Session 6: RNA structure, function and regulation

Lectures

L.06.1

Complex metabolic pathways of mRNA therapeutics *in vivo*

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Though mRNA vaccines against COVID-19 have revolutionized vaccinology and have been administered in billions of doses, we know little about how mRNA vaccines are metabolized *in vivo*. We implemented enhanced nanopore Direct RNA sequencing (eDRS), to enable the analysis of single Moderna's mRNA-1273 molecules, giving *in vivo* information about the sequence and poly(A) tails.
We show that mRNA-1273, with all uridines replaced by N1-methylpseudouridine (mΨ), is terminated by a long poly(A) tail (~100 nucleotides) followed by a mΨCmΨAG sequence. In model cell lines, mRNA-1273 is swiftly degraded in a process initiated by mΨCmΨAG removal, followed by CCR4-NOT-mediated deadenylation. However, when injected intramuscularly into mice, complex modifications occur. Notably, mRNA-1273 is re-adenylated.In macrophages, which are the primary target of the vaccine, mRNA-1273 is very efficiently re-adenylated, and poly(A) tails can be extended up to 200 nucleotides. We further show that the enhanced mRNA stability in macrophages is mediated by TENT5 poly(A) polymerases. Without re-adenylation, antigen production decreases, and specific immunoglobulin production is compromised.
Together, our findings provide an unexpected principle for the high efficacy of mRNA vaccines and open new possibilities for their improvement. They also emphasize that, in addition to targeting a protein of interest, the design of mRNA therapeutics should be customized to its cellular destination.

Oral presentations

O.06.1

Catch me if you can: identification of long noncoding RNAs in vertebrate genomes

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Vertebrate genomes produce tens of thousands of long noncoding RNAs (lncRNAs) – long transcripts with limited protein coding potential. Although an increasing number of lncRNAs is linked to fundamental physiological processes in the cell, >97% of them remain functionally uncharacterized. Understanding biological roles of lncRNAs requires accurate genome annotations describing their precise location, gene boundaries and transcript structures. However, current lncRNA catalogues show evident signs of incompleteness with many gene models being fragmented or uncatalogued. To overcome this issue, the present work aims to advance towards a complete and accurate annotation of lncRNAs in human and mouse genomes. By developing and applying targeted long-read RNA sequencing methodology, this study provides accurate lncRNA annotations at high-throughput rates. Produced transcript models uncover thousands and hundreds of novel, full-length lncRNAs for human and mouse genomes, respectively, also substantially increasing the annotated transcript complexity within targeted loci. Resulting lncRNA catalogues are of quality comparable to present-day manually curated annotations. Moreover, we detected human and mouse lncRNA orthologues in the zebrafish genome using a newly designed synteny-based approach. Improved annotation of mammalian lncRNA orthologues in the zebrafish genome is expected to largely facilitate their functional characterization.

O.06.2

The role of 3' end dynamics in LINE-1 retrotransposon biology

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LINE-1 (L1) retrotransposons are mobile genetic elements posing a mutational danger to modern human genomes and underlying self-immune reactivity. L1s are tightly regulated by multi-layered transcriptional and posttranscriptional processes. To learn about the role of L1 mRNA ends in L1 biology and retrotransposition we comprehensively analyzed the effects temporal or permanent depletions of several proteins involved in post-transcriptional RNA metabolism including XRN1, the major cytoplasmic 5'→3' exoribonuclease, the decapping complex, other factors, and special L1 reporters with defined 3' ends. Our observations suport the crucial role of L1 3' ends, the impact of L1 poly(A) length, dynamics of poly(A) shortening and uridylation, demonstrating the unappreciated role of the 3' end dynamics in L1 biology.

Acknowledgements

This work was supported by the National Science Centre, grant numbers: UMO-2019/33/B/NZ1/02260, UMO-2017/26/D/NZ1/00887, computational grants of the Poznan Supercomputing and Networking Center no. 457 and 523. Sequencing of the 3' RACE-seq libraries NGS was performer by the Genomics Core Facility CeNT UW (RRID:SCR_022718) using NovaSeq 6000 platform financed by Polish Ministry of Science and Higher Education (decision no. 6817/IA/SP/2018 of 2018-04-10).

O.06.3

The functional interplay of conserved RNA-binding proteins for silencing a master regulator

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Regnase-1 is an endoribonuclease that degrades specific mRNAs involved in various biological processes, such as immune homeostasis, development, and cancer. Initially, Regnase-1 was proposed to cooperate with Roquin-1, another RNA-binding protein (RBP), to silence specific mRNAs during T-cell activation. However, studies performed in different cell types suggest that Regnase-1 and Roquin-1 regulate mRNAs independently and through different mechanisms.

