Session 2: RNA and RNP Structures and Mechanisms of Action: from Theory to Experiment

Lectures

L2.1

Structural and biochemical studies of the yeast mitochondrial RNA degradosome

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Mitochondria are essential organelles of eukaryotic cells providing energy through the process of oxidative phosphorylation. The unique feature of mitochondria is that they possess their own genome and gene expression machinery. Regulation of mitochondrial gene expression is greatly achieved by controlling the level of RNA – changing the transcription initiation rate and the rate of RNA degradation. In yeast the main executor of mitochondrial RNA degradation is the mtEXO complex composed of Dss1 3'-to-5' exoribonuclease and Suv3 helicase that act in concert and efficiently remove defective RNAs and excised introns. Both subunits reveal a remarkable functional interdependence involving all the enzymatic functions of the complex – ATP hydrolysis, nucleic acid duplex unwinding and RNA degradation. Crystal structure of Dss1 from *Candida glabrata* reveals it is a unique member of the RNR superfamily of ribonucleases with specialized domains responsible for interactions with Suv3 helicase. The arrangement of both subunits deciphered in the crystal structure of the complex enables the helicase motor to feed the 3' end of the RNA into the catalytic channel of Dss1 for effective degradation. This co-operation of both helicase and nuclease activities within the complex is particularly important for degradation of structured RNAs which cannot be handled by Dss1 on its own and for which the unwinding activity of Suv3 is required.

L2.2

Chemically modified mRNA

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All eukaryotic mRNAs possess the unique structure present at the 5´ end called cap. Cap structure participates in several stages of gene expression and is a good site for site specific chemical modifications which confer new interesting properties to mRNA. Continuing advances in understanding of the biological functions of the cap and the consequences of the disruption of these processes – resulting in serious medical disorders – have opened new possibilities for therapeutic applications of synthetic cap analogs. The medicinal potential of chemically modified mRNA has emerged in several areas. The most promising and the most advanced one is mRNA-based gene therapy. It has been generally assumed that mRNA is not stable enough for therapeutic applications. Significant progress in engineering mRNA properties made such applications possible. The presentation will be focused on recent advances in chemical modification of the cap structure and applications of cap analogs in the studies on cap-dependent processes, which eventually led to improvement of existing methods of protein expression for therapeutic purposes.

L2.3

Selected applications of small-angle X-ray and neutron scattering (SAXS/SANS) in studies of complexes and flexible protein structures

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Transmission electron cryo-microscopy of single molecules has recently become a key technique in the study of protein complexes. However, the general problem in macromolecular studies is still the monodispersity of studied samples, also protein complexes (including complexes with nucleic acids) in which the components are weakly bound or undergo gradual dissociation. Many proteins exhibit also the concentration dependent oligomerisation, that is difficult to study by conventional small angle X-ray scattering (SAXS) method. For such problematic systems, SAXS technique in combination with gel filtration (SEC-SAXS) and MALS, is perfectly suited to study the structure of these macromolecules in solution. Recently, this method has been implemented in synchrotrons (PETRAIII@ DESY, ESRF, Diamond and other). SEC-SAXS, allows also a study of macromolecules that are rapidly degraded. The complementary technique to SAXS is the small angle neutron scattering (SANS), which is not only perfectly suited to the study of large macromolecular complexes, but also does not induce radiation damages. During the lecture our experiences from recent studies with the use of SEC-SAXS and SANS, concerning oligomerisation of HCC, FIP37 (from m6A writer complex), a degradation of human PrP^{C} variants and other examples, will be discussed.
At the same time, it is also worth paying attention to the development of research facilities suitable for bioSAXS in Poland. Recently, the real possibility of construction of the beam-line dedicated to SAXS studies in Polish synchrotron (NCPS SOLARIS) is particularly important. This project will be presented in brief.

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L2.4

Identification and classification of proteins involved in nucleic acids metabolism

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Properly functioning metabolism of nucleic acids secures stability of the genome and proteome in all living organisms. Although proteins responsible for this process have been thoroughly studied, many of the enzymes that contribute to important biological functions related to nucleic acid processing and metabolism are still not known. Using our state-of-the-art methods for distant homology detection and 3D structure prediction we identified several novel enzymes among uncharacterized and poorly annotated proteins, as well as predicted their potential biological roles. We have also revised large and highly diverse superfamilies of nucleic acid processing enzymes that display little sequence similarity despite retaining a common core fold and a few critical active site residues. This provided a global picture of their evolutionary history, sequence-structure diversity and fulfilled functional roles.

