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Revision of MSci Zuzanna Ewa Mackiewicz doctoral dissertation entitled "Investigating cytoplasmic polyadenylation and its role in gene regulation and physiology in *Caenorhabditis elegans*"

The obtained for revision PhD dissertation was performed in the Laboratory of RNA Biology of the International Institute of Molecular and Cell Biology in Warsaw, in frames of the Warsaw PhD School in Natural and BioMedical Sciences. The PhD supervisor is Prof. Andrzej Dziembowski, one of the leading scientist in the field of RNA metabolism and biology focusing on RNA degradation and poly(A) tail processing. The Candidate decided to study cytoplasmic polyadenylation of mRNAs in the model organism *Caenorhabditis elegans* to reveal what is its influence on gene expression regulation and development. Cytoplasmic mRNA tail polyadenylation is performed by three non-canonical poly(A)polymerases GLD-2, GLD-4 and TENT-5. GLD-2 is a well-studied TENT family member involved in germline physiology. GELD-4 is a less-studied member of this family but also involved in germline physiology. The third family member TENT-5 is now studied by Prof. Dziembowski Lab, with important contribution of Zuzanna Mackiewicz. She found, that *tent-5* KO nematodes have a shorter life-span when living on a mildly pathogenic *E,coli* strain because of weak immune response. She also found that TENT-5 localizes close to ER, as do mammalian orthologs of this protein, suggesting that it is involved in the polyadenylation of secretory protein mRNAs or at least mRNAs of proteins targeted to ER.

Here I have the first question: does TENT-5 itself contains ER-targeting sequence? May be it is anchored in the ER membrane facing cytoplasm? Alternatively it can be bound by a specific ER receptor that faces cytoplasm, as it is in the case of SRP ribonucleoprotein, or there are no direct connections of TENT-5 to the ER membrane?

These data are already published and the Candidate started to participate in these studies late, as she joined Dziembowski's Lab. Nevertheless she participated in experiments that allowed to draw the final conclusion on the role of TENT-5 in proper immune response of *C.elegans*.

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The Candidate decided to continue the work on *C.elegans* TENT-5 role in frames of her PhD work.

The aim of this study was (i) to compare the TENT-5 activity towards hermaphroditic and male poly(A) tail metabolism, (ii) to investigate possible functional redundancy between three ncPAPs (GLD-2, GLS-4 and TENT-5); (iii) to elucidate the molecular background of TENT-5 ER -localization and identify potential TENT-5 interactors and (iv) to find the role of NSPC family proteins whose transcripts are the substrates of TENT-5. NSPC proteins (Nematode-Specific Peptides, group C) are produced exclusively in excretory gland cell of yet physiologically unknown function.

According to my opinion all questions have been addressed, some with clear answers and some opening new avenues for further studies.

Before addressing all experiments presented in this thesis I would like to mention that assessments of poly(A) tails length were established using DRS approach (Direct RNA Sequencing) using Nanopore technology that finally enables to define directly poly(A) tail length with one nucleotide resolution. Earlier performed experiments dealing with poly(A) tail length assessment used PCR often introducing biases that did not allow for detecting subtle poly(A) changes.

The Candidate compared mRNA poly(A) tails length between hermaphrodite and male individuals using WT animals. She manually selected male individuals and using male and hermaphrodite populations performed DEG and DRS analyses. She identified a set of male-specific transcripts encoding proteins of unknown functions however, many of them having ER-targeting signals. This suggests that they may be targets of TENT-5 activity as well involved in seminal fluid and sperm production. The Candidate tested the expression pattern of TENT-5 in males and found its enriched localization in male reproductive organs: spermatids, seminal vesicle, and vas deferens. The role of TENT-5 was confirmed by performing DRS analysis for WT and *tent-5* KO male mutants. Indeed, poly(A) tails of mRNAs isolated from WT males were found to have longer poly(A) tails than those isolated from *tent-5* mutants. The longer poly(A) tails were found particularly in the set of mRNAs

