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Review of doctoral dissertation

"The role of phosphorylation of ISGF3 components in the regulation of ISG expression and viral protection"

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under supervision of
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1. Importance of undertaken research

The research problem is part of a valid research direction, aimed at the elucidation of host-virus interactions and the mechanisms of antiviral immune response. Broadening our understanding of this field is essential to developing new preventive and therapeutic strategies.

2. Structure of dissertation and formal evaluation

The thesis has a form a monograph, consisting of 162 pages and is written in English. The structure is well ordered, it includes: title pages in Polish and English, table of contents, introduction divided into subchapters, hypothesis and five goals, materials and methods, results divided into five parts (I-IVB), discussion, a list of 187 references (in alphabetical order), a list of 54 figures, 8 tables and 41 abbreviations. This is followed by a list of 6 publications co-authored by the PhD Candidate; five of them published in the years 2013-2022; the sixth position refers to a manuscript summarizing the results obtained in this dissertation (the manuscript in preparation). There is also information on the funding of the research under 3 NCN grants: PRELUDIUM, in which the PhD Candidate was the PI, and 2 OPUS grants, directed by her Supervisor. The thesis ends with acknowledgments and abstracts (in English and in Polish).

The layout of the dissertation is standard for this type of works. What deserves attention, is the diligence and aesthetics of the preparation in terms of graphics and editing. The content of the work is richly illustrated, and the figures and legends are very informative. Well organized structure and rich illustrations facilitate the perception of this dissertation, which is extensive and complex.

3. Content-based evaluation

The **Introduction** starts with presenting the research problem in a broad context. The Author refers to the threat of infections caused by HIV, Ebola, swine or avian flu, and COVID-19 viruses, which pose medical problems, but also global socio-economic challenges. This broad context allows to appreciate the

importance of the undertaken research topic. The Author then presents the **functioning of the immune system** in a synthetic way, discussing the innate and adoptive immune responses, in both cases describing the role of the cellular and humoral response.

The introduction is further divided into sections, the first of which deals with the general **characteristics of interferons**. The Author presents an overview of the discoveries of IFNs and IFN-related pathways and discusses the mechanisms of stimulation of IFN production by microbial molecules called PAMPs (pathogen-associated molecular patterns) and by endogenous factors termed DAMPs (damage-associated molecular patterns). She then systematically presents IFN-related pathways, describes each group of IFNs (type I, II and III), their coding genes, the cells secreting each of the IFN types and the relevant receptors. She describes the common feature of these pathways, which is the activation of the JAK-STAT signalling, followed by the formation of protein complexes with the participation of STAT, one of them being Interferon Stimulated Gene Factor 3 (ISGF3) complex and the subsequent activation of transcription of Interferon Stimulated Genes (ISGs) involved in antiviral response. Since IFNα signalling is the main focus of this dissertation, the Author specifically describes the **IFN Type-I production**: the role of Toll-like receptors in the recognition of PAMPs and DAMPs, the role of downstream transcription factors, including interferon regulatory factors (IRFs) stimulating the production of IFNα and IFNβ and the positive feedback loop, resulting in the boost of the antiviral response.

Having introduced the information about interferons, the Author presents **canonical IFN-Type I signalling pathway**, devoting the next sub-sections to the detailed description of the structure and function of the Janus-activated kinases (JAKs) and signal transducer and activator of transcription proteins (STATs). She points to the fact that the phosphorylation of specific tyrosine residues in the STATs was originally thought to be key for the regulation of antiviral genes' expression, but current studies suggest that also unphosphorylated STATs can be functional. In this way, she points to the main focus of the dissertation. Separate sections are devoted to the detailed description of STAT1 and STAT2. Finally, the Author presents Interferon Regulatory Factors (IRFs) with separate sections devoted to IRF9 and IRF1.

