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Review of the Doctoral Dissertation by Jacek Nikodem Szymański

Information about the Dissertation

The dissertation entitled "*5′ terminal nucleotide modulates the immunogenicity of*" was submitted by Jacek Nikodem Szymański in the form of a series of two manuscripts: one published and one posted as a preprint. The work focuses on the role of the 5′ terminal nucleotide of RNA in modulating immune responses through cellular receptors of innate immunity. It addresses a highly novel and significant area of RNA biology with strong potential to guide the development of future RNA-based therapeutics.

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 significance of the 5′-terminal nucleotide of RNA in modulating innate immunity. The candidate clearly presents the motivation for the study and provides a well-structured outline of the novelty of the research.

In the context of the candidate's highly mechanistic and biochemical work, the Introduction appears to diverge towards signalling cascade, which is less relevant for this study. At the same time, the author could expand on mechanistic aspects of receptors other than RIG-I, for example, discussing

how MDA5 recognizes long dsRNA sequences. Such a review is largely missing in the literature and would add value by transforming the Introduction into a more mechanistic review article.

Figure 1 omits a recently described source of pathogenic RNA, where viral dsDNA is transcribed by the cytoplasmic fraction of RNA polymerase III, resulting in 5′-triphosphate (ppp) RNAs (https://doi.org/10.1152/physiol.00022.2019).

It is worth noting that the author successfully places the findings in a broader context by introducing *in vitro* transcription (IVT)—a fundamental step in the synthesis of modern RNA-based therapeutics—as a key source of immunogenic contaminants.

Overall, the purpose of the dissertation is clearly defined. The candidate convincingly demonstrates the importance of the research questions and provides a high-quality Introduction.

Results

Manuscript 1: 5' terminal nucleotide determines the immunogenicity of IVT RNAs

Wolczyk M.*, **Szymanski J.***, Trus I.*, Naz Z., Tame T., Bolembach A., Choudhury N.R., Kasztelan K., Rappsilber J., Dziembowski A., Michlewski G. 5' terminal nucleotide determines the immunogenicity of IVT RNAs. *Nucleic Acids Res.* (2025). DOI: 10.1093/nar/gkae1252.

In this work, the authors demonstrated that IVT transcription of RNAs with a 5′-terminal pppA is significantly more immunogenic than those with pppG. This effect is attributed to the strong tendency of pppA-initiated RNAs to promote RNA-templated transcription, resulting in a higher content of dsRNA in IVT products starting with pppA.

The description of the results has good flow and is clear and logical. Using T7 RNA polymerase IVT, ssRNA fragments capable of forming panhandle-like dsRNA structures were synthesized with either 5′-pppA or 5′-pppG initiation. Across human (HEK293, A549) and murine (MEFs, BMDMs) systems, 5′-pppA RNAs consistently induced markedly stronger IFN responses than 5′-pppG RNAs. The authors also confirmed an essential role of the RIG-I receptor in this process by combining cellular and biochemical approaches.

Mechanistic studies revealed that 5′-pppA initiation promotes the formation of dsRNA byproducts, detected via J2 dot-blot, PAGE, and RNA pulldown followed by proteomic analysis (RP-MS). RP-MS revealed dsRNA IP enrichment in dsRNA-binding to proteins (ADAR, PKR, DICER1).

Semisynthetic RNA experiments demonstrated that immunogenicity is driven by these dsRNA impurities rather than the ssRNA itself. This phenomenon extends to longer transcripts, including therapeutic RNA models, where 5′-pppA initiation correlates with higher dsRNA contamination and IFN activation. In the context of this finding, the role of the RIG-I receptor is particularly intriguing, as dsRNA is typically recognized by other PRRs. A discussion of potential explanations for this observation would be highly interesting.

Overall, the findings highlight that the choice of initiating nucleotide profoundly impacts IVT RNA

immunogenicity, emphasizing the need for improved strategies to minimize dsRNA impurities in RNA-based therapeutics and vaccines.

The significant contribution of Jacek Szymański to this work is beyond doubt. This is reflected not only in the co-first authorship but, more importantly, in his substantial involvement in the design and execution of experiments, data analysis, and manuscript preparation.

Manuscript 2: 5′-triphosphate guanosine RNAs recruit GTP-binding proteins to suppress RIG-I/IFN type I signalling.

> **Szymanski J.***, Wolczyk M.*, Trus I., Naz Z., Idlin N., Jackiewicz J., Nowak E., Wuebben C., Hartmann G., Rappsilber J., Michlewski G. 5′-triphosphate guanosine RNAs recruit GTP-binding proteins to suppress RIG-I/IFN type I signalling. *bioRxiv* (2025). DOI: https://doi.org/10.1101/2023.12.22.573000

In this manuscript, the authors investigated how the specificity of the 5′-ppp end is recognized by cellular machinery. The work begins with a bioinformatic analysis demonstrating that Pol III transcripts naturally carry a 5′ppp terminus, typically initiating with 5′-pppG, whereas many RIG-I–activating viral RNAs start with 5′-pppA. This raised questions about whether the cellular machinery can discriminate the identity of the very first nucleotide.