Our study focuses on REGE-1, the *C. elegans* ortholog of Regnase-1, and its functional cooperation with RLE-1, the nematode counterpart of Roquin-1. Although REGE-1 and RLE-1 associate with mRNA independently, both are essential for mRNA silencing. Surprisingly, unlike in mammals, REGE-1-mediated mRNA silencing in *C. elegans* functions independently of SMG-2/Upf1 and mRNA deadenylases. Additionally, our results suggest that other protein partners of REGE-1 may be involved in mRNA silencing, indicating species-specific variations in the requirement for protein co-factors. Interestingly, in contrast to Regnase-1, which regulates a diverse set of transcripts, REGE-1 targets a single mRNA encoding a conserved transcription factor, ETS-4, which affects the transcription of diverse downstream genes regulating various aspects of animal physiology. Our collective results highlight that although REGE-1/Regnase-1 are functionally related from nematodes to humans, the exact mechanisms underlying mRNA regulation vary between species.

O.06.4

Development of a quantitative analysis of tRNA modifications by nanopore RNA sequencing

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Nanopore sequencing has been proven an efficient and reliable method for direct RNA sequencing. The method allows for evaluation of chemical modification present on mRNA. tRNA modifications are typically identified and quantified with high accuracy using liquid chromatography coupled to mass spectrometry (LC–MS) methodologies or NGS-based methods, which despite recent improvements suffer from technical pitfalls (Lucas *et al.*, 2023). New efficient tRNA sequencing methods are still needed.
We have developed a new methodology called Nano-MODe-tRNAseq, a nanopore-based approach to sequence native tRNA molecules that offers quantitative estimation of tRNAs abundance and identification of ACL loop modifications. We designed synthetic, modified tRNAs oligonucleotides for full tRNAs assembling, and use them as internal standards in Nano-MODe-tRNAseq to enhance processing efficiency of raw nanopore current intensity signals obtained for natural tRNAs. An artificial neuronal network external model has been trained and will be utilised to identify specific signals containing elements characteristic to modifications in different tRNAs.

Acknowledgements

Research was funded by (POB Biotechnology and Biomedical Engineering) of Warsaw University of Technology within the Excellence Initiative: Research University (IDUB) programme. Project BIOTECHMED-3 Advanced no. 504/04496/1020/45.010421 founded to MA.

O.06.5

switchSENSE® and RT-IC for biophysical characterization – from small molecules to cells

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Nanopore sequencing has been proven an efficient and reliable method for direct RNA sequencing. The method allows for evaluation of chemical modification present on mRNA. tRNA modifications are typically identified and quantified with high accuracy using liquid chromatography coupled to mass spectrometry (LC–MS) methodologies or NGS-based methods, which despite recent improvements suffer from technical pitfalls (Lucas *et al.*, 2023). New efficient tRNA sequencing methods are still needed.
We have developed a new methodology called Nano-MODe-tRNAseq, a nanopore-based approach to sequence native tRNA molecules that offers quantitative estimation of tRNAs abundance and identification of ACL loop modifications. We designed synthetic, modified tRNAs oligonucleotides for full tRNAs assembling, and use them as internal standards in Nano-MODe-tRNAseq to enhance processing efficiency of raw nanopore current intensity signals obtained for natural tRNAs. An artificial neuronal network external model has been trained and will be utilised to identify specific signals containing elements characteristic to modifications in different tRNAs.

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Posters

P.06.1

New insights into the helicase domain of human Dicer and its biochemical properties: ATPase and RNA rearrangement activities

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Dicer ribonucleases are best known for their important role in microRNA (miRNA) and small interfering RNA biogenesis. They may also be involved in chromatin structure remodeling, apoptotic DNA degradation or production of damage-induced small RNAs, which implies that in the cell Dicer enzymes can interact with many different RNA and DNA substrates. Most Dicers are multi-domain proteins. The presented studies focus on the human Dicer (hDicer) helicase domain (HEL). The hDicer HEL contributes to recognizing pre-miRNA substrates and is suggested to participate in the binding of many different cellular RNAs. However, a comprehensive characterization of the biochemical activities and the substrate specificity of the hDicer HEL towards different nucleic acids have never been reported. In this work, we demonstrate for the first time that the full-length hDicer, through its helicase domain, is capable of ATP hydrolysis. We also show that the hDicer HEL binds only single- but not double-stranded nucleic acids, and that binding of single-stranded RNAs is accompanied by rearrangement of their structure. All of these activities are ATP-independent. Next, we show that the hDicer variant lacking the helicase domain displays a reduced miRNA production capacity in the cell, which is in contrast to the results of the *in vitro* assays. Given the documented importance of the hDicer HEL in antiviral defense, the obtained results may contribute to a better understanding of viral diseases and the role of hDicer in virus-host interactions.

