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Review of the Doctoral Dissertation of Jacek Szymański

The doctoral dissertation "5' terminal nucleotide modulates the immunogenicity of RNA" by Jacek Nikodem Szymański was prepared under the supervision of Prof. Gracjan Michlewski (main supervisor) and Dr Magdalena Wołczyk (auxiliary supervisor). Jacek Szymański was enrolled in the Warsaw PhD School in Natural and BioMedical Sciences and the dissertation was submitted to the Doctoral Committee at the International Institute of Molecular and Cell Biology in Warsaw.

The main goal of the work described in the dissertation was to characterize features of pppRNA that elicit an immune response, in respect to the identity of the first (purine) nucleotide, and especially in the context of recognition by the mammalian RIG-I receptor. The results are novel and are part of the research program of Prof. Gracjan Michlewski's Laboratory of RNA-Protein Interactions. The title of the dissertation fits its topic which is relevant to development of the discipline of biological sciences.

The dissertation is in the form of a collection of two articles (Manuscript 1 published in *Nucleic Acids Research*, and Manuscript 2 which is still a preprint). This is accompanied by an abbreviated form of a written thesis – an introduction, briefly stated research objectives, a short results section, a concluding "summary and future perspectives" section, and a bibliography (whereas the methods section is absent). The mandatory declarations of the Candidate's contributions to the articles are included after each Manuscript. The dissertation is written in English, and uses an appropriate and clear language. There are some minor typos (e.g. "*double-strandness*") and a missing "*(reference)*" in the concluding section, but it does not detract from the readability. The Candidate perhaps tends to overuse the term "panhandle" to describe RIG-I ligands throughout the text, while - as he correctly mentions in the *Introduction* - also natural and artificial non-panhandle structures can fulfil the requirements, and the term "stemloop" or "hairpin" are more commonly used for short RNAs. Overall, the reference to, and choice of, literature is satisfactory and the way the Candidate describes both already published and own work is mature and factual, with clear logic.

The *Introduction* is well written, starting with a description of PPR signalling (illustrated by a schematic Figure 1), then going in more detail into the four main receptor protein families (TLR, RLR, NLR, PKR). Next, important features of RIG-I ligands are described (summarised by Figure 2). The rest of the introduction concerns unintended dsRNA production during in vitro transcription (IVT) by the T7 RNA polymerase. This includes origins of dsRNA byproducts and strategies to reduce their occurrence, for example by T7 RNA polymerase engineering. Overall this section constitutes a sufficient introduction to the topic, though perhaps a schematic figure explaining ways of the dsRNA byproducts formation would have made the content even clearer. The last sentence of the *Introduction* regards dsRNA byproducts and points to the Candidate's first goal of characterizing the immunogenicity of IVT

products. The second research goal is therefore largely omitted in the *Introduction*, although stated next in the *Research objectives* as the studies of the role of the first nucleotide of pppRNA in cells.

The *Results* section describes shortly the contents of the Manuscripts 1 and 2, each accompanied by one schematic Figure summarising the main findings of the respective article. The dissertation aimed at comparing the immunogenicity of pppRNA that differs in the sequence of the first nucleotide (A or G). One approach (Manuscript 1) described increased dsRNA impurities produced during in vitro transcription (IVT) of pppA-RNA (as compared to pppG-RNA) which caused higher immune response when transfected into cells. The second approach (Manuscript 2) explored cellular factors that may lead to a differential immune response to pppA-RNA versus pppG-RNA. Here, the Candidate's work contributed to the discovery that GTP-binding and GTPase proteins are enriched on pppG-RNA, indicating binding that likely protects certain transcripts from recognition by the RIG-I pathway. The *Results* section contains no figures with any of the experimental data analyses. The work of the Candidate encompasses a broad range of methods, ranging from RNA preparation via IVT, RNA splint ligation, through biochemical assays with recombinant RIG-I (EMSA and ATPAse activity), to assays of the immune activation in cells, and also RNA pulldowns and proteomic analyses. This all contributes together to comprehensive stories in both Manuscripts and demonstrates an excellent theoretical and practical background of the Candidate in the biological sciences.

