. Although significant progress has been made in understanding this process in model organisms such as *S. cerevisiae*, much remains unknown about TIS selection in higher eukaryotes. Our project aims to explore how multi-subunit eIF3 and its associated eIFs contribute to the precise selection of the TIS at the molecular level in humans, utilizing cutting-edge genetic, biochemical, and structural methods. Key questions addressed include whether eIF3c collaborates with eIFs 1 and 5 to control AUG recognition in humans, a translatome-wide analysis of TIS-sensitive mRNAs in human cells, and obtaining structural insights into the human mutant 48S pre-initiation complex (PIC) arrested at the TIS.

P24 Quality control during snRNA biogenesis

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Biogenesis of new snRNP particles is a tightly regulated process. We have two classes of snRNAs called Sm and Sm-like. Our project is focused on the biogenesis of the Sm class, which includes U1, U2, U4 and U5. Sm class snRNAs are transcribed by RNA poylmerase II and cotranscriptionally modified with a 7'-methylguanosine cap at the 5' end. This modification is recognized by a number of proteins, such as PHAX or CBC, which are part of the nuclear export complex. The snRNA is transported through the nuclear pore into the cytoplasm where the export complex is dissolved. In the following step, the Sm ring is assembled. This is a crucial step in snRNA biogenesis because if Sm ring assembly does not occur, the snRNA is directed to P-bodies for degradation. The assembly of the Sm ring serves as a signal to TGS1. This enzyme hypermethylates the 7'-methylguanosine cap to a 2', 2,' 7'-trimethylguanosine cap. The presence of the Sm-ring and the trimethylguanosine cap serves as a bipatrial signal for the reimport of snRNA into the nucleus. In the nucleus, snRNP is targeted to the Cajal bodies where the further maturation steps occur.
Aim of our project is to describe how the cell distinguishes between wild type and truncated snRNAs that are able to assemble the Sm ring. Our current data suggest that truncated snRNAs accumulate immature cap types such as the 7'-methylguanosine cap. We also observe that truncated snRNAs interact with innate immunity proteins called IFITs proteins.

P25 Analysis of feature of miRNA precursor in RNA silencing pathway

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RNA silencing pathways employ small RNAs to regulate gene expression and protects against parasitic sequences. The RNA interference (RNAi) and microRNA (miRNA) pathways are two major pathways, which use RNase III Dicer to produce small RNAs. Dicer cleaves RNA hairpins or long double stranded RNA into small RNA duplexes from which one strand is selected and loaded onto an Argonaute protein. This principle of strand selection and the mechanism of Argonaute loading remain poorly understood. One of the postulated principles of strand selection is the so-called 5’ thermodynamic asymmetry rule, which states that the 5’ end of a less stably paired strand is selected. While this rule applies to RNAi reasonably well, more than a half of miRNAs do not follow it.

The goal of the project is to investigate which other miRNA duplex features contribute to the strand selection and AGO loading. Our working hypothesis is that polarization of a miRNA duplex defined by its processing, its three-dimensional structure accommodating bulges and mismatches as well as free nucleotides at RNA strands in the duplex contribute to the process. Therefore, through a combination of computational and experimental approaches, we aim to investigate how these features influence strand selection and Argonaute loading.

P26 Characterization of putative transcription factors in *Bacillus subtilis*

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Bacteria have evolved various mechanisms to regulate gene expression, enabling them to adapt quickly to changing environments. Proteins called transcription factors (TFs) play crucial roles in this regulation. TFs bind to regulatory regions of DNA and either prevent or promote RNA polymerase association with promoter DNA. Despite extensive research, many TFs remain unknown.

Although *Bacillus subtilis* represents one of the most studied and best-understood model organisms, the function of about 20% of its proteins is still unknown or poorly characterized. Using a deep-learning approach, putative TFs were predicted in *B. subtilis*. From the list of predicted TFs, we selected 15 top candidates containing the helix-turn-helix (HTH) motif. The HTH is the most widely used DNA-binding motif in the prokaryotic world.

This project aims to unravel the functions of the 15 selected putative TFs. To get first insights into the cellular roles of these proteins, deletion strains were prepared and evaluated in phenotypic assays. For further analyses, strains with FLAG-tagged TFs under the control of an IPTG-inducible promoter were prepared. To identify interacting partners of our candidate TFs, immunoprecipitation of FLAG-tagged TFs followed by mass spectrometry analysis was performed. Moreover, ChIP-seq experiments are currently underway to further characterize the binding sites of selected TFs across the *B. subtilis* genome. Simultaneously, to associate these binding events with effects on gene expression, transcriptomic analysis of overexpressed TF strains is being performed using RNA-seq.

We will discuss our recent progress, which includes identifying the TF involved in D-cycloserine (a drug used to treat tuberculosis) resistance. In conclusion, this project will provide a better understanding of how organisms mediate responses to varying environmental conditions through transcriptional regulation.

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P27 Adenosine (de)methylation on the crossroads of RNA and DNA synthesis

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Eukaryotic mRNAs are subject to significant chemical modifications that exhibit a multi-layered regulation of transcript metabolism. Among these, N6-methyladenosine (m6A) and N6-2'O-dimethyladenosine (m6Am) are two prevalent modifications in higher eukaryotes [1]. The dynamics of these modifications are tightly governed by dedicated machineries, the perturbation of which destabilise cellular homeostasis. In a recent study using proximity-based labelling approach, we identified that m6A/m eraser protein – FTO – was proximally interacting with proteins in DNA replication and repair pathways [2]. To elucidate this further, in this study we performed subsequent protein-protein interaction assays and genome wide CRISPR screening, where we identified multiple candidates involved at different levels of DNA synthesis. Additionally, we performed functional assays to further investigate the relevance of FTO in these processes. Preliminary results from these functional assays positively correlate with our observations from the high throughput screening approaches, highlighting the importance of the m6A/m modifying enzyme, FTO, in the maintenance of genome integrity.

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P28 Effect of the ADAR1 signalling pathway on HCV replication

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The hepatitis C virus (HCV) is a member of the Flaviviridae family, the genome of which consists of a +RNA molecule. It causes hepatitis C, which infects tens of millions of people worldwide. Although new direct-acting antivirals (DAAs) are highly effective in treating hepatitis C, a preventive vaccine against HCV has not yet been developed. This report examines the relationship between hepatitis C virus (HCV) and the double-stranded RNA editing enzyme adenosine deaminase 1 (ADAR1). As part of the innate immune response, ADAR1 catalyses the conversion of adenosine to inosine, which affects both the stability of the edited double-stranded RNA helix and the information encoded in the primary sequence of nucleotides. In order to evaluate the impact of ADAR1 on HCV replication, an ADAR1 knockout cell line was generated from Huh7.5 hepatocellular carcinoma cells. The findings of preliminary experiments examining HCV replication in the Huh7.5 ADAR1 KO cell line will be presented.

