Review of the doctoral thesis of Ms. Sreedevi Sugunan „Localized translation of nuclear-encoded mitochondrial proteins in zebrafish" conducted under the supervision of professor Agnieszka Chacińska and Dr Cecilia Lanny Winata at the Laboratory of Mitochondrial Biogenesis, Centre of New Technologies, University of Warsaw and the Laboratory of Zebrafish Developmental Genomics, International Institute of Molecular and Cell Biology, respectively.

Although gene expression begins with transcription, there are a variety of mechanisms that cells use to control and tune gene expression post-transcriptionally. Following the transcription the mature mRNA transcripts are transported to cytoplasm for translation on cytosolic ribosomes and then carried post-translationally to the organelles and compartments. However, the synthesis of proteins has also been shown to be localized to specific compartments within the cell, usually close to where the encoded protein is active. Synthesizing a protein at its action site has significant advantages, including the lower probability of malfunctioning at other cellular sites and favorable energy costs of protein transport. It is widely accepted that import of precursor proteins generally occurs in a post-translational manner also in the mitochondria. For some mitochondrial proteins, however, there are clear indications for a co-translational import mechanism what prompts the question about the extent of mitochondrial proteins co-translational synthesis on the mitochondrial surface. This important scientific question was so far not addressed in higher eukaryotic organisms. The gap in this knowledge, in my opinion, is filled comprehensively by the work of Ms. Sreedevi Sugunan supervised jointly by Professor Agnieszka Chacińska and Dr Cecilia Lanny Winata.

The thesis layout is typical and includes the required elements of introduction, description of research material and methods, presentation of results and their discussion. Apart from the table of contents and references, the dissertation consists of 111 typescript pages in total.

In the introduction, the opening subchapter gives a general overview of mitochondrial protein biogenesis and the mechanism of protein import with the emphasis on MIA pathway which is being altered in the model zebrafish organism used in the dissertation. In the second subchapter, the comprehensive and up-to-date description of mitochondrial proteins import modalities are provided highlighting the dominant role of post-translational mode. It is followed by a smooth transition to the description of the co-translational mode of mitochondrial protein import. The possible mediators of the latter mode are mentioned and, as noted, the exact mechanism of localized translation at mitochondria surface remains elusive what gives the rationale to further explore this scientific question. In the third

subchapter the role and possible functions of RNA localization are described. These are supported with the appropriate biological examples of the cell types with a high protein demand at a given compartment, and the advantages of a localized translation, including energy consumed for protein transportation or protein misfolding prevention, to name few.

The subchapter also familiarizes the reader with the state-of-art methods used to study the RNA localization with the emphasis of mitochondria focused approaches including MS2-MCP technique or sequencing based approaches, for which the advantages and weaknesses are highlighted. In the fourth subchapter the mitochondria interaction with other cellular compartments is exhaustively presented underscoring the importance of their physical link with endoplasmic reticulum and cross-talk with the nucleus. The final subchapter provides the overview on the zebrafish model organism, its advantages for studying gene functions and phenotypes following gene knockdowns. Then, the description of mia40 deficient zebrafish model is introduced, which constitutes the model with an altered post-translational mitochondrial proteins import. The aim of the thesis is clearly articulated. To sum up, the introduction to the research topic and the definition of the scientific aim has been described in an exhaustive manner. The extent of individual subchapters of the introduction are appropriate, without unnecessary going beyond the scope of the work. Additionally, I find the artwork on the figures in the introduction chapter as eye-catching and very informative.

The materials and methods chapter is very well written. It contains sufficient descriptions of materials used, zebrafish maintenance and harvesting as well as the genome editing protocols. The details on biochemical experiments and nucleic acid isolations, and processing are appropriate and easy to follow. The mRNA enrichment methods are described thoroughly given the impact of the enrichment method on the transcriptome composition. The RNA-Seq data and functional analyses are presented sufficiently and the invaluable contribution of Dr Uszczynska-Ratajczak to the RNA-Seq data analyses is acknowledged. There were few typos, all I found were in the microliter abbreviation (ul instead µl), but these can be disregarded as unimportant.

The results chapter starts with a description of experimental conditions optimization which ultimately allowed for efficient sub-fractionation into endoplasmic reticulum (ER), mitochondria with associated ribosomes (MARS) and deprived of ribosomes (mito) of zebrafish larvae for downstream analyses. The fractionation efficacy was subsequently evaluated using immunostaining for the respective subfraction markers as well as with RT-qPCR for the presence of associated transcripts. Interestingly, the intervention with translation inhibitors, including puromycin and hydroxylamine, did not

substantially removed the ribosomes from mitochondria surface, what was interpreted that ribosome-mitochondria bond is not entirely dependent on nascent polypeptide interaction with the mitochondria surface. In the following experiments the attempt to isolate MARS fraction and survey associated RNAs from early-stage zebrafish embryos were made but the yield of protein, despite using different magnesium concentrations, was unsatisfactory. **Could the yolk presence interfere with the MARS purification?** In further technical considerations, the 1mM and 5mM magnesium concentrations were tested to find that the latter one better preserves RNA isolated from MARS what was next tested using electrophoresis and through the respective libraries sequencing. **Why these two extreme concentrations were used instead of an incremental testing from this range? How the optimal concentration corresponds to MARS isolates in other organismal models?** In the fourth subchapter of the results section it is convincingly presented that Poly(A) enrichment is superior over ribodepletion approach in the studied model organism. **It would be interesting to see the break on Figure 19B into the fractions and how the samples differ in RNA types (miRNA, snoRNA, mRNA, lncRNA) composition.** I appreciate the thorough scrutinization of RNA enrichment for the zebrafish model since depending on the sample the RNA sample enrichment/depletion protocol may result in significant variations in gene expression profiles.

