Tomasz W. Turowski, PhD, D.Sc.
Group Leader
Laboratory of Transcription Mechanisms
Email: tomasz.turowski@ibb.waw.pl
WWW: turowskilab.ibb.edu.pl

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Review of the Doctoral Dissertation by Agnieszka Maria Czarnocka-Cieciura

Information about the Dissertation

The dissertation entitled "*The Utilization of Advanced RNA Sequencing Technologies to Investigate Post-Transcriptional Mechanisms Involved in the Regulation of Gene Expression*" was submitted by Agnieszka Maria Czarnocka-Cieciura in the form of a series of three articles. The work focuses on the development and application of cutting-edge direct RNA sequencing (DRS) methodologies to study RNA polyadenylation (pA). It serves as a strong example of how innovative methodological approaches can expand the boundaries of our understanding of biological processes.

Evaluation of the Structure of the Doctoral Dissertation, Including Information About Its Individual Components

The dissertation is logically structured, well-organized, and includes all the elements typically expected in a doctoral thesis. The layout supports the clarity of the argumentation, and the writing is generally coherent and professionally edited.

Abstract and Introduction

The Abstract and Introduction effectively highlight the relevance and potential of direct RNA sequencing (DRS) technologies in studying RNA biology, particularly in the context of RNA polyadenylation (pA). The candidate clearly presents the motivation for the study and the novelty of applying DRS to interrogate post-transcriptional regulatory mechanisms.

However, several aspects could be improved to enhance the reader's understanding, particularly for those less familiar with Nanopore-based sequencing technologies. The addition of simple, illustrative figures—such as cartoons or schematic diagrams explaining the principle of DRS—would significantly increase accessibility.

One notable omission is an explanation of the signal detection mechanism used in Nanopore sequencing. Specifically, the introduction does not mention that the nanopore detects electrical signals from short nucleotide stretches rather than individual bases. This is a crucial technical detail that underlies the need for complex, computationally intensive algorithms to resolve sequence data from the raw current signal. Including this information would provide necessary context for

understanding both the capabilities and limitations of DRS.

Furthermore, the claim that Nanopore DNA sequencing provides accuracy comparable to that of Illumina sequencing is not supported by current literature. Recent studies have demonstrated that, while Nanopore sequencing has improved considerably, it still lags behind Illumina in base-calling accuracy, especially for certain genomic regions and sequence motifs. For example, the studies by Stevens et al. (Scientific Reports, 2023, [10.1038/s41598-023-36101-8]) and Bejaoui et al. (BMC Genomics, 2025, [10.1186/s12864-025-11267-9]) offer quantitative comparisons illustrating these limitations. A more cautious formulation of this comparison, supported by up-to-date references, would strengthen the credibility of the introduction.

Additionally, when discussing the cellular surveillance mechanisms targeting aberrant RNAs, particularly the role of the TRAMP complex, an important reference was omitted. The study by Delan-Froino et al. (Nature Communications, 2020, [10.1038/s41467-020-16965-4]) provides key insights into the interplay between TRAMP-mediated RNA decay and post-transcriptional regulation. Including this reference would have enriched the background and demonstrated a more comprehensive engagement with the literature.

Overall, the purpose of the dissertation is clearly defined. The candidate convincingly outlines how the developed methodological framework can be employed to investigate fundamental aspects of post-transcriptional gene regulation, offering potential applications beyond the specific context of polyadenylation. The introduction provides a strong rationale for the study and positions the research within the broader field of RNA biology.

Results

Manuscript 1: Modeling of mRNA deadenylation rates reveal a complex relationship between mRNA deadenylation and decay

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. 2024Dec;43(24):6525-6554. doi: 10.1038/s44318-024-00258-3. Epub 2024 Oct 11. PMID:39394354; PMCID: PMC11649921

The first work, "*Modeling of mRNA deadenylation rates reveals a complex relationship between mRNA deadenylation and decay*" is a quantitative investigation of mRNA deadenylation dynamics in yeast (*S. cerevisiae*), using DRS to monitor poly(A) tail status across the transcriptome under steady-state and stress conditions. A major achievement of this study is the direct calculation of transcriptome-wide deadenylation rates, revealing that, on average, poly(A) tails are shortened at a rate of 10 adenosines per minute. This estimate was made possible by applying DRS to time-resolved samples (chase experiments), demonstrating the power of DRS not only for transcript identification but also for dynamic analyses of RNA metabolism.

To interpret the sequencing data quantitatively, the team developed a custom numerical model based on a modified gamma distribution, enabling robust extraction of kinetic parameters from poly(A) tail length distributions. Although the modelling component was led by Prof. Poznański, its integration into the biological workflow underscores the value of close collaboration between experimentalists and computational modelers. The ability to incorporate mechanistic modelling into transcriptomic data analysis is an increasingly important skill for bioinformaticians, as it allows for a deeper, mechanistically grounded understanding of cellular processes. The model is particularly interesting because it explains deadenylation dynamics through the number of Pab1 proteins bound to the

poly(A) tail. The authors propose a model where a 20-nt poly(A) tail is sufficient for binding a single Pab1 protein and providing decapping protection, while longer tails accommodate multiple Pab1 proteins. Although the modelling was not performed by the doctoral candidate, this close collaboration is considered an asset of the dissertation.

