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Opinion on the PhD thesis of MSc Elżbieta Wątor

The doctoral thesis entitled “Molecular basis of hypusination” by Elżbieta Wątor has a hybrid format of a short descriptive part combined with a set of three manuscripts. Elżbieta Wątor is a first author of all attached articles and her contribution to their preparation was most substantial (60% according to the attached statements). Two out of the three manuscripts are already published in Biomolecules (IF 5.5) and Nature Communications (IF 16.6). The third manuscript has been submitted and will hopefully also be published in the near future. The descriptive part of the thesis is compact but contains all key elements of a scientific report (introduction, aims, results and discussion). Materials and methods are extensively described in the attached articles.

The experimental results presented in the PhD thesis of Elżbieta Wątor are highly impressive and according to the contribution statements she was co-responsible for the writing of all three attached manuscripts. The descriptive part of the thesis introduces the attached papers in a very proficient and comprehensive way. Both parts indicate thorough knowledge of Elżbieta Wątor in the field of eIF5A hypusination and her competence in the techniques used to study the subject of her thesis. The discussion of the presented results confirms her scientific maturity and the ability to put own work into broader perspective.

The thesis presents a detailed study of a rare but important posttranslational modification, namely hypusination of a lysine residue. The modification is introduced exclusively into a specific loop of eukaryotic translation factor (eIF5A). eIF5A was first described as an initiation factor, but it aids the process of translation at a few levels. Hypusinated lysine (K50 in case of the human eIF5A) is required e.g. for the shift of tRNA molecules from peptidyl (P) to the aminoacyl (A) site of the ribosome. It is particularly important for the polyproline stretches that are difficult to translate and may result in ribosome stalling.

The hypusination is introduced by two enzymes, namely deoxyhypusine synthase (DHS) and hydroxylase (DOHH). The first of the two enzymes transfers the 4-aminobutyl group from spermidine (SPD) onto the target lysine residue of eIF5A to form deoxyhypusine and diaminopropane (DAP) as a side product. The reaction requires NAD^+ as a cofactor to activate the substrate via oxidation (introduction of an imine bond) and to stabilize the modified lysine of eIF5A through reduction of the

imine bond. The second enzyme, deoxyhypusine hydroxylase, oxidizes the modification to its final hypusine form. The hypusination pathway is highly conserved in *Eukaryotes* and *Archaea*, but DOHH may be absent is some species and thus it is claimed that the introduction of deoxyhypusine may be sufficient to activate eIF5A in such cases.

The aberrations of the eIF5A hypusination were reported to have pronounced effects on eukaryotic development. Mutations in proteins responsible for the introduction of the modification result predominantly in neurodevelopmental disorders, but the perturbation of the process was also implicated in cancer, stroke and diabetes. According to the thesis the hypusination pathway could thus serve as a target for clinical chemistry. The additional subject of the thesis, namely the enzymes of the hypusination pathway of the parasite, *Trichomonas vaginalis*, responsible for the most common non-viral sexually transmitted infection, were together with tvIF5A suggested as novel drug targets, aimed to combat mounting resistance to the currently used metronidazole and related compounds.

The PhD thesis of MSc Elżbieta Wątor is focused on the detailed characterization of the enzymes introducing the hypusination modification from human and *T. vaginalis*. Elżbieta Wątor has biochemically, biophysically and structurally analyzed human and protozoan DHS. Both wild-type and numerous variants affecting substrate binding and activity were characterized. Structural studies were conducted with the help of X-ray crystallography (MX) and cryo-electron microscopy (cryoEM) and the results were validated in solution with hydrogen-deuterium exchange coupled mass spectrometry (HDXMS). The biophysical characterization was performed predominantly with the aid of microscale thermophoresis, multiangle light scattering, and Foerster resonance energy transfer. The assays of the enzyme activity and catalytic capacity were performed with single turnover fluorescence assay monitoring the redox state of the cofactor and western blot detecting the presence of deoxy(hypusination) with the help of specific antibodies. The experiments are accompanied by extensive positive and negative controls and are very clearly documented.

The most impressive result of the thesis is the elucidation of the structure of human eIF5A-DHS complex by cryo-electron microscopy and of the reaction intermediate with X-ray crystallography. The cryoEM structure confirms the unusual stoichiometry of the complex, anticipated based on the earlier biophysical experiments, with a single eIF5A molecule binding a tetramer of DHS. Intriguingly, all four DHS active sites seem to be catalytically competent. Moreover, apart from a small tilt of the substrate binding subunits, the quaternary structure of the complex does not undergo any substantial reorganization. This provokes the question of the rationale behind the oligomeric state of the complex. Is it just an evolutionary relict or does the tetramer have any catalytic function? The tight DHS dimer should be sufficient to perform the catalysis even in the view of the active sites composed of the residues from two protomers. The tetramer seems to be unnecessary, yet it required for enzymatic activity, as evidenced by the results of a deletion of the N-terminal ball-and-chain fragment holding the two dimers together.

