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Review

of the PhD thesis, entitled: "tRNA modifications in human diseases – Structural and functional consequences of Urmylation in response to Oxidative stress"
authored by Keerthiraju Ethiraju Ravichandran

Thesis evaluation

The presented original thesis elaboration falls into canonical presentation broadly accepted by the Polish research system, with structural organization starting with the abbreviations, abstract, introduction, aim of the study, materials and methods, results, discussion and future perspectives, summary, highlights and conclusions, references, list of figures and tables, and list of publications that arose from this thesis. The form of presentation is very clear and refined, which makes it a well-structured and easy-to-follow thesis.

The first part of the thesis is an introduction providing readers with the current knowledge about the main actor in the dissertation – the ubiquitin-related modifier, Urm1 pathway, mainly responsible for tRNA thiolation. Initially, the Author described the role of the Uba4-Urm1 complex in tRNA modification within the anticodon at the wobble position (U34), especially giving an informative graphical representation of the pathway shown in Fig. 3 and 4. It should be noted that the Author emphasized the dual-purpose of Urm1, linking it with the sulfur-transfer reaction into tRNA (well defined pathway) and ubiquitin-like conjugation to proteins during oxidative stress – a poorly understood phenomenon – *the urmylation*. The Author pointed out that *the urmylation*, being already described as a biological phenomenon, has never been thoroughly explored from the mechanistic perspective, especially the mechanism of Urm1 transfer and substrate specificity represent an unexplored territory. Thus, in the Introduction part, the Author builds the scientific hypothesis, providing numerous examples to validate his statement, linking *the urmylation* with oxidative stress as conditions that may favor *the urmylation* as cellular response and adaptation to stress conditions; the introduction represents a Cartesian approach with a well-developed information-driven trajectory, providing at the same time well-defined objectives to be explored.

In the next part of the thesis, the Author focused attention on the structure and mechanistic details of the Uba4 and Urm1 interplay to decipher the molecular details of the complex interaction and activation. Initially, in the course of the study of the Uba4–Urm1 complex, detailed structure–function analyses of its components were conducted. Thus, the Author solved the structure of the Uba4 protein at 2.2 Å resolution, showing that full-length Uba4 forms an asymmetric homodimer with the AD and RHD domains independently contributing to this unique assembly, indicating that the AD and RHD domains form stable AD-RHD dimers. Next, to understand the influence of nucleotide binding on the Uba4 activity, the effect of different nucleotides was evaluated in connection to the recruitment of Urm1 by Uba4. Using several approaches, such as the GST pull-down approach or co-migration with SEC analysis, the Author showed that the Uba4–Urm1 complex is formed efficiently in the presence of ATP, while other nucleotides were not able to support complex formation. As a continuation

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of this line of the research, the functional test was conducted to map the specific residues of the AD, the RHD, and the linker part of the Uba4 protein, using *in vitro* Uba4 activity assays with functional *in vivo* validation in baker's yeast; especially, the Author examined R18A and R77A mutations, which are located at the nucleotide-binding site; next, the Author analyzed the Uba4–Urm1 complex formation using mutated Uba4 in the GST pull-down approach. He found that the R18A and R77A mutations significantly weaken the complex formation, while other mutations (within the active site or linker) did not exert a negative influence. The Author concluded that adenylation of Urm1 is sufficient for complex stabilization; however, the Author used GST pull-down to detect just complex formation, thus my questions is: is it possible to estimate adenylation of Urm1 using the GST pull-down approach, – Figure 9B? The GST pull-down assay, especially with the Uba4-C202K mutant, laid the foundation for a subsequent study, because it was possible to get the crystal of the covalently bound Uba4-C202K to Urm1, which allowed determination of the structure at 3.15 Å resolution. The structure revealed two Urm1 molecules bound to a dimer of Uba4 AD, unfortunately without well-defined RHD. The structure provided a model defining the interactions between Uba4 and Urm1; especially, it locked the state characteristic for the post-adenylation thioester state, showing how two domains of Uba4 orchestrate recognition, binding of the C-terminus of Urm1.

