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The review of the PhD thesis of Annasha Dutta, M.Sc, entitled: "Small non-coding RNAs in regeneration of *Schmidtea mediterranea*"
Supervisor: Dr. hab. Paulina Jackowiak, prof. ICHB PAN
PhD was completed in the Laboratory of Single Cell Analyses
at the Institute of Bioorganic Chemistry Polish Academy of Sciences in Poznań

Tissue and organ regeneration represents a fundamental biological process essential for maintaining homeostasis and ensuring proper physiological function. This process is critical not only in response to injury and disease but also following intense physical exertion. The regenerative capacity varies significantly among different human organs and across species. Extensive research has been conducted over the years to elucidate the molecular and cellular mechanisms underlying regeneration, as well as to explain the variability in regenerative potential observed both between organs and among different species. However, our understanding of these processes remains incomplete.

Among the organisms exhibiting remarkable regenerative abilities are flatworms, such as *Schmidtea mediterranea*. Notably, *S. mediterranea* harbors genes that are homologous to those in humans, including genes whose mutations are implicated in the pathogenesis of hereditary diseases. This makes *S. mediterranea* an excellent non-mammalian *in vivo* model for investigating conserved molecular mechanisms of regeneration. In this context, the doctoral researcher has undertaken the identification and characterization of short non-coding RNAs (ncRNAs) involved in the regenerative processes of *S. mediterranea*.

The dissertation has a canonical structure. It starts with an abstract written in English and Polish. The following sections and subsections - Introduction, Scope of the Thesis, Materials, Methods, Results and Discussion, and Conclusions - are written in English. The final part of the dissertation is a comprehensive list of references relevant to the work. The dissertation contains 57 tables and 32 figures.

The PhD student begins the Introduction section by emphasizing the critical role of the interplay between specific transcription factors and their downstream effector genes in tissue and organ regeneration in multicellular organisms. She also highlights the differences between embryonic development and adult regeneration. Interestingly, processes such as cell division and apoptosis occur in both contexts. However, what

fundamentally distinguishes these two processes are the gene expression patterns and the distinct cellular origins of the participating cells. The PhD student further discusses the various types of metazoan regeneration and introduces polyploidy as a significant element influencing regenerative capacity.

The subsequent part of the Introduction is devoted to non-coding RNAs, particularly microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), and tRNA-derived fragments (tRFs), with a focus on their roles in the regeneration process. Although the biogenesis of tRFs remains incompletely understood, several enzymes involved in their generation have already been identified. For instance, tRF-1s (16–48 nucleotides in length) are produced through cleavage of the 3′ trailer sequence of pre-tRNAs by ELAC2 (tRNase Z) and its homologs. Specific tRF molecules, such as the 5′ tRNA half Gly-GCC, have been shown to influence key developmental events — such as sperm maturation and preimplantation embryo formation — by repressing the expression of genes associated with endogenous retroelements. An increasing body of evidence suggests that tRFs may play essential roles in maintaining the stemness of specific cell populations in mammals. Furthermore, studies have shown that various tRF types display distinct spatiotemporal accumulation patterns during planarian regeneration. In particular, Figure 5 is a great presentation of the scientific achievements to date illustrating the dynamics of the *Schmidtea mediterranea* transcriptome during regeneration. However, let me remark that in the figure caption it would be good to include references to the literature that was used to prepare this diagram.

Overall, the Introduction is exceptionally well-written, offering the reader a solid and engaging overview of regeneration across diverse organisms. Although certain subsections may delve into a high level of detail, this depth is supported by clear writing. The chapter is enriched with five well-designed figures and six informative tables. The clarity and fluency of the writing not only reflect the Author's strong writing skills but also her deep and critical understanding of the scientific literature. The topic itself is both timely and fascinating.

The overall aim of the study was to identify small non-coding RNAs (sncRNAs) in *S. mediterranea*, followed by the development of a research strategy to investigate the impact of sncRNA pool alterations on the regeneration process - including effects on morphology and anatomy, cell populations, and transcriptome profile - as well as the functional characterization of selected sncRNAs involved in regeneration.

All materials required to conduct the experiments are presented in tables. The Methods section provides a detailed description of procedures, including maintenance, feeding, and segmentation of *Schmidtea mediterranea* into three parts (head, trunk, and tail) to initiate regeneration. The methodology applied in the experiments is both complex and highly advanced. The following techniques were employed in the study: RNAi-mediated target gene knockdown (KD) through the introduction of artificial double-stranded RNA

(dsRNA), fluorescent *in situ* hybridization (FISH) and probe synthesis labeled with digoxigenin, quantitative real-time PCR (qRT-PCR) for gene expression analysis, detection of mitochondrial unprocessed transcripts, detection of sncRNA molecules, and determination of marker gene expression. For flow cytometry, 20–30 whole worms were dissociated into single-cell suspensions. Next-generation sequencing (NGS) was performed at two time points: 3 and 5 days post-amputation (dpa). These time points were selected by the PhD student based on phenotypic observations and qPCR validation. It is important to note that sufficiently large groups of animals were used in the experiments to enable robust statistical analysis, although I must mention that the statistical analyses themselves were carried out by another researcher. At this point, I would like to raise a question: Shouldn't the statistical analyses have been performed by the PhD student? This is a crucial component of experimental work, and every researcher should be capable of performing statistical analysis independently.