In this way, the Author presents all three components (STAT1, STAT2 and IRF9) forming the **Interferon stimulated Gene Factor 3 (ISGF3) complex**, which is the main subject of this dissertation. Consequently, in the next section, she describes the mechanism of ISGF3 formation. She points to the dynamics and robustness of this process, which is vital for a rapid antiviral response. She also describes the possible roles of unphosphorylated ISGF3 components.

The Author further presents the **negative regulation of the IFN Type-I signalling pathway** and explains, how the failures of the suppression of this pathway result in prolonged immune response and contribute to immunological disorders. She describes the various suppression mechanisms, including: endocytosis and liposomal degradation of IFN-receptors, activity of phosphatases that inactivate JAK-STAT proteins, the activity of PIAS proteins (protein inhibitors of activated STATs), SOCS (suppressor of cytokine signalling) proteins and the USP18 (ubiquitin-specific peptidase 18) protein.

The next large part of the Introduction describes the **non-canonical IFN-signalling pathways,** independent of ISGF3 complex, which are complementary ways to provide protection against viruses, important in the light of constant viral evolution. The Author describes the role of STAT2/IRF9 complex in the absence of STAT1 protein, which is a kind of a back-up system evolved in response to viruses able to block STAT1. She presents the studies indicating that STAT2/IRF9 complex is sufficient to combat some viruses in the absence of STAT1 protein. Another form of non-canonical IFN-signalling pathway is that with the participation of **unphosphorylated STAT-based complexes**. The Author describes the role of two complexes U-ISGF3 and U-STAT2/IRF9 in the basal and prolonged expression of ISGs, in the absence and in

the presence of IFN Type-I stimulation. She points to the possible cooperation of classical ISGF3 and U-ISGF3 complex in triggering antiviral response.

Further, the Author presents the overview of **genome-wide studies** aimed at the investigation of IFN Type-I signalling pathway and points to technological advancements, from microarray to genome-wide experiments. She presents the results of studies aiming at the identification of gene expression profiles and the interactions of the components of ISFG3 complex with DNA upon IFN stimulation. She points to the need for the integrative studies combining gene expression profiling with ChIP-seq experiments.

The next section of the Introduction describes the model of a positive feedback loop existing in the regulation of STAT1, STAT2, IRF9s gene expression. The Author points to the complexity of the IFN-dependent responses and to, the still not fully elucidated, role of phosphorylation status of ISGF3-components in antiviral protection. She nicely ends this section with a number of questions indicating research gaps in the field, which this dissertation aims to answer.

In the last section of the Introduction, the Author describes a large group of **Interferon Stimulated Genes** (ISGs). Importantly, only some of these > 300 genes' products have direct antiviral activities, many are involved in apoptosis, pathogen recognition or cell signalling. This section highlights the complexity and different roles of IFN-signalling.

Altogether, the Introduction is comprehensive (takes 47 pages) and it fully serves its role – introduces the reader into the merits related to the research presented in the thesis. It is well structured (from general-to-specific order) and very well illustrated. Some parts of the Introduction seem to be the repetition of the information given in previous sections, but probably this has been done intentionally to describe this complex content in an ordered manner. This prolongs the introduction but contributes to its clarity.

The Hypothesis of the dissertation is well formulated and clear. It assumes that classical ISGF3, U-ISGF3, pSTAT2/IRF9 and U-STAT2/IRF9 complexes cooperate in constitutive and IFN-dependent transcriptional regulation of ISGs and in antiviral responses, in a time-, phosphorylation- and concentration-dependent manner. To test the hypothesis, the Author defined five specific objectives of the dissertation, which are then well addressed in the Results section and in the Discussion.

The description of **Materials and methods** reflects a broad range of techniques used in this research and a number of collaborations with other research units. One might assume that the Author gained valuable experience during her laboratory work and also on the occasion of these collaborations. The methodology section takes 19 pages, it includes 8 tables, 2 lists of in house made buffers, and 5 figures. Some descriptions of the methodology could, however, be a bit more elaborate, which I further describe in point 4: 'Specific comments and questions.' Perhaps a general diagram showing the workflow of presented research would also be useful in this section.

