UNIWERSYTET IM. ADAMA MICKIEWICZA W POZNANIU
Wydział Biologii
Instytut Biologii Molekularnej i Biotechnologii

Zofia Szweykowska-Kulińska, Prof. Dr Hab.
Zakład Ekspresji Genów
Department of Gene Expression

Poznań, 29.02.2020

Review of the PhD thesis of MSci Sreedevi Sugunan entitled "Localized translation of nuclear-encoded mitochondrial proteins in zebrafish"

MSci Sreedevi Sugunan carried out her PhD thesis under the supervision of Prof. Dr. Hab. Agnieszka Chacińska and co-supervision of Dr. Cecilia Lanny Winata. PhD thesis was executed in the Laboratory of Zebrafish Developmental Genomics, in the International Institute of Molecular and Cell Biology in Warsaw and in the Laboratory of Mitochondrial Biogenesis, Centre of New Technologies, University of Warsaw. Both, supervisor and co-supervisor have big experience in studying mitochondria biogenesis and zebrafish development.

The main aim of the study was to find out whether in multicellular organisms co-translational import of proteins to mitochondria takes place. Such studies were carried out using yeast and human cells that revealed that indeed translationally active ribosomes are associated with the outer mitochondrial membrane (OM). Although tight coupling of ribosomes to OM, like it is in the case of ER membrane, was not observed, different experiments performed mainly in yeasts suggested co-translational import of at least some proteins to mitochondria. Mrs Sugunan focused on the identification of the repertoire of nuclear-encoded mRNAs localized on the surface of zebrafish mitochondria and on the fate of RNA localization and localized translation when post-translational mitochondrial import pathway is impaired. According to my assessment Mrs Sugunan successfully identified some mRNAs localized at the mitochondrial OM although their representation was sparse suggesting that the main way of protein import to mitochondria takes place posttranslationally at least in the case of 5 dpf zebrafish embryos. She found also some mRNAs that were present only in the MARS (mitochondria with associated ribosomes) fraction. Moreover, the

ul. Umultowska 89, Collegium Biologicum, 61-614 Poznań
NIP 777 00 06 350, REGON 000001293
tel. +48 61 829 59 50
ibmib@amu.edu.pl

impairment of posttranslational import to mitochondria affected mRNA localization on the surface of mitochondria suggesting a compensatory mechanism at the posttranscriptional level to overcome the defects in mitochondrial biogenesis. I think these are novel and important result.

The PhD dissertation is well written. The Aim of the thesis is clear, the Introduction part is very well written introducing the reader to mitochondria protein import pathways, the role of specific cellular RNA localization, methods to study RNA localization and zebrafish as a model organism.

Studying Introduction part I want to ask the Candidate about protein import to mitochondria. At least earlier papers reported the observation of physical connection between TOM and TIM and suggested the role of this connection in targeting proteins containing mitochondrial targeting peptide (mTP) localization signal directly to the mitochondrial matrix. Is it still recognized as a possible pathway to conduct proteins directly by TOM-TIM complex to the mt matrix?

Materials and Methods part is also well and exhaustively written. Concerning CRISPR-Cas technique I did not find information describing how gRNAs were selected and how many off-targets were found in the zebrafish genome? Were the potential off-targets also tested for the possible mutagenesis? What was the efficiency of the performed CRISPR/Cas mutagenesis?

Results section is also clearly written and contains many interesting results. Mrs Sreedevi Sugunan optimized subcellular fractionation of 5 dpf zebrafish larvae to obtain mitochondrial, microsomal and cytoplasmic fractions. Western blot allowed to assess the purity of fractions: mitochondrial fraction was of relative high purity containing ER elements what is clear taking into the consideration the existence of MAMs (mitochondria-associated ER membranes). However, this fraction did not contain cytoplasmic proteins (GAPDH). Cytosolic and microsomal fractions did not contain mitochondria. Interestingly, mitochondrial and ER fractions contain cytoplasmic ribosomes pointing to the existence of MARS (mitochondria with associated ribosomes) and RER (rough endoplasmic reticulum),

ul. Umultowska 89, Collegium Biologicum, 61-614 Poznań
NIP 777 00 06 350, REGON 000001293
tel. +48 61 829 59 50
ibmib@amu.edu.pl

respectively. In the next step the Candidate isolated mitochondria containing cytoplasmic ribosomes (MARS) or having ribosomes stripped with the use of EDTA (MITO). MARS and MITO fractions still contained ER membranes (the presence of calreticulin) but mitochondria treated with EDTA did not have cytoplasmic ribosomes (no ribosomes or a very small amount of cytoplasmic ribosomal proteins) while MARS fraction was indeed highly enriched in these proteins. It is somehow strange that not all ribosomal proteins seem to be in the 1:1 ratio – why Rps9 is so weakly represented in the MARS fraction while it is strongly visible in the post-MARS ER fraction? On the other site, the presence of Rps9 in post-MARS ER fraction seems also to be underrepresented in comparison to other ribosomal proteins tested. Is there anything known about Rps9 – like that is relatively unstable component of cytoplasmic ribosomes?

