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Review of the doctoral dissertation of M.Sc. Keerthiraju Ethiraju Ravichandran, entitled *tRNA modifications in human diseases – Structural and functional consequences of Urmylation in response to Oxidative stress*

Transfer RNAs (tRNAs) are key molecules participating in protein synthesis. To achieve biological realm, tRNA molecules undergo extensive post-transcriptional, enzyme-catalyzed modifications at a number of base and sugar positions. Currently more than 120 modified nucleosides were identified in tRNAs in all domains of life. The hotspots sites of the tRNA modifications are the 34 position of tRNAs referring to the first anticodon letter, called also the wobble position. The frequency and chemical diversity of modifications identified at this position relate to the biological function of tRNA in codon recognition and maintaining the translational efficiency and fidelity. Some of wobble nucleosides facilitate the anticodon interaction with more than one codon by formation of the specific base pairs between nucleosides positioned at the first anticodon and third codon letters. Similarly, weakening of base pairing can discriminate against binding to near-cognate codons. The cellular-controlled distribution of tRNA modifications can result in the preferential of mRNAs coding for example for stress proteins to facilitate cell survival.

Several recent studies have pointed to a link between defects in tRNA modifications and human diseases including cancer, mitochondrial and neurological deseases. Therefore, defects in tRNA modifications in humans need intensive characterization at the enzymatic and mechanistic level to understand how the lack of such modifications are associated with cellular disorders at molecular level.

Since tRNA thiolation is essential for achieving metabolic homeostasis and cell growth, and changes dynamically in the response to cellular stress conditions, the correlation between disfunctional process of thiolation and severe human diseases was found. Thus, the study on the structural and mechanistic aspects of enzymatic reactions related to tRNA thiolation in eukaryotes make a huge

contribution not only to expand biological knowledge about these processes but also to understand the pathogenesis of human diseases.

Doctoral dissertation, entitled *tRNA modifications in human diseases – Structural and functional consequences of Urmylation in response to Oxidative stress,* performed in Max Planck Laboratory, in Małopolska Center of Biotechnology of Jagiellonian University under the supervision of dr hab. Sebastian Glatt perfectly fits the current research trends. In briefly, research presented in the thesis concerns the molecular explanation of dual activity of the ubiquitin-related modifier 1 protein (Urm1) as a sulfur carrier (SCP) protein in 2-thiolation of wobble uridine in eukaryotic tRNAs and as a Ubiquitin-like (UBL) protein able to conjugate to target proteins in response to oxidative stress.

The doctoral dissertation has a classical layout. It begins with introduction describing how genetic information is transmitted from DNA to protein. Since tRNAs are important players in protein production, their structure and modifications were discussed, focusing on the wobble 5-substituted 2-thiouridines. Next, Student moved to description of the thiolation cascade and protein urmylation. Herein, Student clearly described the state of art and highlighted which areas of knowledge remain elusive or unknown what helped the reviewer to understand the novelty of the doctoral project. The last part of the literature section concerns human diseases associated with the lack or defects of modified uridines at the 34 position.
After short chapter entitled *Aims*, Student moved to chapters relates to his individual research project (Materials, Methods, Results, Discussion and future perspectives). The thesis ends with *Highlights and conclusions*, and the list of the cited references. The dissertation is written in a language typical for scientific papers. I did not notice any typos, mental shortcuts or linguistic errors. However, more attention should have been paid to the record of references to keep one template and full and correct bibliographic data. Figures and schemes are aesthetic and informative what makes easier for the reader to go through.

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In the research Project PhD Student solved several important problems related to the mechanistic aspects of enzymatic reactions of Urm1-Urb4 activation, Urm1-thiocarboxylation and Urm1-SH-initiated urmylation of target proteins in response to oxidative stress.

Several achievements of this work should be emphasized:

- Structural analysis of crystallized Uba4 from *Chaetomium thermophilum* (*Ct*Uba4) and individually purified adenylation domain (AD) and rhodanese-like domain (RHD) were

performed to make an insight into the asymetric Uba4 dimer structure, indicating that homodimerization of AD is critical for the adenylation of Urm1. Of note, it was established that adenylation of Urm1 is sufficient to stabilize the Uba4-Urm1 complex.

