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Review of the doctoral dissertation of Ms. Agnieszka Czarnocka-Cieciura

1. Subject of the review

The doctoral dissertation of Ms. Agnieszka Czarnocka-Cieciura, entitled *The utilization of advanced RNA sequencing technologies to investigate post-transcriptional mechanisms involved in the regulation of gene expression*, was carried out under the supervision of Professor Andrzej Dziembowski (primary supervisor) and Dr. Paweł Krawczyk (assistant supervisor). The dissertation was submitted as part of the doctoral proceedings in the field of biological sciences and conducted at the Laboratory of RNA Biology, International Institute of Molecular and Cell Biology in Warsaw.

The aim of the reviewed dissertation was to investigate the mechanisms of mRNA deadenylation and degradation in yeast, as well as the role of cytoplasmic poly(A) polymerases in regulating cellular phenotypes during gametogenesis in mice. By employing direct RNA sequencing technology (DRS, Oxford Nanopore) combined with computational analysis, the doctoral candidate characterized mRNA degradation dynamics and deadenylation rates under various physiological and stress conditions. In addition, the dissertation points to a potential functional significance of the enrichment of poly(A) tails with non-canonical nucleotides in the context of germ cell maturation.

2. Assessment of originality and scientific contribution

The dissertation addresses a timely and significant research problem in biology, focusing on the interplay between post-transcriptional gene expression regulation, particularly the dynamic

balance between adenylation-deadenylation and RNA translation-degradation rates. In the first part of the introduction to the thesis, the author describes methodological aspects of direct RNA sequencing using Oxford Nanopore long-read technology. She then moves on to discuss the implications of applying this type of analysis to understand the dynamics of poly(A) tail formation during RNA maturation and its cytoplasmic life cycle, including re-adenylation. The final part of the introduction covers the relationship between deadenylation by the complementary Pan2-Pan3 and Ccr4-Not complexes and mRNA degradation. Additionally, this section provides a concise overview of tissue-specific control of poly(A) tail length and its regulation in response to specific cellular stress conditions.

Thanks to this well-structured introduction, the author establishes a conceptual framework that enables a smooth transition to the core part of the doctoral dissertation, which consists of three original research articles in which Ms. Agnieszka Czarnocka-Cieciura played a leading role (as first or second author). The findings are original and represent a significant contribution to the advancement of knowledge in the field of molecular biology of RNA. Main implications and findings of the individual papers are critically discussed in the following sections.

Paper #1 **Czarnocka-Cieciura, A.,** Poznański, J., Turtola, M., Tomecki, R., Krawczyk, P. S., Mroczek, S., Orzeł, W., Saha, U., Jensen, T. H., Dziembowski, A., & Tudek, A. (2024). Modeling of mRNA deadenylation rates reveal a complex relationship between mRNA deadenylation and decay. *EMBO Journal, 43*(24), 6525–6554.

Poly(A) tails, present at the 3′ ends of nearly all eukaryotic mRNAs, are critical for the stability and translation of these molecules. The removal of the poly(A) tail, or deadenylation, is a key mechanism regulating gene expression. The first publication included in the dissertation presents a thorough analysis of the relationship between deadenylation and RNA degradation, considering transcript-specific features, the impact of m^7G cap removal, and the role of poly(A)-binding (Pab) proteins. The study also introduces a mathematical model (gamma distribution, described in two variants – one designed and suitable for estimating half-lives and deadenylation kinetics in large datasets, and a modified version tuned for smaller-scale biological measurements) aimed at understanding the processivity of deadenylation.

The research employed a carefully selected panel of *Saccharomyces cerevisiae* yeast mutants, including strains in which the export of nascent transcripts from the nucleus was blocked (by inhibition of the Mex67 pathway), as well as mutants lacking key RNA metabolism proteins. Analysis of mutants for Pab1, Dcp2, and Xrn1 enabled a detailed characterization of

the relationship between deadenylation and mRNA decapping, and provided insight into the function of the Pab1 protein.

From a methodological standpoint, the study presents an original approach for probing deadenylation dynamics using data obtained through direct RNA sequencing. In my view, one of the most scientifically appealing aspects of this work is the use of a cutting-edge method, innovative both technologically and computationally, to test a hypothesis that had previously been difficult or impossible to address with previous indirect approaches. In particular, the doctoral candidate and her collaborators demonstrated correlations between deadenylation speed, degradation rates, and transcript half-lives. Furthermore, they identified transcript features, such as expression level or poly(A) tail length, that influence kinetics of deadenylation.