Analysis of selected mRNAs that are known to undergo co-translational import into yeast mitochondria confirmed the enrichment of these mRNAs in zebrafish MARS when compared to MITO while the amount of mitochondria-encoded transcripts remained unchanged.

To discriminate between cytoplasmic mRNAs that are actively translated at the mitochondrial surface from the mRNAs associated with mitochondria in other way, Mrs Sugunan performed an experiment aiming to cleave the nascent peptide and release active ribosomes using (hydroxylamine) or terminating prematurely peptide synthesis (puromycin). These reagents were used because in yeast it was shown that the association of ribosomes with mitochondria outer membrane takes place via nascent polypeptide associated complex (NAC). The use of both reagents however, did not remove completely ribosomes from the surface. Analyzing these experiments I started to wonder whether the concentration of Mg^{+2} ions used could still play a role in the persisting association of active ribosomes with the mito OM? On the other hand experiments performed by the Candidate have shown, that upon lowering magnesium ions concentration the quality of RNA drops down as well as potentially bound to outer membrane non-active ribosomes will be stripped. However, maybe it would be worth to play with Mg^{+2} concentration to observe whether it will improve the active

ul. Umultowska 89, Collegium Biologicum, 61-614 Poznań
NIP 777 00 06 350, REGON 000001293
tel. +48 61 829 59 50
ibmib@amu.edu.pl

ribosomes removal from the outer mitochondrial membrane. I would like to ask the Candidate to comment on it during her PhD defense.

From the point of view of zebrafish embryonic development the stage 2,5 dpf seems to be very interesting since at that moment nuclear transcription in embryo starts. This can also reflect the composition of mitochondrially-associated transcripts. The Candidate efforts to purify MARS form this embryo stage have shown, that 5 mM Mg^{+2} is detrimental for biochemical fractionation of subcellular components: the yield of protein was constantly decreasing with the increase of Mg^{+2} concentration. Table 19 shows the effects of $MgCl_2$ concentration on protein yield of subcellular fractions. Protein yield shown in the Tab.19 is given in micrograms without any relation to the amount of starting material.

In Her further experiments Mrs Sugunan compared the quality of mRNA enriched either by poly(A^+) capture or rRNA depletion method. Her studies revealed that the poly(A^+) capture method offers both uniform library composition and high proportion of exonic reads. Thus in further experiments Mrs Sugunan used poly(A^+) approach for mRNA isolation.

In Her next experiments she prepared transgenic zebrafish *mia40*$^{-/-}$ in the transgenic background of *Tg (Xla.Eef1a1:mlsGFP)* which does not produce Mia40 protein – oxidoreductase of mitochondrial intermembrane space involved in the posttranslational import of cysteine-rich mitochondrial proteins and expresses mitochondrial matrix-targeted GFP. The *mia40*$^{-/-}$ mutation is embryo-lethal – at the 10th dpf. *mia40*$^{-/-}$ develops abnormal, enlarged mitochondria in skeletal muscles. Isolated MARS from 5dpf mutant embryos also contained cytoplasmic ribosomes associated with mt OM. I assume, that $MgCl_2$ concentration was the same as in the case of WT 5 dpf MARS – 5mM? She successfully isolated RNA and constructed libraries for transcriptome NGS sequencing. Bioinformatic analysis revealed that different mRNAs are found in MARS and ER fraction. By the way the Author is writing "…differential localization of genes…" what is misleading and does not correspond to reality. In the case of highly enriched category mRNAs Mrs Sugunan found more mRNAs that were depleted than enriched when MARS or ER versus total mRNA were calculated in both WT and *mia40*$^{-/-}$ mutants. In the case of moderately enriched mRNAs one can observe stronger

ul. Umultowska 89, Collegium Biologicum, 61-614 Poznań
NIP 777 00 06 350, REGON 000001293
tel. +48 61 829 59 50
ibmib@amu.edu.pl

