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REVIEW OF PHD THESIS

"The utilization of advanced RNA sequencing technologies to investigate post-transcriptional mechanisms involved in the regulation of gene expression."

By: Mrs Agnieszka Maria Czarnocka-Cieciura
Completed at: Laboratory of RNA Biology, International Institute of Molecular and Cell Biology in Warsaw
Supervisor: prof. dr hab. Andrzej Dziembowski
Auxiliary supervisor: dr Paweł Krawczyk

The PhD dissertation is based on 3 multiauthor research publications in which the PhD candidate was primarily responsible for bioinformatic analysis:

Paper 1 – *co-first author (comprehensive bioinformatic analysis, biological interpretations, figure preparations, paper writing and editing)*

Czarnocka-Cieciura A, Poznański J, Turtola M, Tomecki R, Krawczyk PS, Mroczek S, Orzeł
W, Saha U, Jensen TH, Dziembowski A, Tudek A. Modeling of mRNA deadenylation rates reveal a complex relationship between mRNA deadenylation and decay.
EMBO J. 2024 Dec;43(24):6525-6554. doi: 10.1038/s44318-024-00258-3. Epub 2024 Oct 11. PMID: 39394354; PMCID: PMC11649921

Paper 2 – *second author (bioinformatic analysis of Illumina and Nanopore DRS datasets, figure prepariation, writing and editing)*

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. TENT5-mediated polyadenylation of mRNAs encoding secreted proteins is essential for gametogenesis in mice. Nat Commun. 2024 Jun 22;15(1):5331. doi: 10.1038/s41467-024-49479-4. PMID: 38909026; PMCID: PMC11193744.

Paper 3 – *co-first author (quality control analyses for RNA-seq and Nanopore DRS datastets, analysis downstream of Ninetails, DE analysis and visualisations, figure preparation manusrtipt draft preparation, writing and editing)*

Czarnocka-Cieciura A, Brouze M, Gumińska N, Mroczek S, Gewartowska O, Krawczyk PS, Dziembowski A. Comprehensive analysis of poly(A) tails in mouse testes and ovaries using Nanopore Direct RNA Sequencing. Sci Data. 2025 Jan 10;12(1):43. doi: 10.1038/s41597-024-04226-8. PMID: 39794363; PMCID: PMC11724052.

The contribution of the PhD Candidate to the above mentioned papers is significant.

The thesis explores cytoplasmic mRNA deadenylation and decay in yeast and the role of non-canonical poly(A) polymerases in murine gametogenesis.

The aim of the thesis was to utilize transcriptomic datasets, primarily generated using Direct RNA Sequencing (RDS) on nanopores, to study 3 aspects of post-transcriptional gene expression control:

• the dynamics of mRNA deadenylation and decay in the cytoplasm of *S. cerevisiae*

• mRNA substrates of TENT5 non-canonical poly(A) polymerases during germ cell maturation

in mice

• non-canonical nucleotides in poly(A) tails of mRNAs during mouse gametogenesis

The thesis is well-structured, with a neat and transparent layout. It is composed of an Introduction of 8 pages, half page Purpose of work, Results consisting of the above mentioned 3 papers, along with 1-page description for each of them, and one paragraph explanation of the Candidate's contribution to each paper, followed by a 2.5 page Discussion, 1.5 page Future perspectives and 6-page Bibliography.

The **Introduction** is overall well written, giving the necessary background to understand the presented results, which is what really matters. Of course there is always some room for improvement for future writing. First, some figures and simple graphical representations would have been helpful for the readers. For example it could help in the section introducing adenylating and deadenylating enzymes, the various TENTs, or when explaining how the used algorithms work. Second, the introduction has a slightly fragmented feeling, I would recommend spending more time on linking the paragraphs together.

Questions for the introduction:

1: (top of page 8) "In DRS, during the preparation of the sequencing libraries, a DNA adapter is ligated to RNA, optionally followed by reverse transcription." It seems to me this is a mental shortcut and the author meant no reverse transcription is used for DRS, however could be used in case of cDNA sequencing to increase accuracy?

2: (end of 1st paragraph on page 8) "For nanopore DNA sequencing base-calling accuracy is comparable to Illumina-based methods, reaching 99.9%" Did the author mean single-read or consensus accuracy? Are single-read accuracies of Illumina and Nanopore the same?

The **Purpose of the work** on page 15 is well formulated. However, the concluding sentence "These bioinformatic analyses, together with experimental data and modeling results obtained by others, enabled us to enhance our understanding of post-transcriptional gene expression regulatory mechanisms" is a little vague to my taste – could be possibly be applied to most recent dissertations in Dziembowski lab.

In the **Results section**, the one-page summaries of the research papers gives a very nice framework to the dissertation.