An especially intriguing part of the manuscript is the analysis of deadenylation and decay rates under cellular stress conditions, including heat shock and thiolutin treatment. Authors propose that modulation of deadenylation rates enables rapid transcriptome remodeling, allowing for quicker cellular adaptation to stress.

Paper #2 Brouze, M., **Czarnocka-Cieciura, A.,** Gewartowska, O., Kusio-Kobiałka, M., Jachacy, K., Szpila, M., Tarkowski, B., Gruchota, J., Krawczyk, P., Mroczek, S., Borsuk, E., & Dziembowski, A. (2024). TENT5-mediated polyadenylation of mRNAs encoding secreted proteins is essential for gametogenesis in mice. *Nature Communications, 15*, 5331.

This study focuses on the regulation of gametogenesis by non-canonical cytoplasmic poly(A) polymerases of the TENT5 family, which the research group had previously proven to play important roles in the context of osteogenesis and immune response. In this work, the authors analyzed gamete development in mouse lines with either knockout (KO) of specific TENT5 genes or knock-in (KI) versions containing GFP or HA-peptide tags. The examined genes included *Tent5a, Tent5b, Tent5c,* and *Tent5d*, as well as a double KO (Tent5b/c) and GFP-tagged knock-ins (Tent5c/d KI).

Authors found that in females, deletion of *Tent5a*, *Tent5b/c* double KO, and *Tent5b-GFP* mutations led to infertility, whereas in males, a similar phenotype was observed in *Tent5c* and *Tent5d* KO animals.

Detailed analysis of individual stages of gametogenesis in transgenic mice allowed the identification of the specific developmental points at which defects emerge, depending on the variants of polymerase loss/gain or their combinations. Two observations stand out in particular:

1. The high phenotypic specificity, both at the organismal and cellular levels, across different genetic variants.
2. The influence of varying TENT5 expression levels/activity on polyadenylation of specific mRNAs, without a corresponding global change in poly(A) tail length.

In light of the first observation, the possible existence of TENT5 variants or mutations in the general population, especially in relation to fertility, may be of considerable interest. To explain the substrate specificity, the study puts forward the hypothesis of the importance of subcellular localization, specifically the presence of endoplasmic reticulum-targeting sequences.

In summary, the doctoral candidate convincingly demonstrates that mRNA polyadenylation by TENT5 polymerases is essential for proper gametogenesis in mice, particularly affecting transcripts encoding secreted proteins. In my view, this is an excellent example of a study that bridges fundamental molecular mechanisms (in this case, non-canonical cytoplasmic polyadenylation) with a deep understanding of phenotypic consequences, such as disrupted gametogenesis.

Paper #3 **Czarnocka-Cieciura, A.,** Brouze, M., Gumińska, N., Mroczek, S., Gewartowska, O., Krawczyk, P. S., & Dziembowski, A. (2025). Comprehensive analysis of poly(A) tails in mouse testes and ovaries using Nanopore Direct RNA Sequencing. *Scientific Data, 12*(1), 43.

The aim of this publication was to create a repository on TENT5 poly(A) polymerase mutants in gametogenesis, using direct RNA sequencing with long-read technology. Additionally, the study set out to develop analytical methods suitable for processing such datasets, particularly in relation to homopolymeric and repetitive sequences, such as poly(A) tails.

To this end, the study used samples from **study #2**, obtained during oogenesis and spermatogenesis, when diploid cells undergo meiotic division to produce haploid gametes. This developmental window involves a profound reorganization of the transcriptome, during which post-transcriptional gene regulation plays a pivotal role across various stages of gamete maturation. The resulting dataset provides deeper insights into the complexity of the transcriptome during these dynamic transitions.

The study analyzed Oxford Nanopore direct RNA sequencing data from various Tent5b, Tent5c, and Tent5d mutant mouse lines, along with their respective controls, all derived from the prior study. The input RNA sequencing signals were processed with the *Ninetails* software, which enables the detection of non-adenine nucleotides within poly(A) tails. Interestingly, the authors found that ovarian and testicular mRNAs had a significantly higher proportion of non-canonical nucleotides in their poly(A) tails compared to other mouse tissues. However, due to

the methodological nature of this article, no attempt was made to interpret the biological significance of this finding.

A particular strength of the study is the integration of long-read sequencing data with short-read Illumina RNA-seq from testicular cell populations (wild-type and Tent5c mutants). This allowed a robust analysis of poly(A) tail composition, revealing the presence of uridines at poly(A) tail ends, indicative of mRNA uridylation, a key post-transcriptional mechanism known to be essential for proper germ cell development.