L2.5

Regulation of gene expression by non-canonical poly(A) and poly(U) polymerases

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In eukaryotes, almost all RNA molecules are processed at their 3' ends and most mRNAs are polyadenylated in the nucleus by canonical poly(A) polymerases (PAPs). Recently, several non-canonical poly(A) and poly(U) polymerases have been discovered that have more specific regulatory roles. In contrast to canonical ones, their functions are more diverse; some induce RNA decay while others, especially cytoplasmic ncPAPs, activate translationally dormant deadenylated mRNAs. In this talk I will summarize our recent studies in which we have discovered novel unexpended functions of poly(A) and poly(U) polymerases in humans.

Oral presentations

O2.1

The mitochondrial degradosome complex and its co-factors prevent accumulation of double-stranded RNA in humans

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In humans, the mitochondrial DNA is subjected to bidirectional transcription that generates overlapping transcripts capable of forming double-stranded RNA (dsRNA) structures. We discovered that mitochondria are a major source of cellular dsRNA. Furthermore, we found that the level of mt-dsRNA is restricted by the mitochondrial degradosome, a complex of the ribonuclease PNPase and RNA helicase SUV3. Also, we found that PNPase prevents dsRNA from leaking into the cytoplasm, where it induces a potent interferon response, which can contribute to human diseases. In order to identify other factors involved in mt-dsRNA metabolism we performed the genome-wide siRNA screen and observed several cases of up- and down-regulation of mt-dsRNA. One of the most prominent hits was a mitochondrial poly(A) polymerase, MTPAP. Its silencing led to robust accumulation of dsRNA comparable to inactivation of degradosome components. In vitro reconstitution of dsRNA decay reactions revealed that polyadenylation is a prerequisite of degradation of blunt mt-dsRNAs by the degradosome, which was further supported by *in vivo* experiments.

In conclusion, our study revealed the decay pathway of dsRNA in mitochondria and several other factors involved in mt-dsRNA metabolism. Their further investigation may reveal new aspects of mtRNA metabolism and extend repertoire of mechanisms by which mitochondria control fate of the cell.

O2.2

Long non-coding RNAs as modulators of splicing *in trans* via direct RNA:RNA interactions

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Only a handful of long noncoding RNAs (lncRNAs) have been functionally characterized. In particular, little is known about their engagement in gene expression regulation by coming into lncRNA:RNA interactions. By hybridizing with other transcripts, lncRNAs could be involved in at least several regulatory mechanisms.
Our goal is to investigate splicing-related functions of lncRNAs that are exerted in the context of RNA:RNA duplexes. Recently, based on *in silico* predictions of lncRNA:RNA base-pairings across the human transcriptome, we discovered a great potential for lncRNAs to modulate splicing by means of masking splice sites and/or other splicing signals. In order to select most promising and biologically relevant candidates for experimental tests, we decided to focus on cases where lncRNAs lead to a shift in protein coding capacity of transcripts. We also required that the lncRNAs of interest show higher expression level in the nucleus than in the cytoplasm (using RNA-Seq data) and they represent relatively stable transcripts (established with 4SU-Seq data). At the moment, we are performing laboratory tests on a subset of candidates. One of them is *OIP5-AS1* (*OIP5 antisense RNA 1*), an oncogene in certain types of cancers. Subcellular fractionation of HEK293 cell line and Real-Time PCR have confirmed the lncRNAs are predominantly localized in the nucleus. In line with the predictions, knockdown of lncRNAs with antisense LNA gapmers resulted in changes in splicing pattern of regulated genes. In the next step, it is planned to perform RPA (Ribonuclease Protection Assay) experiments to check whether the observed phenomenon indeed stems from direct lncRNA:RNA interactions.

