INSTITUTE OF BIOORGANIC CHEMISTRY
Polish Academy of Sciences

Noskowskiego 12/14, 61-704 Poznań
tel.: +48 61 852 85 03, secretariat +48 61 852 89 19
fax: +48 61 852 05 32, e-mail: ibch@ibch.poznan.pl
REGON 000849327 VAT no. PL 7770002062
http://www.ibch.poznan.pl

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Dr Habil. Krzysztof Brzeziński, Prof. IBCH PAS
Department of Structural Biology of Prokaryotic Organisms
Institute of Bioorganic Chemistry, Polish Academy of Sciences
e-mail: kbrzezinski@ibch.poznan.pl

Review report on the Doctoral thesis of MSc Piotr Purzycki
entitled
"Structural basis for functional cooperation between proteins involved in the processing of RNA primers during mitochondrial DNA replication"

The dissertation of Piotr Purzycki was prepared under the supervision of Professor Michał Szymański in the Laboratory of Structural Biology, Intercollegiate Faculty of Biotechnology UG and MUG. The research was supported by several external sources, including grants from EMBO and the National Science Centre, Poland, led by the Ph.D. candidate's promoter.

The dissertation investigates mitochondrial DNA (mtDNA) replication. This process differs from nuclear DNA replication and depends on dedicated mitochondrial machinery comprising ribonuclease H1 (RNase H1), 5′ exonuclease G (EXOG), and DNA polymerase gamma (Pol γ). High-fidelity mtDNA replication requires efficient removal of RNA primers to generate ligatable DNA ends, which in turn relies on coordinated action among these three proteins. RNase H1 initiates RNA primer degradation but cannot remove the final one to three ribonucleotides at the RNA–DNA junction, so an orchestrated transfer of the substrate to partner enzymes is required. EXOG then removes these remaining ribonucleotides to complete primer processing. The replicative polymerase Pol γ is also involved in mtDNA replication and interacts with RNase H1; however, the mechanistic significance of this interaction remains unclear. While recent studies have revealed functional interactions among human RNase H1, EXOG, and Pol γ, the detailed mechanism of RNA primer removal in human mitochondria remains incompletely defined. To address this gap, this dissertation characterizes the interplay between RNase H1 and EXOG during terminal primer processing and examines their cooperation with Pol γ during mtDNA replication. Achieving these goals required applying numerous methods at the intersection of molecular and structural biology, biochemistry, and

biophysics, including surface plasmon resonance and biolayer interferometry, AlphaFold-based structural modeling, X-ray crystallography, and cryo-electron microscopy (cryo-EM).

The thesis is divided into several standard parts, including an abstract, a literature review of the topic, aims of the research, a description of the materials and methods applied, and results divided into two parts, each with a summary and a general discussion section. The thesis also contains the candidate's achievements and skills acquired during doctoral studies. The purpose of the work is formulated very clearly and straightforwardly. The literature review is well supported by numerous references from recognized journals and includes both foundational and recent studies. In conclusion, the introduction adequately summarizes the current state of knowledge on the research objects. The approach to setting the main goals, as well as the description of the results and discussion of the work, do not raise my reservations. They prove the Ph.D. student's excellent substantive preparation for scientific work. In my opinion, the most significant achievements include:

1. Characterization of the functional partnership between RNase H1 and EXOG. Surface plasmon resonance mapped the dominant binding interface to the catalytic domain of RNase H1. Biochemical reconstitution demonstrated that this interaction is required for activity, as only the combined action of RNase H1 and EXOG enabled complete *in vitro* excision of RNA primers, whereas neither enzyme alone was sufficient. Notably, EXOG partially restored the deficient activity of a disease-associated RNase H1 variant, underscoring potential clinical relevance. Structurally, crystallization trials were complemented with AlphaFold2 Multimer modelling; although crystallography did not capture the complex, the model pinpointed interfacial residues that were validated by site-directed mutagenesis and biochemical assays.
2. Investigation of the interaction between RNase H1 and Pol γ. Surface plasmon resonance measurements revealed a direct association driven by the RNase H1 catalytic domain. The study showed that Pol γ–mediated DNA synthesis enhanced RNase H1 activity, enabling complete primer removal, whereas a catalytically inactive RNase H1 mutant impeded Pol γ during gap-filling synthesis, consistent with a steric "roadblock" effect. Structurally, cryo-electron microscopy showed substantial conformational heterogeneity, preventing high-resolution localization of RNase H1. To overcome this limitation, AlphaFold3 modelling produced a structurally plausible assembly that aligns with the biochemical evidence and suggests a spatial arrangement that could facilitate coordination during mitochondrial DNA replication termination.

Considering the above, I rate the whole work very highly, which does not change the fact that, as a reviewer of the thesis, I have a few minor comments and a question, which are listed below:

1. From an editorial point of view, the thesis is well organized and well written, and is supplemented with clear, high-quality pictures. A concern is the discrepancy in the number and types of protein variants between the Materials & methods and Results sections. In particular, it corresponds to EXOG-H140G used in crystallization trials (section 6.9) and discussed in the Result section, Pol γA(exo -) used for cryo-EM sample preparation (section 6.15) and discussed in the result section 7.2.1, and RNase H1-D210N used in biochemical and biophysical assays (e.g., section 6.11 and 6.12) and discussed in the result section (e.g., section 7.1.1). However, information on the expression and purification of these variants is elusive. Thus, their source and quality remain unknown.
2. I have noticed that all protein samples used in crystallization trials (section 6.9) were prepared with buffers containing 5% glycerol. A probable reason for that was protein stabilization. On the other hand, glycerol is highly hygroscopic and may affect vapor diffusion if not added simultaneously to the reservoir solution, especially when the protein-to-reservoir ratio is greater than 1. Consequently, a protein concentration in a crystallization drop may be suboptimal, leading to a failed crystallization. Could the candidate comment on this issue?
3. The candidate identified a few crystallization conditions that yielded microcrystals. Has the candidate tried the seeding technique for optimization? Following that, did the candidate try other methods to optimize the crystallization?
4. The candidate used a glycerol-free protein solution for Cryo-EM sample preparations. After glycerol removal, the samples were flash-frozen, then thawed and applied to cryo-EM grids. Numerous proteins and their complexes require the addition of glycerol before freezing, as its absence can lead to denaturation. Therefore, the question arises whether the sample was affected by flash-freezing. Could the candidate comment on this issue?

Despite some minor remarks and comments, I have a favorable opinion of the doctoral thesis of the Ph.D. candidate. The studies performed and presented by Piotr Purzycki are ambitious. In addition, they required the doctoral student's extraordinary skills. Therefore, I hereby declare that the work submitted for assessment meets all the requirements for the doctoral thesis. Thus, I request that the Scientific Council of Biotechnology of the

Intercollegiate Faculty UG and MUG accept Piotr Purzycki for the next steps of the Ph.D. procedure.

The presented research is of high quality. The candidate applied numerous state-of-the-art methods from the intersection of biochemistry, biophysics, molecular and structural biology. It is worth noting that the candidate addressed the problems that arose during the research, identified potential failure causes, and proposed alternative methods to address them. In particular, it relates to structural studies, where experimental methods provided no definitive answers and *in silico* methods must be applied. Notably, the latter results were subjected to scientific criticism, as the candidate decided to validate them experimentally. Additionally, the thesis was written with great care, which greatly facilitated its assessment. Considering the above, I recommend that the Scientific Council of the Intercollegiate Faculty of Biotechnology award this thesis.

Krzysztof Brzeziński