P.06.2

Elucidating the functions of U7 snRNA-dependent lincRNAs in human cells

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U7 snRNA is part of U7 snRNP, a ribonucleoprotein complex required for the 3' end processing of replication-dependent histone pre-mRNAs in the S phase of the cell cycle. During this maturation event, the 5' region of U7 snRNA hybridizes to the highly complementary sequence present in the 3'UTR of histone pre-mRNAs, called the histone downstream element, HDE. Accumulating evidence indicates that U7 snRNA also has another function in human cells that is not related to the S phase of the cycle. We have found long intergenic non-coding RNAs (lincRNAs) containing HDE-like motifs that perfectly match the 5' end of U7 snRNA. The level of these lincRNAs is elevated after the depletion of U7 snRNA (U7 knockdown cells, U7 KD), suggesting that U7 snRNA might be part of the mechanism that represses these genetic element expression. However, the function of majority of lincRNAs is not known. During the conference, I will present the results of the RNA antisense purification (RAP) of two selected U7 snRNA-dependent lincRNAs, using biotinylated complementary oligonucleotides and streptavidin magnetic beads followed by the identification of proteins by mass spectrometry. In addition, I will show the results of high throughput sequencing of RNA isolated from HEK293T U7 KD cells and wild-type cells. Detailed analysis of the pulldown protein fraction and total cellular RNAs will enable to suggest possible functions performed by selected U7-dependent lincRNAs.

P.06.3

Impact of RNA and DNA G-quadruplexes on human Dicer activity

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Guanine (G)-rich single-stranded nucleic acids can adopt G-quadruplex structures. Growing evidence indicates that G-quadruplexes serve important regulatory roles in fundamental biological processes such as DNA replication, transcription, and translation, while aberrant G-quadruplex formation is linked to genome instability and cancer. Understanding the biological functions played by G-quadruplexes requires detailed knowledge of their protein interactome. Recently we have found that both RNA and DNA G-quadruplexes (G4-RNA and G4-DNA) can be bound by human Dicer (hDicer) [1]. Using *in vitro* binding assays, mutation studies, and computational modeling we demonstrated that G-quadruplexes can interact with the Platform-PAZ-Connector helix cassette (PPC cassette) of hDicer, the region responsible for anchoring microRNA precursors (pre-miRNAs). We found that the K_d values of the PPC hDicer•G4-RNA complexes were <10 nM, while the K_d values of the PPC hDicer•G4-DNA complexes were ~300 nM. Consequently, we showed that G-quadruplexes efficiently and stably inhibited the cleavage of pre-miRNA by hDicer. Altogether, our data highlight the potential of hDicer for binding of G-quadruplexes and allow us to propose a G-quadruplex-driven sequestration mechanism of Dicer regulation.

References

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Acknowledgements

Funding: National Science Centre, Poland SONATA BIS 2016/22/E/NZ1/00422, OPUS 2021/41/B/NZ2/03781

P.06.4

MCPIP1 controls transcripts coding for important regulators of embryogenesis

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Monocyte chemoattractant protein-induced protein 1 (MCPIP1), also known as Regnase-1 and encoded by the *ZC3H12A* gene, possesses a PilT N-terminus domain (PIN) that exerts RNase properties. *Zc3h12a* knockout mice develop severe spontaneous inflammatory syndrome and die by 12 weeks old. In addition, evidence indicates that MCPIP1 is involved in cell cycle arrest, apoptosis and regulation of cell differentiation.

In this study, we utilized a zebrafish model to investigate the role of Mcpip1 during embryonic development. Direct comparison of the zebrafish ortholog of the human MCPIP1 protein indicated a similar domain structure and 88% identity within the PIN domain. Firstly, we analyzed expression pattern of *zc3h12a* mRNA during zebrafish development and concluded that it changes dynamically during the first 24 hours post fertilization (hpf). Next, we found that ectopic overexpression of wild-type Mcpip1 but not the catalytically inactive mutant form resulted in an embryonic lethal phenotype (24 hpf). Subsequent RNA-seq analysis of 6 hpf embryos confirmed phenotypic observations. In particular, downregulation of genes related to endoderm, left/right pattern development and organ morphogenesis was observed, in contrast to upregulation of stress-related genes, in wild-type Mcpip1-overexpressing embryos.