Finally, the expression analysis showed that transcripts with uridylated poly(A) tails are highly expressed in spermatids, and that their abundance changes during sperm maturation. These findings highlight the dynamic role of uridylation in regulating mRNA stability and function during gametogenesis, contributing to our understanding of non-canonical RNA modifications in tissue-specific biological processes.

3. Methodological Evaluation

According to the description in the dissertation, Ms. Agnieszka Czarnocka-Cieciura focused her work on the bioinformatic and computational analysis of data obtained through direct RNA sequencing using the Oxford Nanopore platform. The methodological descriptions in the included publications are detailed and thorough, and the final paper in the dissertation even provides open access to a ready-to-use tool (*Ninetails*) for analyzing particularly challenging repetitive sequences, such as homopolymers found in poly(A) tails.

It is worth emphasizing that this type of analysis, both methodologically and computationally, represents a young but highly promising area of transcriptomics research. The work of the host research group, including the studies presented in this dissertation, is clearly positioned at the cutting edge of efforts to understand the relationship between RNA structure and molecular function.

4. Structure and style of the dissertation

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It is worth emphasizing that this type of analysis, both methodologically and computationally, represents a young but highly promising area of transcriptomics research. The work of the hosting research group, including the studies presented in this dissertation, is clearly positioned at the cutting edge of efforts to understand the relationship between RNA composition and molecular function.

5. Original publications

The doctoral dissertation includes three articles published in *The EMBO Journal*, *Nature Communications*, and *Scientific Data*, which strongly attest to the quality of the research and its significance for the scientific community working in the field of RNA biology. In addition to the **three** articles included in the dissertation, the doctoral candidate has co-authored **three** other scientific publications.

6. Specific comments

The presented doctoral dissertation constitutes a coherent, intellectually stimulating, and methodologically robust body of work. The fact that the results have been published in leading journals in the field of molecular and cellular biology limits the scope for critical remarks from the reviewer. At the same time, I would be interested to hear the candidate's thoughts on the following points, which I propose to discuss during the defense following the presentation of the main findings of the dissertation:

- The Paper #1 indicates varying deadenylation kinetics for transcripts differing in expression levels, with transcripts with higher expression exhibiting shorter poly(A) tails and slower deadenylation. What might be the molecular determinants and implications of this phenomenon?
- Which cellular sensors are responsible for detecting poly(A) tails shorter than 20 nucleotides and triggering their rapid degradation?
- Does the developed mathematical model have the potential to detect cellular processes regulated by shifts in deadenylation–decay dynamics? Which pathogenic processes are known to be influenced by such regulation?
- How can the differences in fertility phenotypes between Tent5b KO, Tent5c KO, and Tent5b/c dKO mice be explained, particularly the rescue of fertility in dKO males relative to Tent5c KO, and the inverse phenotype observed in females?
- The role of cytoplasmic poly(A) polymerases appears to be particularly linked to mRNAs encoding secreted proteins. What might be the biological rationale behind this,

and does the candidate anticipate cellular phenotypes related to disruption of this regulatory axis in tissues with high secretory activity?

- Paper #2 did not identify specific sequence motifs in the 3′ UTRs or coding regions of mRNAs being TENT5 targets. On what basis, then, is substrate specificity achieved by these polymerases? The authors suggest that no known regulatory cofactors are involved - did they consider alternative recognition mechanisms, such as interactions with ER-resident proteins or RNA secondary structures?
- What are the candidate's thoughts on potential nucleoside modifications within poly(A) tails? What might be the functional role of uridine residue clustering at the end of poly(A) tails?

These points are intended to spark the scientific discussion during the dissertation defense.

7. Concluding remarks

In summary, the doctoral dissertation by Ms. Agnieszka Czarnocka-Cieciura presents an original solution to a scientific problem and has been prepared at a high substantive and methodological level. It meets the requirements specified in Article 187, paragraph 1 of the Act of 20 July 2018 - Law on Higher Education and Science (Journal of Laws 2018, item 1668, as amended).

Therefore, I recommend to **accept** the dissertation for further stages of the doctoral procedure. At the same time, and in light of the high quality of the work and its significant contribution to understanding post-transcriptional regulation at both the molecular and physiological levels, I also recommend to award the dissertation with a **distinction**.

With best regards,

Maciej Cieśla

Warszawa, 21 June 2025