Additionally, the study validated deadenylation-rate estimates using a simplified approach based on quantile values of poly(A) tail lengths, revealing a strong correlation between mRNA decay and deadenylation rates. These correlations were consistent within functional gene groups and influenced by codon optimality. Notably, the authors observed that these rates shifted during heat stress, highlighting the dynamic regulation of mRNA stability.

A particularly notable finding was that ribosomal protein-coding mRNAs (RPG mRNAs)—which constitute a substantial portion of the yeast transcriptome—underwent both accelerated deadenylation and decay during heat stress. Surprisingly, their degradation could proceed even when deadenylation was experimentally blocked, but only if nuclear export remained active. This suggests that, while deadenylation typically governs the initiation of mRNA decapping, the relationship between these processes is context-dependent and more complex than previously assumed.

Overall, the study provides compelling evidence that DRS, combined with kinetic modelling, can inform fundamental aspects of mRNA metabolism, offering tools and conceptual advances that will benefit both basic research and computational biology.

One caveat of this work is that any perturbation in the synthesis–processing–decay machinery affects other components, as shown, for example, in the cited work by Sun et al. (Genome Research, 2012). It would be important to clarify how such systemic effects were controlled. For instance, thiolutin treatment inhibits RNA synthesis and may thus indirectly suppress decay machinery activity.

The authors demonstrated substantial changes in polyadenylation dynamics upon heat shock. Since such stress affects overall cell metabolism, including energy-related molecules such as ATP, it would be valuable to discuss the relationship between ATP concentration and polyadenylation kinetics.

In this work, the contribution of Agnieszka Czarnocka-Cieciura was substantial, as reflected by her first-author status. Moreover, her individual expertise was crucial for conducting the analysis for the manuscript, further confirming her key role in the study.

Manuscript 2: TENT5-mediated polyadenylation of mRNAs encoding secreted proteins is essential for gametogenesis in mice.

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.

The second work presented in the dissertation, "*TENT5-mediated polyadenylation of mRNAs encoding secreted proteins is essential for gametogenesis in mice*" focuses on the role of TENT5 proteins in mouse gametogenesis. Most of the work involved generating and characterizing mouse lines with either tagged or deleted TENT5 proteins.

TENT5B and TENT5C were shown to be essential for oocyte development. While mice lacking either gene individually remained fertile, the combined knockout resulted in oocyte degeneration and female infertility. Furthermore, introducing a TENT5B-GFP fusion protein in knock-in mice led to a

gain-of-function effect, causing infertility associated with chromosomal abnormalities in ovulated oocytes.

In males, TENT5C and TENT5D were each found to be essential for different stages of spermatogenesis. Knockout of either gene resulted in complete male sterility, indicating non-redundant roles in sperm development. Additionally, deletion of Tent5a reduced overall fertility, although this effect did not appear to result directly from gametogenesis defects.

To gain mechanistic insight, the author analysed DRS data and performed various analyses. Although RNA samples were pooled from groups of animals, the presentation of individual poly(A) tail lengths in boxplot format enabled statistical evaluation. This allowed the identification of several mRNAs with significantly shorter tails in Tent5b/c double knockout samples—mRNAs known to play key roles in oogenesis, such as Zona Pellucida Glycoprotein 3 (Zp3) and Growth Differentiation Factor 3 (Gdf9). The authors concluded that TENT5B and TENT5C polyadenylate a specific subset of mRNAs essential for oogenesis, thereby enhancing their expression.

The analysis of TENT5-regulated mRNAs was systematic and well-planned. Despite several negative results—for example, in CPEB binding motif searches, *de novo* motif searches, and UTR/CDS length analyses—the functional analysis was still informative. However, the statement that "differences in the length of UTR segments, exon length, and GC content (Supplementary Fig. 7C, D), although statistically significant, were negligible" warrants further explanation. Additional questions include whether the author examined other features such as RNA secondary structure, mRNA abundance, translation rate, or translation efficiency.

In this work, the author was a bioinformatician. Her contribution was particularly critical for the DRS analysis, due to the unique methodological requirements. Although the Illumina-based analyses were relatively routine and could have been performed as a service, the candidate's intellectual input in motif searches and customized analyses (e.g., examining UTR and CDS lengths) should be emphasized. While the study was a collaborative effort involving many researchers, Agnieszka Czarnocka-Cieciura's position as second author highlights her significant contribution.

Manuscript 3: Comprehensive analysis of poly(A) tails in mouse testes and ovaries using Nanopore Direct RNA Sequencing.

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 third manuscript, "*Comprehensive analysis of poly(A) tails in mouse testes and ovaries using Nanopore Direct RNA Sequencing*" focuses on identifying non-adenine residues within poly(A) tails. The dataset includes RNA samples from both wild-type and TENT5-deficient mice, covering testes and ovaries, and captures transcriptomic changes caused by the loss of these enzymes. These deficiencies have important biological consequences—most notably, TENT5D dysfunction is linked to infertility in humans, underscoring the clinical relevance of this work.