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encoding ER-targeted proteins. These analyses confirmed the role of TENT-5 in poly(A) tail prolongation of male specific mRNAs encoding ER-targeted proteins. However, no reproduction failures including spermatogenesis were found. The set of mRNAs linked to immune response was also found to be downregulated in *tent-5* mutants both in hermaphrodites and males underscoring the universal role of encoded proteins in immunity. However, it is not clear for me whether poly(A) tails of these mRNAs were also shortened in the mutant background? I asked this question because in her further summary the Candidate wrote that concerning TENT-5 targets a minimal overlap was found between hermaphroditic and male individuals – only two mRNAs encoding proteins probably involved in cholesterol metabolism. Second question, I wonder whether alternative poly(A) sites were found when common hermaphroditic and male transcripts were compared?

Next task of this PhD thesis was to reveal possible functional redundancy between *C.elegans* ncPAPs GLD-2, GLD-4 and TENT-5. To get the answer the Candidate analysed poly(A) tails length in *gld-2* and *gld-4* mutants. This first step was reasonable because literature data for both ncPAPs show they are involved in germline development. *Gld-2* mutants show statistically essential shortening of poly(A) tails in germline-expressed transcripts. Since *gld-2* mutation is lethal, studies were performed on heterozygous animals. Additionally, this study confirmed essential pol(A) shortening of three out eight previously reported GLD-2 substrates - *gld-1*, *oma-2*, and *cpb-3*. For *gld-4* mutants the extent of poly(A) shortening was rather small, confirming that GLD-4 mediates the addition of shorter adenosine stretches than GLD-2. However surprisingly, only for this mutant global shortening of poly(A) tails was observed. GO analysis revealed that GLd-4 substrates represent mainly mRNAs encoding ribosomal proteins and CEY protein family members that are known to be involved in polysomes formation. TENT-5 was also found to add adenosine stretches to transcripts involved in polysomes formation. Double mutant of *gld-4*x*tent-5* analysed for pol(A) tails did not reveal and additional substrates indicating there is no substrate overlap between these two ncPAPs. Final comparison of shortened transcripts between *C.elegans gld-2*, *gld-4* and t*ent-5* mutants revealed minimal overlap for their substrates showing, that these ncPAPs exhibit different

functions during development. I understand that in this experiment hermaphroditic individuals were tested. What about male individuals and GLD-2 and GLD-4 substrates? Is the expression pattern of these two ncPAPs in males known?

Since ncPAPs lack RNA recognition domain they mast have their specific interactors. Three interacting proteins FNDC3A/B, LARP4/5, and ATXN2 were identified for TENT-5C in various types of human cells. The Candidate identified their potential *C.elegans* orthologs, silenced them using RNAi approach and performed Nanopore DRS analysis for mRNA poly(A) tail lengths. The results revealed significant poly(A) shortening for a number of transcripts however, the highest number of affected poly(A) tails was found in the case of *larp5* suggesting its dominant role as TENT-5 interactor. Further detailed studies revealed the possibility that LARP5 and C34F6.10 act in concert with TENT-5. Again I have a question concerning the possible differences between hermaphrodites and males in TENT-5 interactors. Since for all orthologs some mRNAs with shortened poly(A) tail were found I wonder if there might be GO specificity for mRNAs specifically shortened in the case of each interactor, alternatively is there developmental timing for the use of specific interactors? Somehow I miss in the part of discussion the problem of TENT-5 localisation near the ER. Did the experiments on TENT-5 interactors allowed to explain the mechanism underlying TRNT-5 localisation?