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P29 Manipulating MYC mRNP interactions: A novel approach using small druglike molecules

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The complex interactions between RNA and proteins offer a promising approach to drug discovery, especially for diseases that have resisted conventional therapeutic approaches. MYC proto-oncogene is a prime target, involved in over 50% of cancers, and extremely difficult to regulate due to its lack of druggable binding pockets. Our research focuses on modulating the messenger ribonucleoproteins (mRNPs) of MYC by targeting structured motifs within its 3'UTR using small drug-like molecules. To this end, we've developed a novel workflow to study the impact of RNA-targeted compounds on RNA-binding proteins (RBPs).

Our approach began with the selection of MYC 3'UTR fragments that exhibit conformational changes in response to small drug-like molecules based on fluorescent anisotropy data. RNA pulldown experiments on these fragments showed distinct interactomes. Notably, proteins interacting with the most responsive fragment are involved in various biological processes and possess RNA recognition motifs and KH domains. This responsive fragment was chosen as an example to assess how small drug-like molecules induce structural changes in RNA and consequently alter its protein binding partners.

We identified promising compounds based on their regulatory effects, as determined by our team using a functional dual luciferase assay. Subsequent RNA pulldowns of biotin-labeled 3'UTR fragments revealed that different sets of regulatory RBPs are enriched in the presence or absence of these molecules. This finding suggests that the structural changes induced by small molecules could significantly influence the fate of the target mRNA. To validate and extend these initial observations, we expanded our studies to include in vitro-transcribed 3'UTR and are exploring the global effects of these small drug-like molecules on cellular processes.

Our findings indicate that compounds targeting MYC mRNA can interfere with RNA-protein interactions, potentially offering means for modulating MYC expression. This novel approach presents a promising avenue for cancer treatment and may overcome the limitations of traditional drug discovery methods. Our workflow provides a powerful tool for investigating the effects of RNA-targeted compounds on RBP interactions, potentially opening new therapeutic interventions in cancer and other diseases where RNA-protein interactions play a pivotal role. As we refine and expand this method, we anticipate uncovering new insights into the complex world of RNA-protein interactions and their role in disease pathogenesis, paving the way for innovative treatment strategies.

P30 Reverse transcriptase fingerprint method for RNA caps detection

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In 2020, an entirely new class of 5' RNA caps, structured as dinucleoside polyphosphates (Np_nNs), was discovered in E. coli[1]. Free Np_nNs were identified 50 years ago in all types of cells[2]. It is known that their concentration increases under stress. Nevertheless, the intracellular role of Np_nNs remains enigmatic.
Recently, we reported the existence of one of these Np_nN RNA caps, diadenosine tetraphosphate (Ap_4A), in mammalian cell lines[3]. However, nothing is known about the types of RNA bearing Np_nN caps and how they influence cellular reactions to stress. Therefore, it is essential to develop a selective profiling technique that allows for the identification of RNA types capped with Np_nNs.
It is known that reverse transcription of RNA templates containing RNA modifications leads to the synthesis of cDNA containing information on the modification in the form of misincorporation, arrest, or nucleotide skipping events. A collection of such events from multiple cDNAs generated by different reverse transcriptases (RTs) represents an RT-signature (fingerprint) that is typical for a given modification[4]. Recently, machine learning of reverse transcriptase signatures was used to distinguish internal RNA modifications, such as m^6G (6-methylguanosine)[4]. Because nothing is known about the reading and recognition of these NpnN caps by RTs, we aim to exploit this RT fingerprint method for the preparation of cDNA-seq libraries. We have used model RNA bearing different 5` RNA caps (e.g., m^7Gp_3A, TMG, NAD, Ap_4A) as substrates for a combination of commercially available RTs. Subsequently, we prepared a cDNA-seq library suitable for Oxford Nanopore Technology, established in our laboratory. So far, we have observed significantly different behavior of three RTs with various caps through bioinformatic analysis. The next step is to develop a machine learning tool and apply the method to RNA from real samples (*E. coli* or human tissue cells)

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P31 Regulation of gene expression by CDK12 and CDK13

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Transcription of protein-coding genes by RNA polymerase II (RNAPII) is complex and tightly regulated process. Transcriptional cyclin-dependent kinases (CDKs) control transcription by phosphorylating the C-terminal domain (CTD) of RNAPII and other substrates. CDK12 and CDK13 are transcriptional CDKs that associate with cyclin K to fulfil critical roles in transcription elongation and termination and their deregulation is associated with various cancers. They phosphorylate RNAPII CTD on Serine 2 and Serine 5 in vitro, and their inhibition alters CTD phosphorylation in vivo. Previous studies have shown that short inhibition of these kinases impairs the elongation rate and processivity of RNAPII, leading to premature termination of transcription. We showed that long genes, including many DNA replication genes, are highly sensitive to CDK12 inhibition, and their downregulation delays G1/S progression. Despite increased understanding of these kinases, the distinct and overlapping functions of CDK12 and CDK13 in gene expression are still not understood. CDK12 and CDK13 have nearly identical kinase domains but differ significantly in their long unstructured N- and C-terminal regions, which may mediate the binding of different interacting partners and substrates. To identify and compare the interactomes of CDK12 and CDK13, we used a proximity-based labelling method that captures weak and transient interactions. Our latest data from experiments studying the common and unique roles of CDK12 and CDK13 will be discussed.

P32 Manipulation of ADAR1 in Cancer Cell Lines for Neoantigen Discovery

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One of the most widespread RNA modifications found in mammals is the deamination of adenosine to inosine in double-stranded (ds)RNA. This process is catalysed by the family of enzymes known as adenosine deaminases acting on RNA (ADARs). Inosine in dsRNA is recognised as "self" by innate immune RNA sensors[1] whereas non-edited dsRNA molecules are considered as non-self (for instance of viral origin). Unedited dsRNA can lead to the induction of innate immune response. If chronic inflammation is induced it may harm or even kill the cell. In humans, there are two active ADAR enzymes: ADAR1 and ADAR2. Mutations in the gene encoding ADAR1 enzymes have been linked to the development of a fatal childhood neurological disease; Aicardi-Goutières syndrome and to a mild skin disorder; Dyschromatosis symmetrica hereditaria.