Description of **paper 1** is rather technical and the impersonal voice used ("was performed" etc) makes it hard to read. The experiments and results are summarized nicely but I would have like to see a conclusion at the end of that section, in the candidate's own words, especially as this is her first-author work. The paper itself investigates mRNA deadenylation and decay in *Saccharomyces cerevisiae* using DRS direct RNA sequencing. There process are tracked using elegant chase experiments under steady-state and stress conditions. A mathematical model is proposed which estimates the transcriptomic deadenylation rate at 10 A/min. Decay and deadenylation rates correlate, and are consistent within functional transcript groups and linked to codon optimality. The findings indicate that ribosomal protein-coding mRNAs, contributing to 40% of the transcriptome, have accelerated deadenylation and decay under heat stress. Interestingly, degradation can happen even upon deadenylation blocking, depending on nuclear export. Since the EMBO Journal in which the paper has been published, has a transparent peer review policy, one can find that "all three referees found the results of importance and interest, and agreed that the experiments were performed competently". I fully agree with their assessment and congratulate the PhD Candidate for this beautiful piece of work.

Questions for paper 1
3: What is the relevance of your finding of yeast mRNA decay independent of complete deadenylation to higher eukaryotes?
4: What might be the role of nuclear export in decapping?

I have no specific comments or questions to the second paper. It is another well-executed, high-quality piece of work, which shows "that TENT5-mediated polyadenylation of mRNAs encoding secreted proteins is essential for proper spermatogenesis and oogenesis in mice", as the Candidate nicely summarized.

The third paper, in which the PhD Candidate is again a co-first author, provides and analyses new sets of data derived from TENT5 poly(A) polymerase mutant mice. The authors performed poly(A) tail composition analysis, and identified transcripts enriched in uridines at the terminal regions of their poly(A) tails. Interestingly, they found that transcripts with U-rich 3′ poly(A) tails are predominantly highly expressed in spermatids, underscoring the role of uridylation in the dynamic regulation of mRNA stability and function during gametogenesis.

Question for paper 3

5: What do you envisage might be the relationship/crosstalk between TENT5, TUT4 and TUT7 in germ cell development?

The **Discussion** section is rather short, but this is justified by the biological significance of the studies having been discussed in the attached manuscripts. The Candidate instead focuses on methodological advances obtained by DRS – first from the biology perspective (chapter 4.1) then technology (4.2).

The fifth chapter, Future perspectives, I found the most enjoyable read. This chapter to me displays the most scientific maturity, combining a nice discussion of previous work in the field - with ideas for the future, and stating Candidate's own hypotheses and opinions. Using an active voice here also helps readability. It seems to me that perhaps writing a short opinion piece in the near future could be a nice exercise for her – improving her writing mastery and giving more scientific confidence.

Summary

This PhD dissertation studies gene expression regulation in eukaryotic cells (yeast and murine), mainly mRNA post-transcriptional processing by polyadenylation, deadenylation, and mRNA degradation. Methodologically, the focus is on application of Oxford Nanopore's Direct RNA Sequencing (DRS) technology.
The thesis demonstrates the power of DRS in studying post-transcriptional gene regulation, overcoming limitations of Illumina-based methods, such as PCR amplification bias and short-read assembly challenges. In the context of this study, DRS allowed to analyze poly(A) tail lengths, dynamics, and non-canonical nucleotides.
Despite my focus in this review on areas that could be improved, I find this an excellent dissertation.

The key contributions of this thesis are:

1.Methodological: The use of DRS with tools like Nanopolish-polyA, TailfindR, and Ninetails enables precise measurement of poly(A) tail lengths and composition, which was not possible with earlier technologies.

2. Biological Insights: The studies on TENT5 non-canonical polyadenylases and non-adenosine nucleotides in germ cells allow a better understanding of mRNA regulation during gametogenesis. They also have potential implications for reproductive biology and therapeutics.

3. In Vivo Modeling: The dissertation's in vivo analysis of deadenylation rates in yeast (paper 1) provides a deeper understanding of mRNA turnover compared to in vitro studies.

Overall Assessment

All the parts of the thesis: the summarizing chapters as well as the publications included are of high scientific quality, well written, and the contribution of the PhD Candidate are clearly indicated. The conclusions drawn from the papers are convincing and scientifically sound. The cited literature is comprehensive and relevant.

Final Conclusion

I, the undersigned, hereby state that the doctoral dissertation of Agnieszka Czarnocka-Cieciura 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 Agnieszka Czarnocka-Cieciura 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.

In addition, due to the high scientific value and significance of the published work of the Candidate in my opinion the dissertation is worthy of a distinction.

Podpisano przez/ Signed by:
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