- Structural analysis of the $Uba4_{C202K}$-Urm1 complex shown binding of Urm1 to a dimer of the Uba4 AD, while RHDs revealed intrinsic mobility. The C-terminal GG-motif of Urm1 was identified near the linker region. The insight into the structure and interactions between Uba4 and Urm1 indicates close cooperation between two Uba4 regions AD and RHD under linker region control (allowing the movements of RHG) to reach Urm1-thiocarboxylation. To start activation, the Uba4 AD enzyme binds Urm1-OH with ATP-dependent manner. Substrate-bound Uba4 changes its conformation as the linker region moves with RHG to the outside of the AD-Urm1 complex. In this state, RHD is inactive until the thiocarboxylation process takes place. Adenylated Urm-1 reacts with crossover loop positioned C202 to form a thioester (Urm1-GG-C(O)S-AD) intermediate (by thioesterification reaction). The linker region is then released, allowing the RHD domain to be moved towards the C-terminus of Urm1. A nucleophilic attack of the persulfide on the active C397 of RHD results in the acyl-disulfide bond formation (Urm-GG-C(O)-S-S-RHD). Disulfide structure is extremely unstable offering its fast conversion to the thiocarboxylated form of Urm1 (Urm1-SH) which is released to be employed as a sulfur carrier for the Ncs2- and Ncs6-mediated tRNA thiolation.
- The role of Urm1 as UBL enzyme was investigated in the context of mechanistic reactions involved in this process as well as identification of the target proteins which are urmylated in response to oxidative stress. To gain insight into protein conjugation reaction, the in *vitro* experiments were performed with purified Urm1-SH enzyme and target proteins, including alkyl hydroxy peroxiredoxin-1 (Ahp1) exposed to the *tert*-butylhydroperoxide to introduce redox-active cysteines. Also macromolecular crystallography was utilized to determine the structures of unmodified and urmylated *Ct*Aph1 to understand the mechanism of Urm1 conjugation. Proposed mechanism of protein urmylation with Urm-1 involves two steps: 1) recognition of the oxidized, sulfenylated cysteines in target proteins by thiocarboxylated Urm1 (Urm1-SH) resulting in the formation of an acyl disulfide intermediate (Urm1-GG-C(O)S-S-Cys-target protein); 2) nucleophilic attack of lysine, serine or threonine positioned in proximal distance from the Urm1-linked cysteine leading to the conjugation of Urm1 with target protein via amide- or ester-linkage with releasing persulfidated cysteine. Urmylation of target protein with Urm1 does not require additional activation by E2 enzymes or E3 ligases which were found in classical ubiquitin system. Of note, the persulfidated cysteine can be easily reversed to its reduced form under physiological conditions, therefore persulfide modifications play significant role in protection of thiol

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function in cysteines against oxidative stress. Overall, the capacity of Urm1 for cysteines persulfidation in response to oxidative stress should be considered as a cell protection from oxidative damage and aging.

- It was determined that the Urm1 thioesterification with C202 from AD Urb4 domain protects Uba4 against urmylation. It was evidenced that $Uba4_{C202S}$ mutant treated with TBH forms products of Urm1 conjugation showing the ubiquitin-like activity of Urm1 protein. Structural analysis of Uba4-Urm1 system versus known E1-UBL systems revealed their close similarity. The conclusion was gained that Uba4 represents the E1 enzyme in Uba4-Urm1 system and therefore is an ancestor of known E1-activated UBLs proteins which further evolved and expand mechanism of activation with additional E2 enzymes.

Some minor reviewer's remarks:

- pp. 13. The L-shaped tertiary structure of tRNA was defined as an *"L-configuration"*. Could you explain this verbal inconvenience in relation to the L/D configuration of some biomolecules (e.g. L-amino acids occuring in biological systems) ?
- Regarding to the 5-methyluridines and 5-methyl-2-thiouridines present at the 34 position of tRNA anticodons have you any concept why wobble uridines in prokaryotic and mitochondrial tRNAs contain exclusively 5-aminomethyl-type substituents (e.g. mnm5, cmnm5, taum5, inm5) while majority of eukaryotic tRNAs from cytosol possess 5-methyl-type substituents deprived of adjacent nitrogen (e.g. mcm5, ncm5, chm5, cm5) ?
- Is it possible to predict the future fate of urmylated proteins in the cellular system?

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Coming to the end I must emphasize that the research material collected and presented in the dissertation constitutes a significant and valuable contribution to the understanding the Urm1-Uba4 interplay to furnish a Urm-1 tiocarboxylation and Urm-1 urmylation activity under the oxidative stress conditions. Most results obtained by Candidate were published. Student is an co-author of four original papers and one review published in prestigious journals from the JCR list, such as *EMBO J.*, *Redox Biology* or *Microbial Cell*. Student is the first author of one article and the equal first author of two others. He has been awarded three times by the Scientific Community.

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In conclusion, I would like to state that the submitted doctoral dissertation of M.Sc. Keerthiraju Ethiraju Ravichandran fully meets the conditions set out in Art. 187 of the Art of July 20, 2018 on academic degrees and title as well as degrees and title in art. Therefore, I am requesting the High Discipline Council for Biological Sciences of the Jagiellonian University to accept the thesis and allow the PhD Student to enter further stages of the doctoral tract.

Taking into account the Candidate's scientific achievements and a wide range of research that confirmed the student's experience in the field of biochemistry, comprehensive mass spectrometry analysis, protein handling and crystallography, **I recommend this doctoral dissertation to distinction.**

Sincerely,

Dr hab. inż Grażyna Leszczyńska, prof. Uczelni

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