ADAR1 is induced by interferon and in many types of cancer the level of expression of ADAR1 increases due to inflammation.[2] ADAR1 is an essential gene, so ADAR1 knockdown cause increase levels of unedited dsRNA which leads to the activation of intracellular dsRNA sensors such as melanoma differentiation-associated gene 5 (MDA5) and protein kinase R (PKR) resulting in cell death. We hypothesise that in cancer cell cells, excessive or, on the contrary, decreased amounts of ADAR1 could lead to significant changes in the proteome of the cell which could manifest in the production of neo-peptides, e.g. peptides which are present in the cell only due to changes in RNA editing. If presented on the cell surface, these peptides could attract CD8+ T cells. It has already been shown that peptides generated by RNA editing events can be presented by human leukocyte antigen (HLA) molecules and cause a CD8+ T cell response.[3] Therefore, some of the neo-peptides may be used in the future as biomarkers or as therapeutic vaccines. Thus, the aim of this project is to identify novel neo-antigens that arise to changes in RNA editing.

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P33 Effects of the amino acid sequence of the flexible part of the δ subunit of RNA polymerase on transcription

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The δ subunit of bacterial RNA polymerase (RNAP) in Gram-positive bacteria consists of a structured N-terminal domain (NTD) separated from the intrinsically disordered, highly acidic C-terminal domain (CTD) by a positively charged lysine stretch (K-tract). δ is important for regulation of transcription and cell survival in competitive environments. Yet, despite extensive studies of this protein, interactions of its CTD with RNAP and their role in the regulation of transcription are virtually unknown. This project addresses this key aspect of the transcription machinery in Gram-positive bacteria, focusing on *Bacillus subtilis* and *Staphyloccocus aureus* δ subunits. The amino acid (aa) sequence homology between d subunits from these two organisms is 64 %. Differences between these proteins include the absence of the K-tract from the *S. aureus* δ and markedly different aa sequences of their δ-CTDs. We created a panel of chimeric δ proteins from the two organisms with all combinations of NTD, K-tract, and CTD. We then performed *in vitro* transcription experiments with *B. subtilis* RNAP to assess the effects of these domains on the RNAP function. The experiments revealed that *S. aureus* δ does not complement *B. subtilis* δ on RNAP from *B. subtilis*. Furthermore, and most interestingly, CTD from S. aureus was unable to substitute CTD from *B. subtilis*, suggesting that the CTDs, even though they are both unstructured and highly acidic, are not interchangeable. Moreover, the shortening of the CTD of the δ subunit from *B. subtilis* has major inhibitory effects on *in vitro* transcription. These results provide a basis for future comparative studies of these intrinsically disordered protein domains.

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P34 Ribosomal A-site interactions with near-cognate tRNAs drive stop codon readthrough

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tRNAs serve as a dictionary for the ribosome translating the genetic message from mRNA into a polypeptide chain. Besides this canonical role, tRNAs are involved in other processes like programmed stop codon readthrough (SC-RT). There, tRNAs with near-cognate anticodons to stop codons must outcompete release factors and incorporate into the ribosomal decoding center to prevent termination and allow translation to continue. However, not all near-cognate tRNAs promote efficient SC-RT. Here, we demonstrate that those that do, establish critical contacts between their anticodon stem (AS) and ribosomal proteins Rps30/eS30 and Rps25/eS25 forming the decoding site. Unexpectedly, the length and well-defined nature of the AS determines the strength of these contacts, which is reflected in organisms with reassigned stop codons. These findings open a new direction in tRNA biology that should facilitate the design of artificial tRNAs with specifically altered decoding abilities.

P35 Mechanisms of fidaxomicin resistance in bacteria

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Fidaxomicin (FDX) is a narrow spectrum macrocyclic antibiotic produced by fermentation of tiacumicin metabolites by the actinomycete Dactylosporangium aurantiacum. It is currently approved for use in treating Clostridioides difficile infections. FDX acts by targeting RNA polymerase (RNAP), inhibiting the formation of the open complex, an essential intermediate in the transcription initiation pathway. A known mechanism of FDX resistance is mediated by mutations in the FDX binding site on RNAP. Besides this mechanism, however, other genetic determinants of FDX resistance are unknown. We aimed to systematically identify different mechanisms of FDX resistance using the gram-positive model organism Bacillus subtilis. We generated FDX resistant B. subtilis mutants by exposing them to increased concentration of FDX (4-8 µg/mL). Phenotypic resistance to FDX was found to be 16-32-fold higher than the wild-type. Sequencing of the rpoB gene showed few novel mutations near the FDX binding site. Furthermore, we exposed B. subtilis and C. difficile to a sub-inhibitory concentration of FDX and protein levels were compared to untreated controls. In both species, tens of proteins were identified to be significantly upregulated. The phenotypes of respective knockout strains were evaluated for FDX susceptibility. Several proteins were found to be involved in FDX resistance, indicating potential novel mechanisms. The results will be presented and discussed.

P36 Diadenosine tetraphosphate (Ap_4A) serves as a 5’ RNA cap in mammalian cells

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The recent expansion of the field of RNA chemical modifications has changed our understanding of post-transcriptional gene regulation. Apart from internal nucleobase modifications, 7-methylguanosine was long thought to be the only eukaryotic RNA cap. However, the discovery of non-canonical RNA caps in eukaryotes revealed a new niche of previously undetected RNA chemical modifications. We are the first to report the existence of a new non-canonical RNA cap - diadenosine tetraphosphate (Ap_4A) - in human and rat cell lines. 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). Using liquid chromatography-mass spectrometry (LC-MS), we show that the amount of capped Ap_4A-RNA is independent of the cellular concentration of Ap_4A. A decapping enzyme screen identified two enzymes cleaving Ap_4A-RNA - NUDT2 and DXO, both of which also cleave other substrate RNAs *in vitro*. We further assess the translatability and immunogenicity of Ap_4A-RNA and show that although it is not translated, Ap_4A-RNA is recognized as self by the cell and does not elicit an immune response, making it a natural component of the transcriptome. Our finding opens a previously unexplored area of eukaryotic RNA regulation.

P37 New RNAs that regulate transcription in actinobacteria

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Actinobacteria represent one of the largest taxonomic groups within the bacterial kingdom. This phylum includes pathogens, spore-forming soil inhabitants, plant-associated species, and gastrointestinal commensal bacteria. Despite our extensive knowledge of the secondary metabolites, especially antibiotic compounds produced by this phylum, our understanding of the transcription machinery and its regulation remains limited.

We study transcriptional regulation in selected actinobacterial species (*Corynebacterium glutamicum, Bifidobacterium bifidum, Micrococcus luteus*) and I focus on structured regulatory RNAs that bind RNA polymerase (RNAP). Two such RNAs, 6S and Ms1, have been identified so far. Recently, a novel type of similar RNA was discovered in our lab in *C. glutamicum*. It was named CoRP RNA. In *B. bifidum* and *M. luteus* no homologs of 6S, Ms1 or CoRP RNAs have been identified by bioinformatic approaches. Therefore, I will use RIP-seq (RNA immunoprecipitation with Next-generation sequencing) to reveal if any other, new type of RNAP-associated RNA exist in these species.