In the next step, the Author focused on the functional aspect of the Uba4 and Urm1 interplay, namely the phenomenon of attachment of Urm1 to target proteins with Uba4 as a partner. Initially, he tested whether Urm1 can be covalently conjugated to Uba4 *in vitro* and whether thiocarboxylated Urm1 was required. Importantly, he developed an experimental system that allowed obtaining thiocarboxylated Urm1, and the subsequent tests showed that thiocarboxylated Urm1 is required for Uba4 binding, indeed; especially, the appearance of the complex is strongly enhanced by using the Uba4-C202S mutant. The Author also analyzed the effect of mutations, especially within cysteine residues, indicating that redox communication between two cysteines (C202, C305) located in adjacent loop regions represent important interplay. The Author also performed *in vivo* validation using yeast strains harboring various mutations within Uba4, showing that C311 is not important for the Uba4 function. The Author also used rapamycin as a stress factor for the functional study in the yeast strains – why rapamycin, considering that the Author was focused on oxidative stress? Rapamycin belongs to the mTOR inhibitors, and its presence mimics amino acid starvation, and do not induce oxidative stress; thus, were the physiological conditions considered as a stress factors, such as amino acid starvation (compatible conditions to rapamycin treatment), or especially oxidative stress? As a final approach in the characterization of the Uba4 and Urm1 interplay, the Author employed mass spectrometry analysis to map the lysine residue in Uba4, which is the conjugation site of Urm1, using the C202S mutant, identifying several Lys residues close to the active site; the MS data were additionally confirmed by mutational analysis of the identified Lys residues. Summing up, the work of the Author provided us with mechanistic insight into the mechanism of self-conjugation of Uba4 by Urm1; at the same time, he showed the first *in vitro* evidence for the existence of an ubiquitin-like "*urmylation*" reaction. This elegant work was published in the EMBO Journal, and the Author presented a '*Working model of the Uba4-*

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catalyzed reaction cycle of Urm1 thiocarboxylation' shown in Fig. 5 in the article (and in the discussion part of the thesis as well), which is the crowning achievement of the Author, who works in an excellent scientific environment.

In the course of the research, the Author together with the team undertook the next step to characterize the mechanism of Urm1 conjugation to target proteins. The Author focused his attention on Ahp1, a protein that had been reported to undergo 'urmylation' *in vivo*. Importantly, the biochemical details related to the mechanism of the conjugation reaction are undefined. Initially, the Candidate characterized the *in vitro* interaction of Ahp1 with thiocarboxylated Urm1. He used recombinant proteins Urm1-SH (also Urm1-OH) and performed series of optimization reactions to set-up the experimental system, especially using mutated Ahp1 forms, such as C31S, C62S, and C31S-C62S (however, the technology of the preparation of the recombinant proteins has not been mentioned in the thesis). In the main experiment, the Author used Ahp1 mutant forms vs. Urm1-OH or Urm1-SH; interestingly, the *in vitro* reaction of WT Ahp1 had low efficiency in the presence of the oxidizing agent (TBH), a factor which was shown to be an effective stimulant for conjugation *in vivo*. On the other hand, the mutant form Ahp1-C31S very efficiently formed conjugates with Urm1-SH in the presence of TBH (how will the Author explain this surprising observation and *in vivo* and in vitro discrepancies?). Next, the Author examined the influence of other oxidative agents on the 'urmylation' phenomenon, as it was already observed *in vivo*, showing that the formation of the complex occurred in the presence of hydrogen peroxide, peroxynitrite, and diamide, conforming the *in vivo* observations. As a final approach to characterize the Ahp1-Urm1 interplay, the Author showed (using Urm1-SH and Urm1-OH) that thiocarboxylated Urm1 represents the active form for the Ahp1 'urmylation'. The analyses performed by the Author provided the first biochemical *in vitro* insight into the 'urmylation' phenomenon, showing that the Urm1 conjugation reaction requires a thiocarboxylated C-terminus of Urm1, and importantly it is a spontaneous reaction occurring in oxidative conditions and not depending on the canonical cascade of ubiquitin-conjugating enzymes and E3 ligases.