The data reveal key features of mRNA regulation during gametogenesis. For instance, uridylation of poly(A) tails is commonly observed in testicular transcripts—a modification that may signal RNAs for degradation or modulate their stability and translation. In oocytes, where cytoplasmic polyadenylation occurs in discrete waves, this dataset provides a valuable resource for understanding how poly(A) tail dynamics support oocyte maturation and developmental potential.

In this study, the author performed downstream analyses (after Ninetails processing) and visualized poly(A) tail composition from DRS data, as well as conducted differential expression analysis of RNA-seq data. Importantly, Agnieszka Czarnocka-Cieciura also drafted the initial version of the manuscript, confirming her substantial contribution.

Applied aspect of doctoral dissertation

Although the research presented in the dissertation is primarily fundamental, some elements have potential for practical application. Most importantly, the methods developed and applied by Agnieszka Czarnocka-Cieciura represent the cutting edge of bioinformatics for DRS, an area with very few specialists worldwide. Given the importance of mRNA stability measurements in the pharmacokinetics of RNA therapeutics, it is highly likely that the methodologies she helped develop will soon be adopted by the pharmaceutical industry.

Furthermore, two of the studies address the dynamics of polyadenylation during gametogenesis. As infertility becomes an increasingly pressing global issue—and considering that gametogenesis is tightly regulated by mRNA metabolism—this research provides valuable foundational insights.

Discussion, Perspectives, and Bibliography

This discussion highlights how DRS has advanced our understanding of poly(A) tail dynamics in two distinct biological systems: yeast and mouse germline development. While the section effectively summarizes the main findings of the dissertation, it lacks a broader contextualization within the current body of scientific literature. The technical contributions of DRS are well emphasized; however, the opportunity to demonstrate how these findings refine or challenge existing biological models of mRNA regulation is not fully realized. For instance, the model of deadenylation kinetics developed for yeast could have been discussed in comparison with earlier estimates based on traditional methods.

A more integrative discussion that compares the dissertation's results with the wider literature would enhance the case for DRS not only as a powerful methodological advance but also as a tool that delivers unique biological insights. This would help to demostrate that DRS is more than a sequencing innovation—it is capable of reshaping our fundamental understanding of gene expression regulation.

The discussion also addresses current technical limitations and emerging solutions in nanopore signal analysis, particularly regarding homopolymer base calling and the detection of nucleotide modifications. Tools such as Ninetails and BaseNet are beginning to overcome the challenges posed by signal noise and nucleotide context dependence. Moreover, the application of Incremental Learning approaches offers a promising avenue for real-time model updates, paving the way for adaptive and more accurate analyses. These developments position DRS not only as a platform for biological discovery but also as a catalyst for continued methodological innovation.

The author also puts forward several thought-provoking hypotheses. One such hypothesis is that downregulation of ribosome biosynthesis represents an early stress response, given the high energetic cost of ribosome production. In this context, the dissertation's findings on the role of nuclear export in the decay of ribosomal protein mRNAs offer a new perspective on ribosome regulation during stress. Another hypothesis concerns the existence of targeted deadenylation mechanisms—acting as a counterpart to targeted polyadenylation—which may play a selective regulatory role in transcript stability. Additionally, the potential functional significance of non-canonical nucleotide incorporation and distribution within poly(A) tails is proposed as a promising area for future research.

The bibliography is well prepared. However, the literature cited in the Introduction focuses almost exclusively on the dissertation's specific topic. General review articles and broader contextual references would have helped to better frame the research within the field of RNA biology.

Conclusions

Considering both the development and the application of cutting-edge methodologies to biologically relevant research questions, I am convinced that this doctoral dissertation represents an original and valuable contribution to the field. In particular, the work significantly advances our understanding of poly(A) tail dynamics through the innovative use of DRS, offering novel insights and analytical strategies that are likely to shape future studies in RNA biology.

As demonstrated throughout the dissertation, Agnieszka Czarnocka-Cieciura has developed a comprehensive and sophisticated repertoire of bioinformatics tools for DRS analysis. These include raw data processing, accurate poly(A) tail length determination, modelling of mRNA half-lives based on time-course data, trend fitting, data categorization, and clustering—techniques that were applied consistently and effectively across multiple projects.

The candidate's substantial contributions to three experimental studies—as co–first author on two and second author on the third—published in highly respected, peer-reviewed journals, clearly reflect her deep understanding of molecular biology, strong analytical capabilities, and high level of scientific maturity. Her ability to design and execute complex analyses, contribute intellectually to interdisciplinary projects, and take a leading role in manuscript preparation demonstrates her readiness to conduct scientific research at the postdoctoral level.

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.

Dr hab. Tomasz W. Turowski

Podpisano przez/ Signed by:
Tomasz Wojciech
Turowski
Data/ Date: 23.06.2025 12:17
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