tendency in the number of depleted expression mRNAs in the mutant embryos than in WT ones. Is there any explanation for this general observation? Concerning the calculations of mt-rRNA amount in different fractions the Author is writing that some discrepancies can be explained by the annotation of yet unannotated additional 16S rRNA – like gene. I assume that this additional gene is not encoded in the mt -genome? I want to ask about the possibility of incorporation into the zebrafish nuclear genome of large parts of its mt genome. It happens very often in plants where huge fragments of mitochondrial genome can be found inserted into the nuclear genome. What is known about mt-DNA transmission into the nuclear genomes in vertebrates? The same question is also important to explain why in the *mia40*$^{-/-}$ mutants only poor enrichment of mt-transcripts is observed in the MARS fraction while generally these transcripts are upregulated in the mutant. Calculation of MitoCarta 2.0 genes enriched specifically in the MARS fraction led to the conclusion that the number of mRNAs associated with mitochondria surface is smaller than expected. Calculations of mRNAs form MitoCarta 2.0 and associated with MARS and ER showed that the number of genes in *mia40*$^{-/-}$ MARS is ER is much higher than in WT embryos. However, genes annotated within MitoCarta 2.0 represented only ~3% of genes whose mRNAs were identified as associated with MARS fraction. In this category mRNAs (having predicted mTP sequence) associated exclusively with MARS fraction represented interesting involvement in the mitochondria motility. This is an interesting observation suggesting that the specific localization of mRNAs is relevant for their site-specific translation on demand.

Although the results presented in this PhD dissertation provide a lot of interesting and novel data they do not present a final touch in the description of translated mRNAs associated with MARS.

That is why the Author added **a special chapter** to the dissertation in which she describes the preparation of new tools for the identification of MARS associated mRNAs. This technique is based on the preparation of transgenic zebrafish lines containing OM protein (TOM20) fused to BirA and cytoplasmic ribosomal protein fused to avidin. Upon biotin binding one can isolate cytoplasmic ribosomes strongly bound to OM mitochondrial protein. This technique is

ul. Umultowska 89, Collegium Biologicum, 61-614 Poznań
NIP 777 00 06 350, REGON 000001293
tel. +48 61 829 59 50
ibmib@amu.edu.pl

called proximity ribosome profiling. Using CrisR/Cas9 approach She already got zebrafish lines expressing and transmitting upon reproduction TMO20-BirA construct.

Despite the above comments this PhD thesis describes for the first time transcriptome-wide studies on mRNAs associated with mitochondria in zebrafish.

I regret that Mrs Sugunan did not performed any analysis of 5' and 3' UTRs of mRNAs associated with MARS. It is known, that upstream AUGs in 5'-UTR, which modulate translation efficiency of downstream sequences, were found to negatively affect mRNAs associated with mitochondria. Did you looked into the potential 5' upstream AUGs in your transcripts? Moreover, 3'UTR sequence elements were identified as essential elements for mRNA localization at the mitochondrial surface. Thus, both an efficient translation and the presence of *cis*-element in 3'UTR are needed for mRNA localization in the vicinity of mitochondria (for example: S, Salinas T, Maréchal-Drouard L, Duchêne AM. (2017) A genome-scale analysis of mRNAs targeting to plant mitochondria: upstream AUGs in 5'-UTRs reduce mitochondrial association. *Plant J.*). I think such analysis should be also done for zebrafish mRNAs associated exclusively with MARS. In the discussion part a lot of attention is payed to the specific association of cytoplasmic ribosomes to mitochondria and how to proof this association. Are there protocols showing how to get rid of ribosomes from ER membranes? I would assume that if there is co-translational import to mitochondria, like into ER, the way how to remove ribosomes can be similar. Generally I regard **discussion part** as interestingly written, asking good questions for future studies and showing deep understanding of mitochondria biology and biogenesis.

The PhD thesis presented here was performed with many different techniques of molecular biology and zebrafish breeding , required a lot of effort to optimize and obtain pure subcellular fractions of zebrafish embryos, optimize RNA isolation, mRNA enrichment, library construction, and finally analyze and confirm the transcriptome NGSs results. According to my view Mrs Sreedevi Sugunan performed successfully interesting, high-risk and advanced studies that broaden our knowledge on the biogenesis of mitochondria in

ul. Umultowska 89, Collegium Biologicum, 61-614 Poznań
NIP 777 00 06 350, REGON 000001293
tel. +48 61 829 59 50
ibmib@amu.edu.pl

UNIWERSYTET IM. ADAMA MICKIEWICZA W POZNANIU
Wydział Biologii
Instytut Biologii Molekularnej i Biotechnologii

zebrafish and described transcriptome realm associated with mitochondria surface showing that it is not as big as expected.

That is why I ask the Scientific Advisory Board at the Institute of Biochemistry and Biophysics PAS in Warsaw to award MSci Sreedevi Sugunan with PhD degree in Biology.

Zofia Szweykowska-Kulińska

ul. Umultowska 89, Collegium Biologicum, 61-614 Poznań
NIP 777 00 06 350, REGON 000001293
tel. +48 61 829 59 50
ibmib@amu.edu.pl