As a continuation of the project, the Author analyzed the structure of the covalently linked complex Ahp1-Urm1 using a crystallographic approach. He obtained a structural model at 2.5 Å resolution; the model showed a tightly packed hexameric ensemble of the complex, with three homo-dimers of Ahp1 with attached Urm1. The Author noticed structural variations within the Urm1 position on the complex, suggesting some flexibility within this part of the complex. I am wondering whether the Author considered the cryo-EM approach to solve the issue of the structural variations; since the complex has molecular mass close to 100 kDa, it could be suitable for cryo-EM analysis (however challenging) to have a look at structural variations. An interesting issue is represented by the docking part of Ahp1 for Urm1. In the crystal structure, the Lys63 residue from each Ahp1 molecule forms a covalent isopeptide bond between the lysine side chain and the C-terminus of Urm1; interestingly, using site-directed mutagenesis, the Author showed that multiple Lys residues can be targeted by Urm1. Moreover, the Author did 'Benedictine work' (with plenty of protein forms with mutated Lys – so-called lysine-less-Ahp1-forms, as well as other residues) showing that other residues, like Ser and Thr, can work

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as alternative attachment sites. Thus, how can the Author explain the homogeneity in the crystal structure, showing that the Lys63 residue is the only docking place? Next, the Author turned his attention to the thiocarboxyl group of Urm1, which is essential for the conjugation reaction, focusing on the fate of the sulfur atom. He used Urm1-SH carrying radioactive sulfur (^{35}S) at its C-terminus and analyzed the distribution of the sulfur atom upon the Ahp1-Urm1 complex formation, detecting efficient transfer of ^{35}S from Urm1-^{35}SH to Ahp1 with the use of the well-established SDS-PAGE method and verified it by MS analysis, showing that the catalytically active peroxidatic cysteine in Ahp1 underwent S-sulfhydration at the C60 position. Importantly, the work was supplemented with crystallographic analysis centered on the redox-active cysteine residues in Ahp1, confirming the biochemical data that the thio-modification of the cysteine in Ahp1 indeed takes place during the conjugation reaction with Urm1-SH. All these data obtained based on the examination of the 'urmylation' phenomenon allowed the Author to propose the mechanistic model of 'urmylation', indicating that upon oxidative stress, Urm1-conjugation is directly associated with cysteine persulfidation of the substrate. I am wondering what is happening *in vivo*, during the adaptation phase of the cell to stress conditions, because in a majority of cases, post-translational modifications represent a reversible reaction, thus the question arises of what is happening to the 'urmylated' proteins.
Next, as a continuation of this line of research, the Author examined additional substrates for Urm1; based on the available MS/pull-down data, he chose several proteins, purified them, and analyzed the 'urmylation' reaction. He showed that several proteins, for example GAPDH, may undergo 'urmylation' upon the addition of an oxidizing agent *in vitro*, also confirmed by the MS analysis, mutational of the peroxidatic cysteine (for example in GAPDH), which abolished the conjugation reaction. Additionally, using isotope labeling coupled with MS, the Author confirmed the transfer of the sulfur atom during Urm1 conjugation. Thus, the Author showed that the mechanism of Urm1-SH-dependent 'urmylation' of several substrates represents a universal scheme. Interestingly, in the next step, the Author asked the question whether any other protein harboring the thiocarboxylated C-terminus can conjugate to target proteins. So, the Author used ubiquitin-SH or SUMO-SH, and they were able to attach for example to Ahp1, in the same experimental conditions as for Urm1-SH. Additionally, the Author tested whether the conserved C-terminal GG motif is required for the conjugation reaction, showing that almost any amino acid at the C-terminus permitted the conjugation reaction with thiocarboxylated C-termini; also, he showed that the β-grasp fold within Urm1 is not required for specific conjugation, as shown with the GFP-SH proteins that can be attached to target protein. Thus, this cluster of experiments raises the question about the specificity of the 'urmylation' - whether Urm1-SH can recognize the target proteins upon oxidative stress in a specific way. As the last line of the Author's research, an *in vivo* approach was employed to cast more light on the biology of Urm1 and its role in tRNA and protein thiolation. In the background of this thesis, the most relevant issue that I would like to point out is the Urm1 involvement in protein modification, especially persulfidation of cysteines. With the aid of collaborators, the Author analyzed a set of yeast mutant strains, including *Δurm1*, and showed that protein conjugation and cysteine persulfidation by Urm1 are independent events for tRNA