P38 Unveiling new Dicer-miRNA interactions via Cryo-EM

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RNA silencing is a complex group of cellular pathways involved in the sequence-specific regulation of gene expression by double-stranded RNAs.[1] One of the critical steps in these pathways is mediated by the protein Dicer,[1] an RNase III enzyme that cleaves small RNA precursors into shorter double-stranded RNA molecules, which are then loaded on Argonaute protein. The recent advances in structure biology techniques have enabled numerous studies of Dicer-RNA complexes,[2] providing previously inaccessible insights into RNA substrate processing by Dicer. However, the structural details of critical events such as the initial recognition and binding of pre-miRNA, and the release of product miRNA remain elusive in full-length mammalian Dicer studies. To answer some of the questions that have remained unresolved in the field for over two decades, we aimed to capture new conformations adopted by the mammalian Dicer-miRNA complex using cryo-electron microscopy.

Two additional distinct conformations of miRNA bound to the PAZ domain of mouse Dicer were successfully identified and resolved. These structures provide further insights into the dynamic mechanism of miRNA precursor processing by Dicer. The uncovered Dicer-miRNA interactions may be of significance in substrate recognition, release and formation of the RNA-induced silencing complex.

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P39 Even small subunits can do great things

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Some bacterial species can survive in hostile and even extremely adverse conditions due to their ability to sporulate. This process depends on precisely temporally and spatially regulated gene expression, which is mediated by RNA polymerase (RNAP), the central enzyme of transcription, and a cascade of alternative σ factors. Here we describe the effects of small, non-essential subunits of RNAP, δ and ω, on sporulation in the model Gram-positive bacterium *Bacillus subtilis*. The absence of genes encoding these subunits resulted in a significant decrease (to 10^{-2}) of sporulation. RNA-seqs and proteome analyses then revealed the main processes and regulators affected – starting already in the early stages of sporulation. This was confirmed by high resolution microscopy (SIM) that showed defects in the asymmetric septum formation. Subsequently, *in vitro* experiments identified the mechanistic aspects of the sporulation-specific transcription regulation that were impaired by the absence of δ and ω. A model of their involvement in the sporulation process will be presented and discussed.

P40 Diadenosine tetraphosphate (Ap_4A) serves as an RNA cap

Jiří František Potužník, Ondřej Nešuta, Anton Škríba, Barbora Voleníková, Maria-Bianca Mititelu, Flaminia Mancini, Valentina Serianni, Henri Fernandez, Kristína Spustová, Jana Trylčová, Pavel Vopalensky, Hana Cahová

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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 (m^7G) RNA cap and its modifications are a key component of this recognition process. While the m^7G 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 (Ap_4A). Free Ap_4A 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 Ap_4A-RNA capping is not dependent on the intracellular concentration of free Ap4A. We further identify two enzymes that are capable of cleaving the Ap_4A-RNA cap in vitro, NUDT2 and DXO and we show that Ap_4A-RNA is not translated. Interestingly, we also show that even though free Ap4A has been linked to the innate immune response and its dysregulation, Ap_4A-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, Ap_4A-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.

P41 Global analysis by LC-MS/MS of N6-methyladenosine and inosine in mRNA reveal complex incidence

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The precise and unambiguous detection and quantification of internal RNA modifications represents a critical step in understanding their physiological function. Only a handful of marks can be detected by reverse transcription and sequencing, some of which require additional chemical conversions of isolated RNAs. The detection and quantification of m6A, m6Am and m1A modifications remains one of the major challenges in the field. A second intriguing and timely question that remains to be addressed is the extent to which individual marks are co-regulated or can potentially affect each other. Here we present a methodology and study of the detection and quantification of several key mRNA marks in human total RNA and mRNA. We show that the adenosine demethylase FTO primarily targets m6Am marks in non-coding RNAs in HEK293T cells. Surprisingly, we observe little effect of depletion of FTO or a second adenosine demethylase, ALKBH5, on m6A mRNA levels, indicating that FTO and ALKBH5 have a minor impact on total m^6A levels in steady-state mRNAs. Interestingly, we show that m^6A and A-to-I RNA editing are only linked in certain human cell types.

P42 Understanding the role of methyltransferases in rifampicin resistance

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Antibiotic resistance is a growing global concern, necessitating novel strategies of action against it. Crucial for development of such strategies is understanding of the resistance mechanisms available to bacteria. This study investigates the role of methyltransferases in rifampicin resistance. *Bacillus subtilis* was treated with a sub-inhibitory concentration of rifampicin followed by proteomics analysis. Upregulation of several proteins was detected, including proteins known to be involved in rifampicin resistance, validating the approach. Protein previously unsuspected to play roles in this process were also identified, such as uncharacterized putative methyltransferases. With minimal existing research on methyltransferases in the context of rifampicin resistance, they present a unique opportunity for novel discoveries. They could potentially modify DNA, RNA, proteins, or antibiotics by attaching a methyl group and therefore affecting the gene expression, or the localization, stability, activity or structure of the target molecules. Our research thus focuses on two enzymes. The first is DnmA, a DNA methyltransferase responsible for genome-wide N-methyladenosine modifications and the second is YbxB, for which the target is unknown. *ybxB* has an evolutionarily conserved genome localisation upstream of *rpoB* (coding for the β subunit of RNA polymerase), which is the mutational hotspot for rifampicin resistance. This colocalization suggests a relationship between the two. This study aims to explore the roles of the mentioned methyltransferases by conducting phenotype analysis, which includes growth curves, spot assays, and mutational rate analysis. We will also perform co-immunoprecipitation to detect the *in vivo* interacting partners under rifampicin stress and examine differential DNA methylation by long-read sequencing. Understanding these mechanisms will help us develop new strategies to combat rifampicin resistance.