The work presented in the Manuscripts clearly constitutes an original approach to scientific questions, and presents novel findings with significant implications, and the Candidate has made major contributions to these works as indicated by a main author position. However, in *Results*, the Candidate writes almost exclusively in the plural "*we*" or in the passive voice, only once at the beginning stating "*I* employed IVT". This might be the most appropriate from for scientific articles, but in a dissertation it unfortunately obscures the amount of original work contributed by the Candidate. It would have been advisable to point to the exact data and Figures in the manuscripts that were produced and analysed by the Candidate. In the *Candidate's Contribution Statement* for Manuscript 2, the Candidate states he designed and conducted "all RNA pulldown experiments" and analysed "all proteomic data". This suggests that the remaining tasks and contributions were a shared or partial work (e.g. that other collaborators also performed EMSA etc.), especially in the Manuscript 1 which has 3 shared co-first authors, suggesting their significant contributions. Again, this is quite natural in original research articles which most often are collaborative works. In a dissertation however, it is expected to make it quite clear as to which results were produced and analysed by the Candidate on his own, so that his capacity for independent work can be judged fairly. Luckily, the Candidate's contribution statements claim that the Candidate not only performed experiments but also designed assays, analysed data and participated in the writing of the article (hopefully, in the first draft of the manuscript, though this is not explicitly stated). In summary, although the quality of work contained in the Manuscripts is excellent, and the Candidate's scope of the methods is impressive, it would be more beneficial for the Candidate to describe the extent of his original contribution in more detail.

The "*Summary and future perspectives*" section is perhaps the most revealing of the Candidate's potential for scientific reasoning. The Candidate clearly identifies the significance of his work for the field, in both applied and basic research aspects. Manuscript 1 demonstrates that pppA-RNA

production by IVT is more prone to dsRNA formation than pppG-RNA, and therefore more immunogenic, which might be of note for the industry producing mRNA therapeutics. However, some large-scale protocols use co-transcriptional capping by using di- or trinucleotide cap analogs, instead of pppRNA production followed by enzymatic capping. The Candidate correctly states that future work should address whether the increased dsRNA byproducts formation also holds true for co-transcriptionally capped RNA starting with A. Manuscript 2 describes a new mechanism in the regulation of the RIG-I pathway by the choice of the starting nucleotide, and the selective binding of proteins "working on GTP" to pppG-RNA. Such binding may mask the 5' ends of some endogenous transcripts and prevent any unwanted initiation of the RIG-I/IFN pathway. The Candidate suggests that elevated GTP levels in the cells during viral infection may free the bound pppG-RNA to enhance the immune response. These findings are of relevance to the innate immunity field and the development of immunoregulatory therapeutics and strategies. It will be exciting to see in what final form and where the Manuscript 2 will be published.

I have some questions and suggestions to which the Candidate may respond during the defence:

1. On the formal side, one concern is that Figure legends do not state the source of the illustration – Figure 3 is part of the Manuscript 1 (published), Figure 4 is part of Manuscript 2 (a preprint). The Candidate should have cited his own articles as the source, since the publisher's licence has likely been agreed on. From the appearance, some of the Figures might have been prepared using BioRender, which should have been stated and appropriately cited both in the articles and in the thesis. Please include proper references to both articles and tools (according to the signed licences) during the PhD defence and in future presentations and publications.
2. During presentation, for each experimental result shown (not all data can be shown, of course), please indicate clearly which piece of data was produced and analysed on their own by the Candidate and which in collaboration with a team member and to what extent.
3. I am missing the discussion of the mechanism that leads T7 RNA polymerase to produce more dsRNA byproducts for pppA-RNA, as well as the mention of promoter class. Would the Candidate speculate that this is due to less efficient transcription initiation from the different class of T7 promotor used (which one?)? Which unintended events are likely to blame? Please discuss the use of different classes of T7 promoters used to produce 5'-pppA RNA or 5'-pppG RNAs.
4. It would be interesting to comment on whether A-starting RNA (including m^6A) would have a known advantage in translation or other processes that could explain its selection and prevalence in some viral RNAs, balancing out the increased risk of immune recognition.

I evaluate this PhD dissertation positively. The Candidate presents a sound and original approach to each of the scientific problems and research objectives. The results described in the dissertation are of high relevance to the field of innate immunity and RNA therapeutics.

I, the undersigned, hereby state that the doctoral dissertation of Jacek 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 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.

Date 19.12.2025

Signature

Maria Górna

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