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thiolation. However, the *in vivo* experiments did not provide conclusive evidence of Urm1 as an important element in resolving oxidative stress in the cell. As mentioned by the Author, hydrogen sulfide (H_2S) is a factor responsible for the major cellular protective response against oxidative stress to protect cysteine residues by persulfidation; thus, from the energetic point of view, H_2S utilization by the cell could be much more efficient than 'urmylation'; in line with this remark, the protein sulfhydrome (in human cells) has been already established (Mol. Cell. Proteomics 2020, 19(5):852-870), showing a plethora of proteins undergoing persulfidation upon oxidative stress, including a significant number of ribosomal proteins (with GAPDH as a prominent example as well). In this respect, which mechanism can be predominant in persulfidation, considering that the sulfhydrome represents a significant fraction within the cellular proteome? Thus, the main question is: what is the biological role of 'urmylation' - persulfidation and/or conjugation to protein and what are the biological consequences of Urm1 attachment to the target protein? To sum up, the Author provided an elegant mechanistic insight into the *in vitro* 'urmylation' phenomenon, keeping an eye on the process on the atomic scale, showing that the Urm1-SH can transfer the sulfur atom from the C-termini to the cysteine side chain of the target protein, and at the same time being linked to the target proteins by the unique E2/E3-independent mechanism. The work was also very positively accepted by research community, being published in respected scientific the EMBO journal.

Concluding, Keerthiraju Ethiraju Ravichandran has approached the metabolic cascade, that is responsible for modification of the uridine base at the wobble position on the tRNA, but the project was focused on different biological aspect of the pathway and devoted to resolving the so-called 'urmylation' phenomenon. First of all, by solving the Uba2/Urm1 structure, the Author defined the mechanism of Urm1 thiocarboxylation at its C-terminus, dissecting the reaction at the atomic level and providing at the same time a universal metabolic scheme for other UBL systems; secondly, he build a detailed mechanistic model showing the conjugation reaction of Urm1-SH to the target protein in a nano-scale, explaining the dual-functionality of the protein as a Sulfur Carrier Protein and also as a Ubiquitin-Like Protein, laying the foundation for the mechanistic understanding of the post-translational modification of the proteins by Urm1. It should be emphasized that the Author showed remarkable experimental expertise; he applied and used successfully numerous biochemical methods at the protein-engineering level and especially the structural analyses based on protein crystallography and structure determination.

Final conclusion

The thesis presents high quality research data, where the scientific problem was clearly defined and successfully resolved. Through numerous biochemical and structural analyses, the Author provides a set of experimental evidence exhaustively characterizing the metabolic pathway related to the Urm1-dependent post-translational modification, solving the issue on the atomic scale. The thesis is well written, showing that the Author has high capability and research skills. The information provided in the introduction and discussion sections indicates that the Author has profound knowledge and is able to interpret the data and build a general

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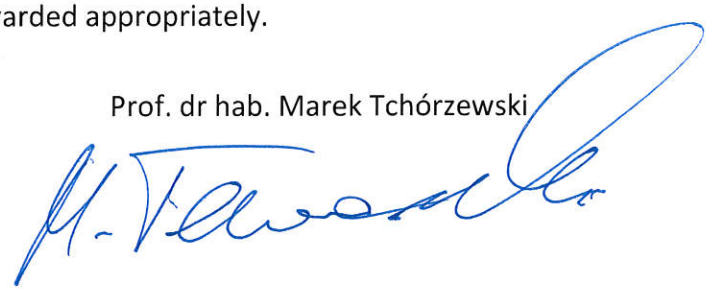
biological picture in a very professional way, taking us beyond the current state of the art, at the same time formulating his own view.

The doctoral dissertation meets the conditions specified in Art. 187 of the Act of July 20, 2018 on academic degrees and titles in science and arts (Journal of Laws 2018, item 1668 as amended). Therefore, I recommend that the Biological Sciences Discipline Council of Jagiellonian University in Kraków admit Keerthiraju Ethiraju Ravichandran for the subsequent stages of the doctoral proceedings.

Concurrently, given the exceptionally high quality of the research and generated knowledge taking us beyond the current state of the art and providing a new view on mechanisms of the post-translational modification of proteins, I recommend that the research effort made by the doctoral student should be awarded appropriately.

Lublin 24.03.2023

Prof. dr hab. Marek Tchórzewski



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