P43 Tackling the molecular function of the human RNA demethylase ALKBH5

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ALKBH5 is an m6A mRNA demethylase identified in metazoa, some plants and some unicellular eukaryotes [1]. In humans, dysregulation of ALKBH5 is associated with multiple cancers and developmental disorders. On cellular level, is believed to be responsible for removal of m6A methyl groups from mRNAs which in turn affects mRNA processing, export, localization, stability and translation. Despite a large number of studies on ALKBH5, we are still lacking good understanding of its molecular function. Our recent interactome analyses revealed that ALKBH5 interacts with the core exon junction complex (EJC) components, EJC peripheral proteins, and several mRNA export factors. This is in agreement with previous study showing that ALKBH5 depletion leads to poly(A) RNA accumulation in the cytoplasm [2]. Here, we aim to tackle the mechanism of ALKBH5 in pre-mRNA splicing and mRNA export. To this end, we investigated ALKBH5 genetic interactions by CRISPR-Cas high-throughput genetic interaction screen. We observed pronounced lethality for EJC factors such as CASC3 and Y14 further supporting the role of ALKBH5 in mRNA export to the cytoplasm. The transcriptomics analysis of ALKBH5 knockout (KO) HEK293T cells revealed dramatic expression changes in polymerase-II transcripts compare to control WT cells and we also observed strong splicing defects. Overall, our findings emphasize the importance of ALKBH5 in mRNA processing.

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P44 Loss of ADAR1 protein induces changes in small RNA landscape in hepatocytes

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In recent years, numerous evidence has been accumulated about the extent of A-to-I editing in human RNAs and the key role ADAR1 plays in the cellular editing machinery. It has been shown that A-to-I editing occurrence and frequency are tissue-specific and essential for some tissue development, such as the liver. To study the effect of ADAR1 function in hepatocytes, we have created Huh7.5 ADAR1 KO cell lines. Upon IFN treatment, the Huh7.5 ADAR1 KO cells show rapid arrest of growth and translation, from which they do not recover. We analyzed translatome changes by using a method based on sequencing of separate polysome profile RNA fractions. We found significant changes in the transcriptome and translatome of the Huh7.5 ADAR1 KO cells. The most prominent changes include negatively affected transcription by RNA polymerase III and the deregulation of snoRNA and Y RNA levels. Furthermore, we observed that ADAR1 KO polysomes are enriched in mRNAs coding for proteins pivotal in a wide range of biological processes such as RNA localization and RNA processing, whereas the unbound fraction is enriched mainly in mRNAs coding for ribosomal proteins and translational factors. This indicates that ADAR1 plays a more relevant role in small RNA metabolism and ribosome biogenesis.

P45 Enzymatic Synthesis of Base-Modified XNAs Using Engineered Polymerases

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Nucleic acids find applications in the fields of biotechnology and medicine, but in their natural form, they suffer from inherent narrow chemical diversity and susceptibility to degradation by nucleases. The efforts aimed at overcoming these drawbacks center around the alteration of key structural elements of nucleic acids, with the resulting xenobiotic nucleic acids (XNAs) serving as promising alternatives to the canonical genetic polymers. Modifications at the 2’-position of ribose were determined to be crucial in minimizing nuclease cleavage, while also further altering properties of the XNA. The use of 2'-modifications is common practice in most existing therapeutic oligonucleotides; however, there is only a handful of works exploring enzymatic synthesis of dually sugar- and nucleobase-modified XNAs. In this work, we focus on the enzymatic construction of nucleobase-modified 2’F XNA, utilizing primer-dependent engineered thermophilic DNA polymerases. Most notably Taq Stoffel fragment mutant (SFM 4-3) polymerase was used, which is known to accept 2’-modified NTPs [1], as well as nucleobase-modified rNTPs as substrates [2]. Four 2’F NTPs were synthesized, each bearing a different modification on the nucleobase of either hydrophobic, aromatic, anionic or polar nature. The base-modified 2’F NTPs were used in the development of enzymatic synthesis of hypermodified XNAs, starting from synthesis of XNA containing one base-modified 2’F NTP, followed by combinations of two, three and even four base-modified 2’F NTPs. In future experiments, the conditions of primer extension and isolation of XNA will be fine-tuned to allow synthesis of various sequences as well as the study of the properties of the base-modified XNAs.

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P46 Base-modified RNA for translation and CRISPR-Cas studies

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Around 150 types of RNA modifications have been identified in the nature and it is known that some modifications can improve its biological effects. Most of those modifications are found in the tRNA and ribosomal RNA, but also there are modifications in the mRNA that can affect the secondary structure and improve translation efficiency.[1] Moreover, modification in the sgRNA are studied in order to improve efficiency and specificity for the Cas9. Previously our lab reported the efficiency of the T7 polymerase to incorporate different small modifications.[2] In this work, a library of 8 nucleoside triphosphates, with methyl and ethyl modification in the position 7 in case of purines or 5 for pyrimidines, have been synthetized and incorporated by *in-vitro* transcription using the T7 polymerase. Modified single-guide RNAs have been synthetized and used for studying CRISPR-Cas cleavage efficiency and its stability in Human serum. Furthermore, modified mRNAs were synthetized encoding Gaussia and Renilla Luciferase and evaluated for translation efficiency *in-vitro* using the Rabbit Reticulocyte System and for the stability in HeLa S3 cells after transfection, by qPCR and measuring the luminescence at different time points.

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P47 Molecular Insight into 5' RNA Capping with dinucleoside polyphosphates by bacterial RNA Polymerase

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The discovery of a novel class of 5´ RNA caps, dinucleoside polyphosphates (Np_nN), in bacteria has opened new questions about RNA structure and role of non-canonical capping (1). We demonstrated that Np_nNs, also known as alarmones, are incorporated into RNA by RNA polymerase during transcription and this incorporation is dependent on the metabolic status of the cell. However, the regulatory mechanism governing the incorporation of Np_nNs into RNA remains unknown. In this work, we present a detailed molecular insight into how Np_nNs serve as non-canonical initiating nucleotides (NCINs) during the initiation phase of transcription using *Thermus thermophilus* RNA polymerase. During transcription initiation, RNA polymerase binds to the DNA promoter, forming the RNAP-promoter open complex. To investigate the interaction between Np_nNs and RNA polymerase, we employed four distinct DNA templates with varying -1 and +1 nucleotide combinations, facilitating the base pairing of six different Np_nNs ($Ap_{3-4}A$, $Ap_{3-4}G$, $Gp_{3-4}G$) at the 5'end of RNA. As result, we observed the production of the capped RNA product with the expected length of 15 nucleotides. Interestingly, various combination of Np_nNs and DNA templates, resulted in different RNA yields and the unexpected production of a 16-mer capped RNA, in addition to the 15-mer. To understand the incorporation mechanism of Np_nNs using various DNA templates, we used cryo EM technique to resolve the structure of *Thermus thermophilus* RNA polymerase transcription initiation complex. The structure analysis unveiled the interaction of NpnNs at the -1 and +1 positions, providing crucial insight into the regulation of NCINs incorporation in RNA.

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P48 LC-MS analysis of RNA and DNA modifications

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This work presents a comprehensive overview of protocols and LC-MS methods specifically designed for detection of modifications in nucleic acids.