Posters

P2.1

Structural determinants of Ty1 genomic RNA dimerization and packaging in the LTR-retrotransposon Ty1

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RNA dimerization is the non-covalent process by which retroviruses carry two copies of genomic RNA (gRNA) into the virion particle. The dimerization process is crucial for several important steps in the retroviral life cycle: selective packaging of the genome, regulation of translation, enzyme switching during reverse transcription.Ty1 of *Saccharomyces cerevisiae* is an interesting example of active LTR-retrotransposon. Ty1 family shares critical features with retroviruses in genome organization and replication. The Ty1 gRNA in virus-like particles (VLPs) is also dimeric, but sequences that are necessary for dimerization and packaging have not been precisely defined. We applied high-throughput selective 2' hydroxyl acylation analysed by primer extension (SHAPE), a technique that allows structural interrogation at each nucleotide, to map the secondary structure of the Ty1 RNA in dimer state. We have indicated palindromic sequences that are involved in the genome dimerization process. Our data inform a model in which two intermolecular PAL1/PAL2 interactions in the 5' UTR of Ty1 RNA are critical for dimer maintenance and stability *in vitro*. The mutational analyses confirm the importance of identified *cis*-elements. We also show that Ty1 Gag acts similar to retroviral Gag polyproteins and promotes dimerization of Ty1 RNA, and annealing of primer tRNAiMet *in vitro*.

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P2.2

Regulation of LTR-retrotransposons life cycle

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Long-terminal repeat retrotransposons are transposable genetic elements that comprise a significant fraction of many eukaryotic genomes. An active LTR-retrotransposon Ty1 is the most abundant mobile genetic element in the *S. cerevisiae* genome. Ty1 has pronounced functional similarities to retroviruses and its structure is analogous to that of retroviral proviruses. Both groups replicate via an RNA intermediate and insert their double-stranded DNA into the host genome.

Unlike retroviruses, Ty1 retrotransposition is not infectious and Ty1 never leaves the cell. *S. cerevisiae* maintain a low copy number of the Ty1 in their genomes trough the mechanism called copy number control. This mechanism depends on Ty1 Gag derived restriction factor – p22. Our recent data indicate that p22 inhibits normal Ty1 VLP assembly and maturation through interactions with Gag. Ty1 Gag and p22 share a region crucial for RNA binding and chaperone activity. Data obtained from chemical probing of ribonucleoprotein complexes show that the Ty1 Gag and p22 recognize the same nucleotide sequences and thus they may compete for interactions with Ty1 RNA. Inhibition of Gag function by the p22 abolishes the packaging of Ty1 gRNA in VLP. Moreover, using SHAPE analysis of Ty1 RNA inside VLPs we have indicated significant changes in reactivity in the region engaged in dimerization with increasing amount of p22.

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P2.3

Low- mass RNA Ladder and its potential application in analysis of small-molecule RNAs

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DNA and RNA Mass Ladders are commonly used for nucleic acid size estimation and approximate quantification. They are particularly helpful in determining the mass of individual species during their electrophoretic separation and as such are widely applied in analytical techniques in biotechnology, biology, medical diagnostics and gene therapy. Nevertheless, commercial availability of RNA Mass Ladders is much lower than DNA Mass Ladders, especially in a low mass region of up to 100 nt. Still, it is exactly in this range that many biologically relevant RNAs - either the subject of research themselves or used in indirect analyzes - are located, e.g.: siRNA, miRNA, scRNA, tRNA, snoRNA and a portion of snRNA. It is therefore in direct response to the market demand that we have developed Low Mass RNA Ladder with component oligoribonucleotides' length from 10 to 80 nt, to be used in electrophoresis in agarose or polyacrylamide gels.

Component RNA oligonucleotides with the following lengths: 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70 and 80 nt have been synthesized by phosphoramidite method using the solid support approach. Due to the fact that RNA is much more susceptible to degradation than DNA in standard conditions, an entirely new method "FS-cap" has been developed to increase the stability of each oligonucleotide. Importantly, our method does not affect the migration of oligonucleotides during electrophoresis. Moreover, a stabilizing buffer has been developed to reduce the degradation of RNA oligonucleotides upon storage. Last but not least, a non-fluorescent dye was covalently attached to two of the oligonucleotides from the Ladder, to allow visual follow-up of RNA separation.