My only minor comment to this otherwise perfect and comprehensive dissertation is related to the presentation of the catalytic mechanism of DHS. In a thesis dedicated to the detailed elucidation of the molecular mechanism of enzymatic activity, I would love to see a chemical scheme of the proposed reaction, including expected charged forms of its substrates and products, and the electron flow

arrows. The optimal pH of the reaction is very basic (9.3). Is this pH physiological? How is it assured in the cellular environment? What is the expected protonation state of the reaction components in the active site in view of the new set of relatively high resolution crystal structures? Are the local pH perturbations in the protein active site expected to affect the protonation of the components?

The part of the reaction that I would be particularly interested to see in more detail comprises the transfer of the 4-aminobutyl moiety from the substrate spermidine to the target lysine of eIF5A. The reaction is believed to occur in a few steps. The substrate is first activated by oxidation with the help of a NAD cofactor. The modification is then transferred to the lysine residue of the enzyme (K329 in human DHS), and then to the lysine of the substrate. Finally, the imino group is reduced and the deoxyhypusine modification gets stabilized. Elżbieta Wątor with her newly determined structure of the DHS-EIF5A complex is in an ideal position to discuss the relative locations of the reaction components in the active site. I would expect that the temporarily modified lysine of the enzyme has to be located away from the NADH cofactor so that the intermediate does not get reduced and stabilized. The lysine of the substrate should in turn be optimally positioned for the reduction of the imine bond. Elżbieta Wątor in her thesis suggests that this is assured by the conformational changes of Trp327. However, she also hypothesizes that the reaction could in principle occur simultaneously at two active sites on the opposite sides of the tetramer. Is it plausible that the tetrameric form of the enzyme is necessary for the oscillation of the active sites between forms that would favor modification handover between the two lysines and prevent the back reaction?

The thesis includes extensive study of DHS mutations that are linked to neurodevelopmental disorders. Interestingly, the medical condition is reported to be very rare, and one of the two mutations is always observed in the patient samples. I was puzzled by the fact, that despite the importance of the process for the eukaryotic development, the diseases connected with it are so rare. In view of the homozygous deletion of DHS encoding gene in mice being embryonically lethal, would it imply that all other mutations are prevented because of the lethality? I understand that the presence of the N173S mutation ameliorates phenotypic consequences that are otherwise too severe to be recorded, but why does it have to accompany the other clinically relevant variants? What is the frequency of the variants in the population? Is a single copy of functional DHS sufficient to prevent developmental disorders?

The thesis emphasizes the potential therapeutic use of the inhibitors of the human and in particular *T. vaginalis* enzyme and reports the ongoing high-throughput fragment screening. If this is not under potential patent restriction, I would be interested in a more detailed comparison of the human and *T. vaginalis* enzymes and the discussion of how the candidate structural differences between the two enzymes could be exploited for specific drug design purposes. The results of this part of the thesis indicate that the hypotheses of DHS from *T. vaginalis* having double deoxyhypusination and hydroxylation activity are very likely wrong. This result, together with the absence of the DOHH candidate in this species and also the sufficiency of eIF5A deoxyhypusination for its activity reported for some other species suggests that this modification might also be the final one in *T. vaginalis*. This provokes the question on how the binding sites for the modified eIF5A on the ribosome compare between this species and higher eukaryotes. Is there a difference in the hydroxyl group binding location? Could this difference be exploited for clinical applications?

The structural biology studies presented in the thesis have been performed in a very precise and detailed way. I have only one question related to the methodological part of the work. The diffraction data for deoxyhypusinated DHS has been collected at ambient temperature. Why was it necessary? Is the intermediate sensitive to flash cryocooling? I would expect that it should rather be affected by radiation damage that is expected to be more extensive in the absence of the liquid nitrogen protection. I was also impressed by the lack of difference in the data collection statistic and average B factor of the RT structure in comparison to the other ones collected presumably at 100 K. Why is the change in data collection protocol not reflected in data collection and refinement parameters?

Despite my very minor concerns I find the work of Elżbieta Wątor very impressive. Apart from the articles forming the basis of the thesis she has substantial further publication record. She is a co-author of three other articles published in Genetics and Molecular Biology (IF 2.1), Biochimie (IF 4.1) and Int. J. Mol. Sci. (IF 5.6), a first author of a FEBS Letters publication (IF 3.5) and a co-author of a manuscript currently under review in Cell Reports. According to google scholar her work has been cited more than 100 times, which is remarkable at this career stage. Her PhD studies were supported by the grants to young scientists from NCN, NAWA, DAAD and a Scholarship for Women in Science from L'Oreal. Very few students can be proud of being awarded by so many funding bodies.

In summary, in my opinion the doctoral dissertation of MSc Elżbieta Wątor fulfills all statutory requirements contained in Art. 187 of the Act of July 20, 2018 (Law on higher education and science (Journal of Laws of 2023, item 742, as amended), and qualifies for the next stages of the procedure for awarding a doctoral degree. Therefore, I recommend to the Scientific Council of Jagiellonian University to accept the reviewed doctoral dissertation and to proceed with further steps necessary for awarding Elżbieta Wątor a doctoral degree, including public defense. Due to the outstanding quality of the presented thesis, I suggest to the Scientific Council to award it with a distinction.

With kind regards,

Dr hab. Honorata Czapinska