Our workflow includes DNA/RNA isolation, enzymatic digestion by Nuclease P1, followed by dephosphorylation of nucleotides using Shrimp alkaline phosphatase.

In the case of internal modifications, the digestion and dephosphorylation steps are followed by ultrafiltration and reverse-phase chromatography with HSS-T3 column.

For the analysis of 5'-RNA caps, samples are purified using solid-phase extraction (SPE). The SPE method is often used as one of the best options for sample preparation to extract the analyte of interest from complex samples. The benefits include reduced matrix effects, concentration of small amounts of analyte, reduced interference during co-elution, reduced data variability, and ultimately increased sensitivity of the analysis. A special mixed-mode weak anion exchanger sorbent was used, allowing successful removal of nucleosides and isolation of desired 5´-RNA caps. For subsequent separation, we have used HILIC (hydrophilic interaction chromatography) as an alternative to ion-pairing chromatography.

High-resolution mass spectrometer Xevo G2-XS qTOF (Waters) is used for untargeted or qualitative detection. For targeted and quantitative analysis, Xevo Absolute Triple Quadrupole mass spectrometer (Waters) is more common choice.

Examples of the analysis includes some of the most widespread internal modifications and both canonical and non-canonical 5'-RNA caps present in bacterial and mammalian cell cultures.

P49 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*, Ap_4A 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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P50 Regulation of initiation of translation during T cell activation

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T lymphocytes regulate many aspects of our adaptive immunity, including responses to pathogens, allergens and tumours. If they are not properly regulated, they can also become dangerous to us and cause a tumour disease - lymphoma. A better understanding of these cells, from their origin to their differentiation, is essential for the diagnosis and treatment of these diseases. During T-cell activation, a dramatic increase in protein synthesis must be precisely controlled and regulated. The stimulation of translation is due to an increase in the rate of initiation caused by the regulation of the activities of initiation factors (eIFs).

In eukaryotes, members of the eukaryotic initiation factor 4A (eIF4A) family, of which there are three different isoforms, are essential for translation. The eIF4A1 and eIF4A2 isoforms are involved in translation initiation. Changes in the expression levels of eIF4A1, eIF4A2 have been observed in different types of malignancies and are closely associated with the clinicopathological characteristics of tumours.

Based on our results, we found that eIF4A2 is surprisingly up-regulated compared to eIF4A1. In this part of our project, we will focus on performing eIF4A2 knockdowns in primary T cell lines, where we will investigate the influence of knockdowns on T cell activation.

P51 Splice site diversity and abundance of non-canonical introns in diplonemids (Diplonemea, Euglenozoa)

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Non-coding introns are a unifying feature of protein-coding genes in virtually all extant eukaryotes, with most lineages following the canonical intron structure. However, euglenozoans, unicellular flagellates encompassing free-living euglenids, human pathogenic kinetoplastids, and the highly diverse and abundant marine diplonemids, represent a notable exception. Euglenozoan genomes range from extremely intron-poor kinetoplastids to euglenid genomes containing both canonical and non-canonical introns. Here, we present a comprehensive analysis of splice sites and spliceosomal components in six species of understudied diplonemids. All examined diplonemids contain a nearly complete set of spliceosomal snRNP components indicating the presence of a functional U2-type spliceosome. However, the majority of introns in the closely related Artemidia motanka and Namystynia karyoxenos are non-canonical and lack conserved GT-AG terminal dinucleotides typical for U2-type introns. We further show that non-canonical introns are capable of extensive base-pairing, which brings intron ends into close proximity. Our analysis suggests an independent evolution of the canonical and non-canonical splice sites in these planktonic protists. In summary, we show that while the splicing apparatus is conserved in diplonemids, the splice sites vary considerably among individual species.

P52 Mycobacterial HelD is a global transcription regulator and is associated with increased rifampicin resistance in clinical isolates

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Mycobacterial HelD protein, also named HelR (encoded by *MSMEG_2174* gene in *Mycobacterium smegmatis*), interacts with mycobacterial RNA polymerase (RNAP) and affects rifampicin resistance in *Mycobacterium abscessus*.

Using ChIP-seq, we detected HelD on a subset of CarD- and RbpA-occupied promoters. Next, we analyzed the transcriptome of a *helD* deletion strain using RNA-seq. Globally, HelD increased transcription in exponential phase and decreased transcription in stationary phase, during which we observed reduced levels of CarD, RbpA and GTP. GTP is the initiation nucleotide for majority of *M. smegmatis* transcripts.

Our model suggests that HelD removes stalled RNAPs from highly expressed genes' promoters during exponential growth, increasing transcription, while in stationary phase, HelD also removes RNAPs waiting for CarD, RbpA, or GTP, decreasing transcription. HelD is more widespread among rapidly growing species of mycobacteria, which correlates with a higher transcription rate in these species, while slow growing mycobacteria lack HelD homologs.

In addition, we show that HelD presence in the genome correlates with reduced susceptibility to rifampicin in several nontuberculous mycobacterial species. HelD is beneficial for mycobacteria not only to support rapid growth during exponential phase, but also to overcome rifampicin treatment indicating a dual role of this transcription regulator.

P53 Identification of Hfq-dependent RNA transactions in *Bordetella pertussis* by RIL-seq

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The requirement of the RNA chaperone Hfq for virulence of *B. pertussis* suggested that Hfq-dependent small regulatory RNAs (sRNAs) are involved in modulating gene expression in this re-emerging pathogen. High-throughput sequencing (dRNA-seq) revealed hundreds of putative sRNAs. However, the identification of mRNA targets directly regulated by these sRNAs is a limiting step in their functional characterization. Therefore, we applied a method combining experimental and computational approaches called RIL-seq. This method is based on *in vivo* ligation of Hfq-bound sRNAs-target mRNAs pairs, immunoprecipitation of Hfq-RNA complexes, sequencing of ligated chimeric RNAs and computational analysis. Thus, this method allows for global capture and mapping of *in vivo*-formed Hfq-dependent duplexes between sRNAs and target mRNAs. Our RIL-seq analysis revealed putative targets of several sRNAs, including Pred285. The abundance of this sRNA is strongly decreased in the absence of the Hfq protein and the sRNA shares 60% identity with the last 46 nt of the *E. coli* sRNA MicA. MicA is a well-characterized sRNA involved in outer membrane remodeling under stress conditions. In support, RIL-seq data and *in silico* predictions indicate that Pred285 can interact with 5´UTR regions of mRNAs encoding the outer membrane proteins BP0840 and BP0943 (OmpA). In addition, the mutant strain displayed moderate defects in cytotoxicity towards human macrophages and biofilm formation.