Final experiments of this PhD thesis dealt with the explanation of NSPC proteins function that show really unique expression pattern (found exclusively in single nematode excretory gland cell) that are the most characteristic TENT-5 targets. The Candidate performed very interesting approach to reveal excretory gland cell functions, namely she performed blue-light ablation that specifically inactivated excretory gland cell expressing *minisog* gene. Next, the Candidate independently developed a high-throughput platform for ablation performed with the use of LED advertising board. Surprisingly, ablation did not induce any observable phenotypic changes. Moreover, transcriptomic data of ablated nematodes show only the downregulation of NSPC transcripts. Thus the author concluded that the only role of these excretory gland cells was NSPC proteins production. These results led the Author to knock-

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out all 18 members of NSPC gene family using CRISPR/Cas9 approach. However, worms still did not reveal any phenotypic changes. I want to know whether you looked into excretory gland cell morphology? Did they look unchanged? Transcriptomic data of this *nspc-ko* mutant together with *tent-5 ko* revealed the connection of NSPC proteins with defense response genes connecting again to TENT-5 role in nematodes immune response. A family of *PMK* family genes exhibited significantly upregulated expression. These proteins are known to regulate defense response genes. All these data led the Candidate to perform a test for mutants resistance against pathogenic agents like *P.aeruginosa*. And again disappointment, there was no difference in lifespan between WT and mutant individuals! However, the Candidate discovered that WT animals excretory gland cells were enriched in cholesterol, as it was found for male and hermaphroditic overlapping TENP-5 substrates suggesting that NSPC regulate cholesterol biosynthesis.

Literature mining led the Author to test DAF-2/DAF-16 insulin signaling pathway since this pathway strongly affects the amount of NSPCs. Moreover, NSPCs resemble in their structure insulin. Further transcriptomic experiments led to the conclusion that NSPCs act upstream of DAF-16. The Author concluded that NSPC proteins may act as neuropeptides that help to regulated various signaling pathways, including insulin DAF-2/DAF-16 pathway. Further experiments are needed to prove NSPC role as neuropeptides.

Experiments performed in frames if this thesis are very well done using cutting edge technologies, all experimental controls have been performed. Experiment analysis required advanced bioinformatics tools. The Candidate coped very well with these all analyses. What I really like in this thesis is the effort to connect poly(A) tail length metabolism linked to TENT-5 activity with animal physiology. Experiments performed in this thesis show that TENT-5 is a very specialized enzyme with a rather narrow range of substrates. Nevertheless, it is clear that its activity is connected to immune response, to spermatogenesis, to cholesterol biosynthesis and most likely to physiological control via neuropeptides. One paper has been published with Zuzanna Mackiewicz authorship in Science Advances and two are deposited in bioRxiv. In both papers Zuzanna Mackiewicz is the first author. Statements provided by the

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Candidate clearly show her dominant role in designing and performing experiments in the last two papers. I am sure both of them will be well published but this of course takes time and will require discussions with the reviewers. There is an immense work behind the results obtained in frames of this dissertation that required a lot of laboratory and bioinformatic skills from the Candidate. The entirety of the doctoral thesis presented to me for evaluation constitutes a valuable contribution to the understanding of the molecular biology and physiology of hermaphroditic and male individuals of *C.elegans*.

I regard the Candidate dissertation as fulfilling the requirements to be awarded with the doctoral degree.

I, the undersigned, hereby state that the doctoral dissertation of Ms. Zuzanna Mackiewicz meets the requirements specified in Article 187 of the Act of July 20, 2018 – Law on Higher Education and Science (c.t., Journal of Laws of 2024, item 1571, as amended). I hereby recommend to the Doctoral Committee of the International Institute of Molecular and Cell Biology in Warsaw to admit Ms. Zuzanna Mackiewicz to the subsequent stages of the procedure for the conferment of the doctoral degree in the field of natural sciences, in the discipline of biological sciences.

Considering good quality of experimental work, advanced bioinformatic tools applied in the study and interesting results that push forward our understanding of *C.elegans* physiology linked to RNA metabolism, I suggest to award this PhD thesis with the appropriate reward.

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Signed by / Podpisano przez:
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