In conclusion, our studies in the zebrafish model suggest that MCPIP1 may function as a safeguard of early embryonic development.

Acknowledgement

This study was supported by the MNS 5/2021 (to WS).

P.06.5

N4BP1 is a novel ribonuclease found in P-bodies

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N4BP1 (*NEDD4 Binding Protein 1*) is a ribonuclease that consists of K-homology domain (*KH domain*), UBA (Ubiquitin Associated) domain, NYN (N4BP1, *YacP-like Nuclease domain*) domain and CoCUN (*Cousin of CUBAN*) domain. N4BP1 was described as an E3 ubiquitin ligase NEDD4, however, recent data show that this protein regulates immunological response, signal transduction to NF*κ*B (nuclear factor kappa-light-chain-enhancer of activated B cells) and degradation of viral mRNAs. The aim of this project was to identify binding partners of N4BP1 with subsequent validation of obtained results. We performed immunoprecipitation of N4BP1 followed by mass spectrometry analysis to find N4BP1 binding partners. Our results revealed numerous proteins interacting with N4BP1. Most of them are the components of P-bodies engaged in mRNA decapping. We validated these interactions using Western blot and immunofluorescence staining. Furthermore, we generated plasmids coding for different deletion variants of N4BP1, to find the region responsible for the interaction with key P-bodies components. We found that the KH domain, especially its RNA-loop-binding N-terminal fragment is crucial for such interaction. In conclusion, our results reveal a new function of N4BP1. Given its ribonucleolytic activity, N4BP1 may turn out to be a crucial factor for mRNA decay occurring in P-bodies.

P.06.6

Identification of long non-coding RNAs (lncRNAs) and study of their biological significance in mitochondria of *Arabidopsis thaliana*

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Long non-coding RNAs (lncRNAs) are non-translated RNA comprised of more than 200 nt, involved in regulating gene expression. So far, lncRNAs in plant mitochondria are poorly characterized, and there is still much to learn about them. To identify lncRNAs in plant mitochondria, we analyzed our previously published mtRNA-seq data for wild-type (WT) and *rps10 Arabidopsis thaliana* plants, the latter with deficiency of the mitoribosomal S10 protein and showing the accumulation of transcripts derived from sequences outside known genes. We identified seven putative intergenic lncRNAs with high (>200) coverage per nucleotide. For further analysis, we chose two with the highest coverage per nucleotide (1000) in the *rps10* plants – NCO transcript, which was identified and characterized earlier by Holec et al. (2006), and novel lncRNA, which we called "BP transcript". First, we confirmed the accumulation of these lncRNAs in *rps10* compared to WT by RT-qPCR and Northern-blot analysis. Additionally, we determined the ends of the BP transcript by circular PCR. Using the mitoTALEN approach, we knocked out NCO and BP transcripts in the WT and *rps10* mitochondrial genomes. Data from genotypic and phenotypic analyses of transformants will be presented on the poster. The selected plants will be used to analyze the function of NCO and BP transcripts in plant mitochondria.

Acknowledgements

This work was supported by Grant OPUS-21 (2021/41/B/NZ3/00571) from the National Science Centre, Poland, for HJ.

P.06.7

Subcellular localization of ncRNAs in primary mouse astrocytes

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Non-coding RNAs (ncRNAs), including microRNAs, long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs), are essential regulators of gene expression in the central nervous system (CNS). Astrocytes, a heterogeneous group of glial cells, are implicated in synaptic neurotransmission, blood-brain barrier formation, neurogenesis and neuroplasticity. Astrocyte activation is a hallmark of pathophysiological changes observed in many of CNS diseases, but how regulatory ncRNAs contribute to this process is poorly understood. Central to the function of ncRNAs is their subcellular localization. We tested the subcellular fractionation (SF) methods of mouse primary astrocytes and chose the best-suited approach for the separation of cytoplasmic and nuclear, and then nucleoplasmic and chromatin compartments. SF was validated with Western Blot for presence of marker proteins specific to individual compartments and qRT-PCR for the enrichment of maker transcripts. *Malat1* lncRNA was observed at high levels in the nuclear fractions, and conversely, *Gapdh* mRNA was expressed highly in the cytoplasmic fractions. The astrocyte-specific *Gfap* mRNA and *Gfap* pre-mRNA were assayed in the obtained fractions, as we expected, enriched in the cytoplasmic and nuclear fractions, respectively. We are investigating subcellular localization of several selected ncRNAs (miRNAs, lncRNAs and circular RNAs) in mouse astrocytes. We plan to expand the project by applying the same methodology to reactive astrocytes.