P54 Elucidating the role and the position of RACK1 within stress granules by its ribosome binding-deficient and glycosylation-deficient mutants

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RACK1 is an evolutionarily conserved seven-bladed WD40 repeat protein, which has a role as a scaffold protein in eukaryotic cells. We probed for an alteration of overall cellular morphology and fitness, translation status and related signalling, stress granule (SGs) dynamics and composition upon RACK1 depletion and the expression of RACK1 mutant variants.

In particular, we used CRISPR/Cas9-based RACK1 deleted mammalian epithelial cells and ribosome-binding deficient and glycosylation-deficient RACK1 mutant variants. We complemented the obtained data using siRNA-based depletion of RACK1 and the expression of the mutant variants in mammalian fibroblasts.

First, we characterized the impact of RACK1 depletion on mammalian cell morphology, including cytoskeleton organization, growth and polarization. We showed that RACK1 depletion leads to cell polarization and spreading defects, resulting in altered morphology, cytoskeletal organization and mass distribution in both epithelial and fibroblast cells. In connection, the cells display a defect in cell proliferation and cell cycle progression.

Second, we monitored the changes in translation, related signalling, and SG dynamics that resulted from RACK1 depletion. We did not observe any pronounced effect of RACK1 depletion on translation-related stress signalling and SGs core composition and dynamics.

Finally, using ribosome-binding deficient and glycosylation-deficient RACK1 mutant variants, we clarified the position of RACK1 within SGs. We showed that RACK1 binding to ribosome correlates with the level of its glycosylation, but neither the ability of RACK1 to bind ribosome nor its glycosylation is required for the sequestration to SGs. In connection, we identified SG's core constituent, G3BP1 protein, as a stress-specific binding partner of RACK1. Together, these data specify the position and the role of RACK1 within SGs and clarify conditions for its sequestration.

P55 Searching for new factors involved in RNA tailing and decay

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In eukaryotes gene expression is regulated at several levels, one of them being mRNA stability with RNA tailing marking transcripts for further processing or degradation. Mammalian cells encode several types of 3’ to 5’ exoribonucleases with specific targets and functions. Our lab has previously identified the DIS3L2 protein as a processive 3’ to 5’ exoribonuclease with specific affinity to 3’ terminal oligoU extensions on different, even highly structured, RNAs in the cytoplasm [1][2][3]. We reasoned that its activity *in vivo* must be regulated to prevent unspecific disastrous degradation of cellular RNAs via posttranslational modifications or putative additional protein factors. To date, DIS3L2 was shown to interact with Xrn1 in an RNA-dependent manner [4]. To search for additional regulatory cofactors, we biotinylate proximal proteins of known TUT-DI3L2 pathway players using inducible expression of these proteins fused with promiscuous biotin ligase miniTurboID [5] in HEK293T cell line. The biotinylated proteins are then purified with streptavidin and identified by LC-MS/MS. These candidates are to be validated and put in a broader context of ongoing studies in the lab.

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P56 Development and application of chemiluminescent, fluorescent and colorimetric deoxyribozymes

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Deoxyribozymes are DNA molecules that catalyze reactions. They can be useful alternatives to protein enzymes for a number of reasons: DNA can be chemically synthesized at low cost, is stable over a wide range of conditions, can typically be denatured and refolded without losing activity, and can be readily engineered using artificial evolution. Our group is particularly interested in developing dexoyribozymes that can generate different types of signals. Here we provide an overview of recent work related to three deoxyribozymes discovered in our group: the chemiluminescent deoxyribozyme Supernova, the fluorescent deoxyribozyme Aurora, and the colorimetric deoxyribozyme Apollon. These deoxyribozymes generate robust signals using a fast and easy workflow, and can be engineered to only generate signals in the presence of specific inputs. One of our sensors is activated by RNA cleavage, and was used to identify inhibitors of a ribonuclease from SARS-CoV-2 in a high-throughput screen. Our work highlights the potential of catalytic DNA, and shows how it can be used to solve real world problems.

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P57 HIV-1 Infection Reduces NAD Capping of Host Cell snRNA and snoRNA

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Nicotinamide adenine dinucleotide (NAD) is a critical component of the cellular metabolism and serves as an alternative 5' cap on various RNAs. However, the function of the NAD RNA cap is still under investigation. We studied NAD capping of RNAs in HIV-1-infected cells because HIV-1 is responsible for the depletion of the NAD/NADH cellular pool and causing intracellular pellagra. We demonstrate that reducing the quantity of NAD-capped RNA by overexpressing the NAD RNA decapping enzyme DXO results in an increase in HIV-1 infectivity. By applying the NAD captureSeq protocol to HIV-1 infected and uninfected cells, we revealed that various snRNAs and snoRNAs lost their NAD cap when infected with HIV-1. Due to the crucial role of U1 snRNA in RNA splicing and telescripting, we investigated the role of the NAD-capped U1 in HIV-1 infection and found that the presence of the NAD cap decreases the stability of the U1/HIV-1 pre-mRNA duplex. Currently, we are investigating the differential splicing of HIV-1 mRNA and other cellular mRNAs under conditions with altered NAD-capping of U1 (HIV-1 infection, DXO overexpression and DXO knock-down) to connect the alternative capping of U1 RNA to cellular phenotype.

P58 Sweet life: What the antibiotic rifampicin and sugars have in common

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Rifampicin (RIF) is an ansamycin antibiotic targeting bacterial RNA polymerase. Alone or in combination with other antibiotics, it is used to treat several bacterial infections, including infections caused by *Mycobacterium* species, methicillin-resistant *Staphylococcus aureus* and others.

We analysed the proteome of the model bacterium *Bacillus subtilis* to identify proteins differentially expressed under RIF stress, to discover and elucidate novel mechanisms providing antibiotic resistance. Among the significantly upregulated proteins, we found components of the phosphoenolpyruvate phosphotransferase system (PTS). PTS is a crucial metabolic pathway found universally in eubacteria but absent in eukaryotes, making it an ideal target for antimicrobial agents. This system involves a series of proteins that undergo sequential phosphorylation to facilitate the simultaneous phosphorylation and uptake of various sugars.

The functioning of PTS is tightly regulated on the transcriptional level through the phosphorylation of the central enzyme HPr and its binding to transcriptional regulator CcpA, a mechanism called Carbon Catabolite Repression (CCR). We identified enzymes important for CCR to impact RIF resistance. Additionally, we noticed a remarkable homology between several PTS proteins and domains of RIF phosphotransferase (an enzyme conferring antibiotic resistance by converting RIF to inactive phospho-rifampicin). Such homology suggests a potential direct interaction of PTS with RIF. Our findings indicate that the CCR regulator also acts as a transcription factor directly or indirectly governing the general cellular response to RIF and significantly affecting RIF sensitivity in *Bacillus subtilis*.