To address this, the authors synthesized dsRNAs bearing either 5′-pppA or 5′-pppG and demonstrated that, in human and murine cells, 5′-pppA dsRNAs induced stronger RIG-I signalling and IFN responses than 5′-pppG. This was further confirmed using reporter mice and by the loss of response in 5′-p controls.

Biochemical assays revealed a higher RIG-I binding affinity for 5′-pppA dsRNA, although ATPase activity remained unchanged, suggesting enhanced binding without proportional activation.

Moreover, the authors performed proteomic RP-MS studies showing enrichment of protein interactors associated with 5′-ppp-terminal RNAs. This analysis identified that GTP-binding proteins (NUDT16, RAN, RANBP1) preferentially interact with 5′-pppG dsRNA, reducing its availability for RIG-I. This finding was further validated in cellular systems by demonstrating that guanosine supplementation disrupted these interactions and diminished the activation gap between 5′-pppA and 5′-pppG RNAs.

Collectively, these findings indicate that 5′-pppA dsRNAs more effectively stimulate innate immunity, while GTP-binding proteins modulate recognition of 5′-pppG RNAs. The study uncovers a novel mechanistic insight into dsRNA recognition by RIG-I, placing it within a broader cellular context.

Similar to MS1, the significant contribution of Jacek Szymański to this work is beyond doubt. This is reflected in the co-first authorship and his substantial involvement in experimental design and execution, proteomic data analysis, and manuscript preparation.

Applied aspect of doctoral dissertation

This research provides critical insights into the design and safety of RNA-based therapeutics and vaccines. By demonstrating that the identity of the 5′ terminal nucleotide profoundly influences the immunogenicity of in vitro transcribed (IVT) RNAs, the work highlights a previously underappreciated source of variability in innate immune activation.

The discovery that 5′-pppA initiation promotes the formation of dsRNA byproducts, which drive strong RIG-I–mediated responses, underscores the need for improved IVT protocols and purification strategies to minimize these contaminants. Furthermore, the identification of cellular GTP-binding proteins that selectively interact with 5′-pppG RNAs reveals an additional layer of regulation that could be exploited to fine-tune immune responses.

These findings have direct implications for mRNA vaccine development, gene therapy, and antiviral RNA design, where controlling immunostimulatory properties is essential for both efficacy and safety. Ultimately, this work provides a mechanistic foundation for optimizing RNA synthesis and formulation to achieve predictable immune profiles in clinical applications. It is an outstanding example of how fundamental research can directly inform decision-making in drug design and optimization pipelines.

Discussion, Perspectives, and Bibliography

The results are thoughtfully discussed in the context of alternative strategies for generating the 5′ end, such as 5′ capping or the use of dinucleotide primers.

Another noteworthy aspect is the difference in GTP-binding affinities compared to ATP binding. This demonstrates that the PhD candidate approaches the problem not only from an *in vitro* perspective but also considers a broader biochemical context, including GTP-binding proteins and cellular GTP concentrations.

The perspectives are briefly described, but provide a well-rationalized and forward-looking direction, focusing on the use of native RIG-I agonists transcribed by RNA polymerase III.

The bibliography is well prepared. The literature cited in the Introduction is well selected and sufficiently broad to demonstrate the author's comprehensive knowledge. An editorial error is present on page 23: the word "reference."

Conclusions

Considering all aspects of the presented work—including the important biological questions, rigorous methodology, diverse RNA- and protein-based techniques, scientific novelty, and translational relevance—I am convinced that this doctoral dissertation represents an original and valuable contribution to the field, with significant contribution from Jacek Szymański.

The candidate's substantial involvement in two experimental studies—both as co–first author, one of which was published in a highly respected, peer-reviewed journal—clearly reflects his proficiency in the subject matter.

The dissertation is well balanced, containing all essential elements while avoiding shortcuts and overstatements. Altogether, the work presented for review demonstrates Jacek Szymański's ability to apply sophisticated techniques and contribute analytically and intellectually to complex projects, which confirms his readiness to conduct scientific research at the postdoctoral level.

I, the undersigned, hereby state that the doctoral dissertation of Jacek Nikodem Szymański 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 Jacek Nikodem Szymański 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 the scientific quality and importance of the work, I recommend that this doctoral dissertation be distinguished.

Dr hab. Tomasz W. Turowski

Podpisano przez/ Signed by:
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Turowski
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