P.06.8

The dynamics of miRNAs expression in the postnatal mouse pituitary gland and insights into cell-type specific miRNA regulation in pituitary cells

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Pituitary gland (PG) is a neuroendocrine organ that produces hormones essential for maintaining homeostasis of the entire organism and influencing metabolism, growth, reproduction and stress responses. Hormone production is maintained by 6 endocrine cell types within PG, each of which secretes a specific hormone. The regulation of hormone production and secretion on a cell-type basis is not well understood. Especially, how non-coding RNAs contribute to the regulation of gene expression programs in the PG is just beginning to emerge. We hypothesize that cell-type specific miRNAs are essential contributors in posttranscriptional gene expression regulation in PG. Here, we present preliminary results of miRNAs profiling in murine PG at three different postnatal stages: P1, P30, and adult mice. We used NanoString miRNA assay to quantify global miRNA expression levels. We analyzed expression patterns of a few already known miRNAs that are essential for pituitary function. We identified miRNAs that are dynamically regulated over postnatal PG development and created a resource of stage-specific miRNAs. Using TaqMan assay and qRT-PCR we measured expression of selected miRNAs and some of long-noncoding RNAs that are known to influence miRNA stability. These tests were done both in PG tissues and in a catalogue of PG cell lines representing different secretory cell types. In the future, we aim to explore the cell-type specificity of miRNAs and miRNAs targets in PG cells in more detail.

P.06.9

The importance of sulfur and selenium atoms in wobble uridines of bacterial tRNAs for reading NNA- and NNG-3'-ending codons; the thermodynamic stability of RNA duplexes with S2U and Se2U modifications

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To date, sulfur- and selenium-containing uridines, including 5-methylaminomethyl-2-thiouridine (mnm5S2U) and 5-carboxymethylaminomethyl-2-thiouridine (cmnm5S2U) and their 2-selenouridine analogs mnm5Se2U and cmnm-Se2U, were found in bacterial tRNAs specific for lysine, glutamate, and glutamine. Two of these iso-acceptors ($tRNA^{Lys}_{UUU}$ and $tRNA^{Glu}_{UUC}$) occur in bacteria as single tRNA molecules and are responsible for the recognition of two synonymous 3'-purine codons, NNA and NNG.
In our studies on modified uridines in transfer RNAs, we previously proposed a two-step biosynthetic pathway for Se2U tRNAs [1] and showed that 2-selenouridines are sensitive to oxidative stress [2]. We also hypothesized that mnm5S/Se2U can adopt a zwitterionic form to bind G efficiently according to a "new wobble mode" [3]. In this work, we present the results of our pH-dependent thermodynamic studies with 5-unsubstituted U/S/Se- and mnm5U/S/Se-containing RNA duplexes. The results suggest a very specific role of 2-selenouridines for tRNA function to ensure reading of the 3'-G-ending codons.

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Acknowledgemens

This work was supported by The National Science Centre of Poland (UMO-2018/29/B/ST5/02509) to B.N.

P.06.10

Studies on the affinity of the bacterial 2-selenouridine-tRNA synthase (SelU) enzyme for its prenyl pyrophosphate substrates

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The SelU is responsible for the synthesis of the two nucleoside modifications in the 34th position of bacterial tRNAs specific for Lys, Glu, and Gln: 5-substituted 2-selenouridines (R5Se2U) and 5-substituted *S*-geranyl-2-thiouridines (R5geS2U). Recently, we demonstrated that the conversion of 5-substituted 2-thiouridines (R5S2U) to R5Se2U is linear, with R5geS2U being an intermediate [1].
Using *in vitro* enzymatic S2U-RNA→prenyl-S2U-RNA conversions, we have shown that among the prenyl pyrophosphate substrates such as GePP, IPP, DmaPP, FPP and GeGePP naturally occurring in cells, only GePP is accepted by SelU to give a prenyl-S2U-RNA product [2]. Moreover, MST affinity studies confirmed that SelU binds preferably GePP with a *K*d value of ~4.7 μM, while other prenyl pyrophosphates were not recognized as substrates. *In silico* analyzes also confirmed that GePP is the best binding substrate for SelU.
Our studies confirmed that the SelU enzyme in bacteria exclusively selects GePP as a prenylation substrate. This result is in contrast to the previously discussed hypothesis that SelU selects its prenylation substrate based on the length of its carbonyl chain and the thermodynamic stability of the prenylated – RNA duplex [3].

References

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Acknowledgement

This work was supported by The NCN of Poland (UMO-2018/29/B/ST5/02509) to B.N.