This project aims to uncover the unknown functions of PTS proteins in rifampicin resistance of bacteria.

P59 The Role of Long Non-coding RNAs in BCR-mediated CLL Activation

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B Cell Receptor (BCR) plays a pivotal role in providing maturation and survival signals for B cells. However, dysregulation of the BCR pathway is a fundamental characteristic observed in numerous B cell malignancies, including chronic lymphocytic leukemia (CLL).

Despite the absence of recurrent mutations in the BCR-related genes, BCR inhibitors lead to a universal clinical response in CLL patients. We and others have shown that short non-coding RNAs, namely microRNAs, can (dys)regulate the BCR signaling propensity, but it remains unclear if long non-coding RNAs (lncRNAs) play a role in BCR activation. We hypothesized that lncRNAs could be involved in BCR-mediated CLL cell activation.

We conducted a differential lncRNA expression analysis in CLL cells from patients treated with BCR inhibitors to address our hypothesis. We cross-validated these findings in intraclonal CLL subpopulations characterized by high BCR activity (CXCR4 ^{dim} CD5 ^{bright}) versus low BCR activity (CXCR4 ^{bright} CD5 ^{dim}). We identified a BCR-regulated lncRNA that consistently correlated with BCR activity in primary CLL samples.

The studied lncRNA was upregulated upon BCR activation (BCR crosslinking with anti-IgM) in CLL cells and this was blocked *in vitro* by BCR inhibitors (ibrutinib/idelalisib). In line with this data, the lncRNA was also downregulated in CLL patients undergoing BCR inhibitor therapy (n=6, p=0.003, fold-change=3.7). CLL patients with higher expression of the lncRNA have longer survival than those with relatively lower levels (n=100, p=0.04, HR = 2.28; median survival of 9.7 vs. 16.8 years).

To understand these observations, we transcriptionally repressed the lncRNA using dCAS9-KRAB system in CLL-derived MEC1 cell line. Notably, the engineered cells showed higher BCR responsiveness, as evidenced by the increased calcium flux (FLUO-4 assay) following BCR activation. RNA profiling of the lncRNA-depleted MEC1 cells showed a decreased expression of numerous genes regulating BCR signaling and increased expression of genes involved in mitochondria respiration metabolism. Preliminary data suggest that although the cells transcriptionally enhance metabolic gene expression, the lncRNA-depleted cells have lower mitochondria respiration fitness. Altogether, these results indicate a possible mitochondria respiration deficiency, which the cells try to compensate transcriptionally.

P59 The Role of Long Non-coding RNAs in BCR-mediated CLL Activation

To reveal the mechanism by which the lncRNA affects these processes, we performed RNA pull-down followed by mass spectrometry analysis. A class of proteins related to mRNA control and translation was pulled together with the lncRNA. Upon subjecting the engineered cell lines to transient puromycin exposure, initial observations indicated that lncRNA-depleted MEC1 cells exhibited a significant reduction in *de novo* protein synthesis compared to control cells.

In summary, the studied lncRNA is directly regulated by the BCR activation and potentially acts in a negative feedback loop to limit BCR-mediated CLL activation while simultaneously modulating protein translation required in activated CLL B cells.

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P60 Unraveling the Role of Splicing Factors in Autosomal Dominant Retinitis Pigmentosa: Insights from a Human Retinal Organoid Model

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Autosomal dominant retinitis pigmentosa (adRP) is a retinal disorder characterized by the progressive loss of photoreceptors. It is therefore not surprising that most mutations causing adRP are found in proteins critical for photoreceptor function, such as Rhodopsin. What is unexpected is that mutations in ubiquitously expressed splicing factors including Prpf8 are the second most common cause of adRP. The mechanism by which mutations in splicing-related proteins lead to retinopathy and why the resulting phenotype is limited to the retina remains unclear. Here we present the development of a human retinal organoid model to elucidate the underlying disease mechanism. We utilized CRISPR/Cas9 genome editing to introduce the adRP-associated mutation Y2334N into the *PRPF8* gene in human induced pluripotent stem cells, followed by their differentiation into three-dimensional retinal organoids. We found that while the cellular composition of these retinal organoids remains unaffected, the formation of photoreceptor outer segments seems to be impaired. Transcriptome analysis revealed minor changes in differential gene expression but affected splicing of neural and retinal disease-associated genes. Our data indicates that splicing perturbation of specific genes drives the RP phenotype in retinal cells.

P61 Screening for novel proteins involved in resolution of G-loop mediated transcription-replication collisions

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DNA replication is an essential cellular process ensuring faithful and complete duplication of the genome. However, the progress of replication machinery can be stalled by numerous obstacles, including transcription complexes moving along the same DNA template and secondary structures of DNA such as G-quadruplexes (G4), which are planar stacks of guanine tetrads. The head-on collisions between transcription and replication complexes (TRCs) promote annealing of nascent RNA transcript into the DNA duplex behind the RNA polymerase, leading to the formation of an RNA:DNA hybrid and displaced single-stranded DNA (ssDNA). These structures are referred to as R-loops. Extruded G-rich ssDNA in R-loop is prone to formation of G4. Coexisting RNA:DNA hybrid and G4 mutually stabilize each other in a structure referred to as G-loop and further exacerbate replication fork stalling. TRCs are a major source of replication fork stalling and consequent genome instability in precancer and cancer cells, however the mechanisms resolving TCRs are not fully understood. Our goal is to identify proteins that are involved in removal of G-loops and the restart of stalled replication to maintain genome stability.

To identify novel factors involved in G4 metabolism, we used proximity-based labelling followed by mass spectrometry analysis (MS). We established a new cell line U2OS T-REx APEX2-BG4 that is inducibly expressing BG4 antibody tagged with ascorbate peroxidase (APEX2). The BG4 antibody is a specialized single-chain variable fragment antibody specifically designed to recognize and bind to G4 with high affinity. In cells expressing APEX2-BG4 and preincubated with biotin-phenol, brief hydrogen peroxide treatment triggers APEX-mediated generation of biotin-phenoxyl radicals and covalent labeling of the proximal proteins. These biotinylated proteins are isolated from lysed cells by streptavidin beads and identified by MS. We have identified several proteins that are significantly enriched in the proximity of G4 structures upon conditions promoting G-loop dependent replication stalling (depletion of MSH3 protein) compared to unperturbed conditions. Currently, we are working on functional analysis of identified proteins with respect to their role in G-loop metabolism, resolution of TRCs and suppression of genomic instability.

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