Following the optimization part of the results section the actual data is presented. It starts with showing that the Mia40 mutant larvae mitochondria, which have altered proteins' import, are decorated with MARS what later enabled direct comparisons of mitochondria associated RNAs to its wildtype counterpart. It is worth mentioning at this point that all respective pair-wise comparisons and resulting differentially expressed transcripts were also validated in RT-qPCR on independent biological replicates further assuring data reproducibility. The transcriptome comparisons were made for the ER and MARS fraction separately against the intact larvae (the whole) using the adj. p. value threshold < 0.05 and two abundance criteria, namely high and moderate enriched. The comparison showed a relatively high number of transcripts differentiating the fractions from the whole also for the Mia40 mutant, irrespectively of the fold change threshold. **In this chapter the referencing to the genes instead transcripts should be avoided since the latter ones are investigated, this exchange is misleading. I wonder if there were the instances of RNAs that were not detected in the whole but present in fractions?** Next, by focusing on the mitochondrial encoded transcripts in the RNA-Seq dataset and by performing subsequent RT-qPCR validation, it was found, contrary to the expectations, that MARS fraction in Mia40 mutants is not enriched in these transcripts. At the same time some of them were

elevated, when compared to WT, although the differences were not exceeding 1.5 fold. **How the presence of these transcripts outside of the mitochondria could be explained based on the previous findings presented in the literature?** To narrow down the analyses to the nuclear-encoded mitochondrial proteins, the differentially enriched transcripts were intersected with the MitoCarta2.0 database that comprises genes encoding proteins with the strong evidence of the mitochondrial localization. This analysis revealed that the genuine nuclear-encoded mitochondrial transcripts, accounted for 12% of all MARS enriched in WT, while in the Mia40 mutant the fraction was 24%, indicating a distinct increase in nuclear-encoded mitochondrial transcripts on mitochondria surface upon MIA pathway dysfunction. **When showing the numbers of differentially genes and intersections it is more compelling to use the Venn diagrams instead of column figures. Furthermore, it would be worth showing the intersection of MARS and ER fraction enriched transcripts in WT and Mia40 mutant samples.** In the large scale transcriptomics studies the functional analyses are used to translate the lists of differentially expressed genes into meaningful biological processes and terms to further generalize the consequences of an experimental intervention. Such analysis performed with the Gene Ontology (GO) for MARS enriched MitoCarta transcripts revealed common and specific GO terms for WT and Mia40 mutant, which were mostly mitochondria related. Similar analyses executed for MARS enriched non-MitoCarta transcripts uncovered overrepresentation of transcripts encoding intermediate filament family proteins. In the last part of the results the successful attempt of CRISPR-Cas9 mediated genome editing was made to generate the zebrafish model with BirA fused to an outer mitochondria membrane protein that would allow performing the proximity-specific ribosome profiling. To conclude, Ms. Sreedevi Sugunan has fully achieved the goals of her work with the presented results. Although, the data are overall descriptive in nature, the transcriptomics profiles provide a solid basis for generating and testing further research hypotheses, which has been skillfully presented with an example of non-MitoCarta transcripts exploration. Additionally, the BirA knock-in model will serve as an invaluable tool for further research in that area.

The discussion is very well written. The first section thoroughly discusses the technical considerations undertaken in the protocols optimization stage of the thesis, acknowledging the limitations of fractionation–sequencing approaches and considering mitigation strategies to improve the fractionation. In the second section, the phenomenon of a strong mitochondria-ribosomes bond is discussed highlighting the unknown, and worth further exploration, mechanistic basis of this association. **In the next section it is stated that there were no common chemical or structural similarities**

between the set of proteins that are presumably produced from mitochondria associated RNAs, however, it is known that the length of poly(A) tails correlates with a relative mRNA stability, and I wonder if it is possible to infer about the poly(A) tails length from the sequencing data to investigate if there is an enrichment of long poly(A) tails among the fractioned RNA to the whole. Next, the observation on mRNA enrichment of mitochondrial surface following MIA pathway deficiency is interpreted as a cellular response to the import deficiency stress. I find this conclusion important and compelling as it is also supported from the functional analyses that pointed an enrichment of proapoptotic transcripts in MARS upon MIA deficiency that ultimately could drive the lethal phenotype in the zebrafish model. To sum up, Ms. Sreedevi Sugunan has comprehensively examined the results against the scientific literature and suggested future directions of research on RNAs localization on the mitochondrial surface . The conclusions presented correspond with the aim.

My final evaluation of Ms. Sreedevi Sugunan's doctoral thesis is very positive. I believe that the dissertation handed to me for evaluation constitutes an interesting, independent, original and extensive scientific study of great value. The work presented is interdisciplinary, as it combines the expertise in the mitochondria proteostasis, the zebrafish development, next-generation sequencing libraries preparations, functional analyses of transcriptomic dataset and genome editing technology. Therefore, I can consider Ms. Sreedevi Sugunan, without the doubt, as an experienced molecular biologist. I hope that she will continue her scientific work with passion. The dissertation meets the requirements set out in art. 13 of the Act on the Academic Degrees and Academic Title, and the Art Degrees and Title as of 14 March 2003 (DZ.U. of 2017, item 1789 as amended). Therefore, I recommend to the Scientific Council of Institute of Biochemistry and Biophysics of the Polish Academy of Sciences allowing Ms. Sreedevi Sugunan advancing to the further stages of her doctoral proceedings.

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