PhD Thesis Review
Biotechnology Discipline Council, Intercollegiate Faculty of Biotechnology UG&MUG

Candidate's name and surname: **Piotr Purzycki**

PhD Thesis Title: ***Structural basis for functional cooperation between proteins involved in the processing of RNA primers during mitochondrial DNA replication***

Thesis Supervisor: dr hab. Michał R. Szymański, prof. UG

Field: Exact and biological sciences

Discipline: Biotechnology

Reviewer: dr hab. Maria Górna, prof. UW

The PhD thesis of Piotr Purzycki regards the molecular machinery responsible for RNA primer removal in human mitochondrial DNA replication, namely RNase H1, Exonuclease G and DNA Polymerase γ. This is a very relevant topic, since the molecular mechanisms in these fundamental processes are still being discovered and determined among the several proposed modes of action. Moreover, mutations in these factors are involved in a number of mitochondrial diseases, and may contribute to further pathologies in neurodegenerative or metabolic disorders. A deeper understanding of mitochondrial DNA replication is therefore also important to enable new therapeutic approaches.

The Dissertation follows a classical format, with main parts such as Introduction, Aims, Materials and methods, Results (Part 1 &2), Discussion etc. Formally, the thesis is very well prepared and carefully illustrated by 39 Figures. I was particularly impressed by how well the Thesis was written – the language and flow are clear and concise, the data are presented with logical order and sufficient citations, and the reasoning of the Candidate is well explained. This is especially felt since the topic of the Thesis is quite complex, involving intricate molecular mechanisms and their alternative proposed models. The fluency of the Candidate in these complex biological pathways of mitochondrial nucleic acids metabolism is evident in the Introduction and Discussion sections. The Candidate's grasp of the appropriately chosen and state-of-the-art methodology can also be seen in the "practical" sections of the Dissertation. Combined with the high level of reasoning shown, undoubtedly the Candidate has demonstrated a substantial skillset and knowledge sufficient to be awarded a Doctoral degree.

The objectives of the Thesis aimed to clarify the cooperation of RNase H1 with EXOG and with Pol γ using purified proteins and model substrates. These included physical association studies using biophysics methods (SPR, BLI, SEC, co-sedimentation). Functional interactions were studied via activity assays. Finally, structural studies aimed to delineate interfaces and complexes formed. These aims were mostly achieved and the Candidate's work provided novel insights into several aspects of mitochondrial biology. Successful purification of protein constructs - full-length proteins, selected domains or point mutants - enabled downstream characterization. For example, the pathogenic RNase H1 mutant A185V (but not V421I) showed decreased protein stability and RNA cleavage activity. The CTD of RNase H1 was found to be chiefly responsible for interactions with EXOG and also with Pol γ. However, crystallization trials and AlphaFold-directed point mutagenesis were unsuccessful in further delineation of the RNase H1-EXOG interface. More luck was to be had with cryo-EM of RNase H1 and Pol γ – here, their complex with a RNA/DNA hybrid was stable enough to be purified by SEC, and the additional density in cryo-EM map suggested where RNase H might be roughly located on Pol γ, though the insufficient resolution prevented atomic model building for this complex. Since obtaining experimental structural models is inherently a risky process, this is at no fault of the Candidate, who demonstrated multiple attempts to optimise both crystallization and cryo-EM trials. The most valuable are the results of the functional assays performed by the Candidate. The combined activity of EXOG and RNase H1 led to efficient removal od residual RNA from a hybrid substrate. The Candidate's work also demonstrated interplay of the RNase H1 and Pol γ: gap-filling by Pol γ was able to stimulate RNase H1 activity to excise full RNA content from the shared hybrid substrate. Conversely, catalytically inactive RNase H1 was shown to stall Pol γ by presenting a physical obstacle. The Candidate suggests that these two alternative pathways of RNA primer removal might be complementary – when less than

4 nt RNA is present in the hybrid, RNase H1-Pol γ cannot engage productively, but RNase H1-EXOG can still act on it. In summary, the Candidate has shown two alternative partners of RNase H1 that can provide different pathways of RNA primer removal. These results contribute novel insights into the mechanisms of DNA replication termination, and expand our knowledge of mitochondrial biology.

A minor comment (a pet peeve of this Reviewer) – the fraction numbers in Figure 35B and in the text should be replaced by the retention volume instead, as no reference to the start or volume of fractions is indicated anywhere and the numbers remain largely uninformative.

A few questions come to mind that could be addressed by the Candidate during the defence:

1. Perhaps the weakest part of the Dissertation, but fortunately only a very minor part of it, are some of the results of CD spectroscopy. For example, Figure 11E shows melting curves that have too much noise, yet the Candidate draws conclusions of an over 10°C difference in T_m of the two constructs (HBD and HBD-linker). It would be good to show the fitting of the model used to calculate T_m here, but in general it would be even better to use a more concentrated sample giving a stronger signal, and to perform replicates of the measurement to obtain experimental errors. Some of the current conclusions from the CD data should be softened and rephrased.
2. Given the large experimental errors of SPR measurements in Figure 14, the binding constants of RNase H1, CTD and CTD-linker might be regarded as basically the same, and the rest also needs to be taken with caution (error sizes on par with parameter values). How does the Candidate address the inconsistent response size – for example, the largest for EXOG-CTD but the smallest for EXOG-CTD-linker?
3. At times, the Candidate refers to RNase H1-EXOG are "stably interacting". SEC purification of a stable complex could be more conducive to crystallization. Has the Candidate attempted SEC purification of the RNase H1-EXOG complex?
4. Has the Candidate followed up on RNase H1-EXOG prediction with the newer AlphaFold3 tool - would it predict a different interface or recapitulate the AlphaFold2/Colab models?
5. BLI assays of RNase H1 and Pol γ sequential binding to the hybrid substrate lack one-protein-only controls. Has the Candidate considered whether (as opposed to a ternary complex formation) RNase H1 and Pol γ could bind independently to the unsaturated substrate?
6. The BLI results would benefit from more replicates and fitting of the kinetic parameters. Has the equipment or material availability limited the Candidate's work in this aspect?
7. Has the Candidate tried masked refinement of the Complex I from CryoEM of RNase H1-Pol γ?
8. Having the two partners at hand (EXOG and Pol γ) that both could interact with CTD of RNase H1 – has the Candidate tested if their interactions were mutually exclusive or permissive?

I, hereby, declare that the reviewed PhD thesis by Piotr Purzycki meets the criteria pursuant to art. 187 of Act of 20 July 2018 The Law on Higher Education and Science (Journal of Laws of 2018, item 1668, as amended) and request that the Biotechnology Discipline Council of the Intercollegiate Faculty of Biotechnology UG&MUG accepts Piotr Purzycki for further stages of doctoral proceedings in the field of exact and biological sciences, in the discipline of biotechnology.

Ja, niżej podpisana stwierdzam, że recenzowana rozprawa doktorska Piotra Purzyckiego spełnia warunki określone w art. 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2018 r. poz. 1668 z późn. zm.) i wnioskuję do Rady Dyscypliny Biotechnologia Międzyuczelnianego Wydziału Biotechnologii UG i GUMed o dopuszczenie Piotra Purzyckiego do dalszych etapów postępowania ws. nadania stopnia doktora w dziedzinie nauk ścisłych i przyrodniczych w dyscyplinie biotechnologia.

06.02.2026

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Reviewer's signature