Subject PhD thesis Piotr Purzycki
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To the Intercollegiate Faculty of Biotechnology UG&MUG

With great interest I have read the doctoral dissertation by PhD candidate Piotr Purzycki entitled **'Structural basis for functional cooperation between proteins involved in the processing of RNA primers during mitochondrial DNA replication'.** The subject of this dissertation deals with proteins and mechanisms involved with some of the final stages of mitochondrial DNA (mtDNA) replication. Mitochondria in most eukaryotes, including in humans, are essential for life as they provide most of the cellular energy via the process of oxidative phosphorylation. As a remnant of their endosymbiotic origin, mitochondria still contain their own genome, so called mtDNA. MtDNA is small but nonetheless codes for 13 protein subunits of 4 of the 5 oxidative phosphorylation complexes. Its maintenance (replication and repair) and expression therefore are absolutely essential for cellular and organismal function and survival. This has become clear by the occurrence of many, and often very severe, mitochondrial disorders, either involving direct mtDNA mutations or involving proteins involved in mtDNA maintenance and expression. One of the essential processes of mtDNA maintenance is the copying or replication of this genome. All cells in the human body, except for red blood cells, contain hundreds to thousands of copies of mtDNA. Mitochondria use dedicated proteins, coded for by the nuclear genome, to continuously make these copies. For example, there is the exclusively mitochondrial DNA polymerase (Pol gamma) that is the enzyme that makes the actual copies and a similar exclusive replicative DNA helicase called Twinkle. Both enzymes and several others have been identified as proteins/genes involved in a variety of human diseases when found mutated in patients. For polymerase gamma alone more than 300 different disease mutations have been identified in many patients around the world. To understand how mutations in the various replication proteins affect the mtDNA copying process, researchers try to understand the working of these proteins. On the one hand by obtaining detailed structural information, preferably at atomic resolution, and on the other hand by studying the broader mechanisms of the copying process. Surprisingly, even though mtDNA is tiny compared to the nuclear genome and the number of proteins believed to be involved in the copying process is also quite small, there still is considerable controversy regarding the mechanisms of the copying process. In addition, even if we know that some steps in the process must occur, we do not know the mechanistic details of how they occur and/or which proteins are involved. It is one of these steps that this thesis is particularly concerned with. Almost all DNA replication processes need an initiation event that involves the synthesis of a, usually short, stretch of RNA (not DNA). This stretch of RNA, called the RNA primer, annealed to the DNA molecule that needs to be copied, serves as a docking point for the DNA polymerase that can start the copying process from this point onwards. However, at the end of the copying process, the small stretch of RNA needs to be replaced by DNA to make a complete DNA copy. If this process fails it will result in incomplete, and more importantly unstable, copies of mtDNA that will be prone to mutation and/or degradation, thus emphasizing the importance of this process and its study. This thesis has studied both the proteins and mechanisms by which the final steps of mtDNA replication (by removal of the RNA primers) take place and has provided important insights in this process.

The dissertation presented by the candidate starts with a comprehensive and well written overview of the mechanisms and proteins involved in mtDNA maintenance, demonstrating detailed knowledge of the current state of the research field including its controversies and open questions/gaps that still need to be resolved. The main subject of the thesis, which concerns the termination process, is often underrepresented or overlooked in reviews dealing with mtDNA copying and the candidate has in a very insightful manner presented this subject in the introduction.

Following an overview of the methods used to address the research questions, the main body of the thesis are two studies relating to primer removal and termination of mtDNA replication. In the first study the candidate has examined the potential interaction between two proteins believed to be involved in the RNA primer removal (being RNaseH1 and EXOG) and providing detailed mechanistic insight how they achieve this. With the second study the candidate has assessed the interaction between RNaseH1 and the mtDNA polymerase during RNA primer removal. Both studies have clearly been a substantive amount of work with which the candidate has shown the ability to conduct a comprehensive scientific study in the field of molecular and structural biology. With both studies the candidate has shown the capability to apply a breadth of different methods to address the questions at hand, to discuss the outcomes in a comprehensive manner and to adapt methodology based on the obtained results, whether positive or negative.

As with any well conducted study, the results should also raise new questions, while also methodological questions can be raised. I look forward discussing a number of issues with the candidate. To start with methodological questions, I would be curious regarding some of the choices that were made to purify protein complexes for structural studies using cryo-EM. For example, for RNaseH1 and EXOG the candidate has chosen to isolate a complex of the two proteins without a DNA substrate while for the RNaseH1-POLG structural study it was chosen to assemble on a substrate. Additional questions concern the use of further purification steps, even though pure and isolated proteins and substrates were used to assemble complexes. Second, I would be interested to discuss the use of Alphafold to predict protein-protein interactions in heterologous complexes, and the question how robust these predictions are and how Alphafold trained the algorithm for this particular purpose. Have they shown that it can with high confidence predict known interactions? And, what is the success rates of these predictions? In relation to the more mechanistic aspects of the experiments, particularly those that address the interaction and activities of RNaseH1 and POLG on gapped substrates it will be interesting to discuss the possible influence of POLG strand-displacement synthesis on RNaseH1 activity on any remaining ribonucleotides. Also, the effects of gap-length on interaction of both proteins will be an interesting topic for discussion, as well as possible reaction monitoring with one-to-three NTPs instead off none or all four. Finally, it will be interesting to discuss the actual order of events during the replication cycle in mitochondria and the possible redundancy between the POLG-RNaseHI and EXOG-RNaseHI interaction and mechanisms in primer removal. In actual *in vivo* mtDNA replication, it will take considerable time before a POLG-RNaseHI driven mechanism is required for primer removal at termination, as the replication fork rate in mitochondria is believed to be very slow. This will allow an EXOG-RNaseH1 module to process the primer, long before POLG reaches this junction, which could imply that the EXOG-RNaseHI is the main primer processing module, while the POLG-RNaseHI module is a fail-safe in case EXOG-RNaseHI action was incomplete or failed altogether.

To conclude, the results presented in this thesis provide important and novel insight into the mechanism of RNA primer removal and termination of mtDNA replication, and the possible interplay between different protein factors involved. It therefore represents a body of work of high academic standards and therefore the doctoral dissertation meets the requirements set for doctoral dissertations by The Higher Education and Science Act dated 20 July 2018 (Polish Journal od Laws od 2018 item 1668, as amended). Therefore, I am applying to the Council of the Biotechnology Discipline for admission of Piotr Purzycki to further stages of the doctoral procedure.

Yours sincerely,

Hans Spelbrink