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P2.4

Bioinformatic construction of PUM1 and PUM2 RNA-Regulons in TCam-2 cell line

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Posttranscriptional gene regulation (PTGR) is one of mechanisms ensuring proteome balance, while its disruption may cause severe diseases including infertility and cancer. PUM are PTGR proteins containing PUF RNA-binding domain which recognizes UGUAHAUW motif within 3'UTR and recruit specific ribonucleoprotein complexes to direct selected mRNA pools towards translation, storage or degradation. Although human PUM1 and PUM2 recognize same UGUAHAUW motif, the question whether they control separate or common mRNA pools (form separate RNA-regulons) is unresolved. To address this in the context of germ cells, we used RIP-Seq (RNA co-immunoprecipitation and sequencing), siRNA-Seq (siRNA silencing and sequencing) and Co-IP/MS (co-immunoprecipitation and mass spectrometry) to identify PUM1 and PUM2 mRNA targets and PUM protein cofactors, in TCam-2 cell-line representing human male germ cells. We obtained mostly different mRNA pools regulated by PUM1 (299) and by PUM2 (94) involved in different biological processes. We also obtained mostly different PUM1 (28) and (31) PUM2-bound protein cofactors, majority of them implicated in RNA processing. Gene ontology analysis seems to indicate that PUM1 and PUM2 (together with their protein cofactors) regulate RNA processing in distinct cellular pathways. Namely, PUM1 regulon possibly controls oncogenesis related-, while PUM2 regulon germ-cell specific pathways.

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P2.5

The activity and function of MCPIP2 – the most enigmatic member of the ZC3H12 family of proteins

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Regnase-1/ZC3H12A/MCPIP1 is the best-described member of the ZC3H12 family of proteins containing three more members. All members of the ZC3H12 family contain an NYN/PIN-like domain and a CCCH-type zinc finger. Although the role of Regnase-1 as a crucial negative regulator of inflammation is well established the knowledge about the other members of the ZC3H12 family is very scarce. MCPIP2 is the most enigmatic one – there is no published study concerning the activity or function of this protein.

Bioinformatic examination indicates that all amino acids within MCPIP2 corresponding to the ones that are crucial for Reganse-1 RNase activity are conserved. Microscopic analysis indicates that MCPIP2 forms small granules in the cytoplasm. Studies using reporter constructs reveal that MCPIP2 is able to decrease the luciferase activity when the sequences of the 3'UTRs of Regnase-1 substrates like *IL-6* or *IER3* are attached to the luciferase coding sequence. The ability of MCPIP2 to decrease the luciferase signal strictly depends on the presence of a functional NYN/PIN-domain. The observed decrease in luciferase activity is the result of luciferase transcript degradation as revealed by qPCR. Moreover, MCPIP2 requires the presence of a specific stem-loop structure within the substrate mRNA for target recognition. Overexpression studies indicate that MCPIP2 is a negative regulator of proliferation and that this activity also requires a functional NYN/PIN-domain.

P2.6

Isolation and biophysical studies of proteins from IFIT and FASTKD families

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RNA-binding proteins are involved in post-transcriptional regulation of gene expression, for example on translation level. This makes them a very important group of biomolecules and a hot research topic in the search for new drugs and gene therapy tools. We study proteins of two families, FASTKD and IFIT, that contain non-canonical RNA-binding domains. Human proteins of those families were obtained by heterologous expression and chromatographic purification. The proteins will subsequently be characterized by their structure, using molecular biophysics techniques like circular dichroism (CD) and attenuated total reflectance fourier-tranform infrared spectroscopy (ATR-FTIR). Such information may not only contribute to better understanding the function of RNA-binding proteins, but also to the search for specific biotechnological applications of investigated proteins.

P2.7

Structure-function studies of RNA-binding IFIT proteins

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Interferon-induced proteins with tetratricopeptide repeats (IFITs) are family of RNA binding proteins, which sequester viral RNA and prevent translation of viral proteins, as part of the vertebrate innate immune response. IFITs form larger complexes which stoichiometry and function is not fully understood. We study complexes assemblies by size-exclusion chromatography with multi-angle light scattering. Using interaction analysis methods such as microscale thermophoresis (MST) and isothermal titration calorimetry (ITC) we are checking RNA preference of IFIT complexes *in vitro*. We are also interested in using properties of IFIT proteins in diagnostics and biotechnology applications.