To initiate the research and achieve the intended objective, the PhD candidate developed a research strategy. This strategy aimed to disrupt the pool of small non-coding RNAs (sncRNAs) by silencing a specific ribonuclease, followed by assessing its impact on regeneration through an initial phenotypic evaluation.

Ribonucleases were identified through analysis of the Online Mendelian Inheritance in Man (OMIM) catalog of human genes and genetic disorders. The PhD candidate focused on 11 planarian homologs of RNases implicated in Mendelian diseases and involved in sncRNA metabolism. It was found that five genes encoding ribonucleases - RNASEH1, RNASEH2A, RNASEH2B, PARN, and ELAC2 - were upregulated in neoblasts. Ultimately, four RNases - Smed-RNASEH1, Smed-PARN, Smed-ELAC2, and Smed-RNASET2 - were selected for knockdown using an RNA interference (RNAi) gene silencing strategy. Artificial double-stranded RNA (dsRNA), synthesized with the AmpliScribe T7-Flash Transcription Kit, was introduced into the worms via microinjection.

However, some methodological aspects remain unclear and require clarification:

- How was the dsRNA concentration determined?
- Were preliminary tests performed to optimize the dosage?
- Was only a single dsRNA variant used per gene?

Successful knockdown was achieved for the genes encoding Smed-ELAC2 and Smed-PARN. In the case of Smed-ELAC2, the phenotypic changes were particularly striking — nearly 70% of worms regenerating from Smed-ELAC2 knockdown tail fragments were either eyeless or exhibited a cyclopic phenotype up to approximately 7 days post-amputation (7 dpa). In addition, abnormal ventral nerve cord development was observed at the phenotypic and anatomical levels. Advanced screening did not reveal any significant morphological differences in cells representing X1 (neoblast), X2 (progenitor), and Xins (differentiated) cell populations between Smed-ELAC2 KD, GFP mock, and WT controls. However, the PhD candidate found that the

knockdown of Smed-ELAC2 led to the discovery of multinucleated cells (MuNs) in planarians, containing from 2 to 8 nuclei per cell. Using imaging flow cytometry and fluorescence-activated cell sorting (FACS), the PhD candidate showed that MuNs are large homeostatic cells with a potential epidermal progenitor-like identity.

Further studies revealed that Smed-ELAC2 silencing leads to defects in mitochondrial tRNA (mt-tRNA) processing and dysregulation of genes involved in developmental processes and cell proliferation. Changes were also detected in the pool of small non-coding RNAs (sncRNAs). Overall, miRNAs constituted the largest proportion of sncRNAs, followed by tRNA-derived fragments (tRFs). In the case of tRFs, 24 different tRNAs gave rise to differentially accumulated fragments. Among these, the PhD candidate observed a significant reduction in the 5′ tRNA half Gly-GCC upon Smed-ELAC2 knockdown, indicating that this ribonuclease is directly involved in the biogenesis of this molecule. In the final experiment, the PhD candidate showed that the 5′ tRNA half Gly-GCC has functional relevance in the context of planarian regeneration and specifically downregulates the expression of the SMESG000048842.1 gene *in vivo*, suggesting it may act as a non-canonical miRNA.

Final Conclusions

This doctoral dissertation constitutes an exemplary case of how the mechanism of action and functional role of a protein can be elucidated in a stepwise and systematic manner. The PhD candidate has generated substantial new insights into the role of the Smed-ELAC2 protein in the regenerative processes of *Schmidtea mediterranea*. She has proposed a plausible mechanism of action and identified molecular components involved in this process that are dependent on Smed-ELAC2. These findings were made possible through the application of sophisticated research methodologies and meticulously designed, technically demanding experimental procedures.

The results, together with their thorough and mature interpretation, provide a comprehensive and coherent evidence on the function of Smed-ELAC2 at both the cellular and organismal levels. This work reflects a high degree of scientific rigor and intellectual maturity. In addition to demonstrating outstanding technical and methodological expertise, the candidate has shown a commendable ability to formulate a clear scientific question and address it through a logical, well-structured research approach.

The dissertation makes a significant contribution to the field of regenerative biology, advancing our understanding of the mechanisms underlying regeneration in *Schmidtea mediterranea*, and offers promising perspectives for extending these findings to the study of regenerative processes in humans.

I therefore recommend proceeding to the further stages of the doctoral defense. Considering the exceptionally high quality of the dissertation - including the breadth of methodological approaches, the number of experiments performed, and the scientific achievements - I propose to award the Author of this dissertation with an appropriate distinction.

Przedstawiona do recenzji rozprawa doktorska spełnia warunki określone w Ustawie z dnia 20 lipca 2018 roku prawo o szkolnictwie wyższym i nauce (Dz.U. z 2018 r. poz. 1668 ze zm.) oraz w Sposobie postępowania w sprawie nadania stopnia doktora w Instytucie Chemii Bioorganicznej PAN w Poznaniu (uchwała Rady Naukowej ICHB PAN nr 40/2025/Internet/RN_140 z dnia 18 marca 2025 r.) i wnioskuję do Rady Naukowej Instytutu Chemii Bioorganicznej PAN o dopuszczenie **mgr Annashy Dutty** do dalszych etapów postępowania o nadanie stopnia doktora. Ze względu na skalę i stopień trudności przeprowadzonych eksperymentów oraz jakości uzyskanych wyników wnioskuję do Rady Naukowej Instytutu Chemii Bioorganicznej PAN o wyróżnienie pracy doktorskiej i stosowne nagrodzenie Doktorantki.


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