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P2.8

Studies on the oxidative damage of wobble 5-methylcarboxymethyl-2-thiouridine in transfer RNAs

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The 5-substituted 2-thiouridines (R5S2U) present in a wobble position of specific transfer ribonucleic acids ($tRNA^{Lys}$, $tRNA^{Glu}$, $tRNA^{Gln}$) play an important role in proper decoding of genetic information in the process of protein biosynthesis. Recently, we have found that in oxidizing environment 2-thiouridine (S2U) alone, built into an oligo RNA or tRNA chain is desulfured, i.e. the sulfur atom is removed from the molecule, and the products of this reaction are uridine (naturally occurring in RNA) and a deprived of sulfur atom 4-pyrimidinone riboside (H2U). For the latter the hydrogen bond acceptors and donors pattern of which is different from that of uridine and 2-thiouridine. The presence of H2U can be considered as damage, since tRNAs containing the H2U unit may not act properly (e.g. may allow for the formation of mutant proteins or inhibit the protein synthesis). H2U-tRNA is also unstable in the cellular environment and can be easily cleaved at the modification site.
In the presented studies, we show that oxidative damage of S2U-tRNA occurs not only in a test tube (*in vitro*), but also in living cells, e.g. yeast cells. Experiments carried out in *Saccharomyces cerevisiae* yeast cells cultured under oxidative stress conditions confirmed the presence of products of S2U-tRNA desulfuration (mcm5H2U and mcm5U) in the mixture of tRNA-derived nucleosides.

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P2.9

Escherichia coli tRNA 2-selenouridine synthase (wtSelU) and its G67E mutant (mutSelU G67E): catalysis of conversion of bacterial 2-thiouridine-tRNA to its 2-seleno-analog

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The sulfur- and selenium-modified uridines (S2U and Se2U) present in the wobble position (first position of the anticodon) of specific tRNAs ($tRNA^{Lys}$, $tRNA^{Glu}$, $tRNA^{Gln}$), play a fundamental role in decoding of genetic information.
Bacterial tRNA 2-selenouridine synthase (SelU) is responsible for introducing into tRNA two post-transcriptional modifications: 2-selenouridine (Se2U) and a recently discovered *S*-geranyl-2-thiouridine (geS2U). By using anticodon-stem-loop tRNA fragments (17-mers) containing S2U, Se2U, or geS2U units, we found that *in vitro* wtSelU converts S2U-RNA to Se2U-RNA in a two-step process involving S2U-RNA geranylation and subsequent selenation of the resulting geS2U-RNA. No direct S2U-RNA→Se2U-RNA replacement was observed. These results suggest that *in vivo* the S2U→Se2U and S2U→geS2U transformations in tRNA, so far claimed to be the elementary reactions occurring independently in the same domain of the SelU enzyme, should be considered a combination of two consecutive events – geranylation (S2U→geS2U) and selenation (geS2U→Se2U). Additionally, we demonstrated the G→E mutation introduced into 67 position of wtSelU polypeptide chain increases low intrinsic geranylation activity of the wild type enzyme (from 10% to 70%), but abolishes its selenation activity towards S-geranyl-2-thiouridine.

Reference:
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P2.10

Thermo-sensitive fluorescent dye for nucleic acid labeling

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Biomolecules (nucleic acids, enzymes, antibodies etc.) is a general term used for molecules and ions that are present in organisms and essential to some typically biological processes. Biomolecules grow ever more present in drug discovery programmes, because they hold promise to be drug candidates of the next generation – more efficient and showing fewer side effects. Our understanding of their activity and mechanism of action during preclinical and clinical drug development is partly due to application of various molecular imaging techniques (like MRI, PET etc.) to non-invasive assessment of biological and biochemical processes in living subjects. Among all types of molecular imaging reagents, fluorescent dyes stand out as a particularly important class due to their wide applicability, low toxicity and high sensitivity. E.g. test based on fluorescence detection are used in diagnosis of Alzheimer's disease. [1]. We have successfully developed a thermo-sensitive fluorescent dyes for molecular imaging applications, based on a 2-pyridinyl scaffold [2] and characterized their chemical structure as well as optical properties. We have also developed methods to conjugate them to nucleic acids in clean and efficient manner. A unique feature of these species is that they are thermolabile: following the intramolecular cyclization mechanism induced only by temperature rise, they can be easily removed from a biomolecule. Cleavage rate was found to be dependent on certain parameters, such as solvent (aqueous or non-aqueous medium), pH values, and electron distribution in pyridine rings.

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