The **Results** section consists of 53 pages and is divided into five parts (I-IVB), presenting the outcomes of experiments addressing the role of different ISGF3 component-based complexes and their phosphorylation status in canonical IFNα signalling pathway, in the absence of STAT1 protein and in the basal conditions. These parts correspond with the five specific objectives of this thesis.

In the first part, the Author describes the results of experiments performed in 2 cell lines (2fTGH and Huh7.5) in basal conditions and at different time points upon stimulation with IFNα. She starts with **Western blot** analysis, aimed at the identification of the patterns of protein expression of STAT1, STAT2, and IRF9, as well as the pattern of phosphorylation of STAT1 and STAT2. These results are combined with the results of **genome-wide gene expression analysis** (validated by q-PCR) of the same samples and the same timepoints, to identify genes upregulated upon IFNα stimulation, and with the results of **ChIP-seq**

analysis (for selected time points) to study the binding of STAT1, STAT2, pSTAT1, pSTAT2 and IRF9 upon IFNα stimulation. The integrative analysis of these results enabled to describe the transient patterns of the expression of ISGs and to describe the involvement of pSTAT1, pSTAT2 and IRF9 (forming a classical ISGF3 complex) as well as the involvement of unphosphorylated STAT1 and STAT2. An important aspect of this research is the investigation of IFNα-dependent and time-dependent ISG expression and inclusion of multiple timepoints upon IFN stimulation. This provided original results indicating the role of ISGF3 complex in sustaining prolonged ISGs' expression, up to 72h. What is more, a cell-type-dependent pattern of ISGF3 phosphorylation, expression and chromatin binding was observed in two studied cell lines. Finally, **antiviral assays** were used, which demonstrated that even low levels of antiviral genes' expression (observed at later timepoints) were enough for viral protection and that this protection was IFN-dose dependent.

In subsequent sections of the Results, basically the same experimental workflow was used. Additionally, the experiments with use of with **JAK Inhibitor** I (JII) were performed to investigate the role of phosphorylation of STAT1 and STAT2. The most important findings indicate that 1/phosphorylation is a key factor for prolonged expression of ISGs, both in wild-type and STAT1-deficiency conditions; 2/classical ISGF3 is the predominant complex mediating ISG expression in an IFNα- and time-dependent manner; 3/ STAT2/IRF9 complex can take over the role of ISGF3 in the absence of STAT1; 4/ U-ISGF3, together with classical ISGF3, could be involved in IFN-dependent and independent transcription od ISGs, while the role of U-STAT2/IRF9 is excluded. All the results are well illustrated with 37 figures. The description of the results is very clear and well balanced with relevant comments on the meaning of the results. While the major discussion of the findings is appropriately included in the Discussion section.

The **Discussion** is also well structured and adequately refers to the objectives of the thesis. It take 16 pages, is illustrated with 4 figures and informative legends. The Author smoothly combines and interprets the results obtained in experiments using Western blots, RNA-seq, qPCR, ChIP-seq, viral protection assays. She also refers to methodological issues, which might influence the results, e.g. the quality of used antibodies from different suppliers. She nicely discusses her results in the light the results of other researchers, including previous findings of the group she belongs to, and all these elements are well balanced. The Author highlights, which of her findings (the findings of the group) are novel and how they change or extend the current state of knowledge in the field, e.g. the role of ISGF3 in sustaining ISG expression (up to 72h) in contrast to the previous perception of the ISGF3 as a transcription factor acting only during the early IFN-dependent response. In case of some of the results, which do not fully resolve the issues in question, the Author proposes the possible interpretations, but clearly states their speculative nature. Importantly, she also highlights the limitations of her research, indicating questions that still need to be answered and sketches further directions to be taken, e.g. ChIP-Seq experiments on STAT1-deficient cells upon JAK inhibition. Finally, based on the data presented in this dissertation and the available literature data, the Author proposes 4 models of complex mechanisms that regulate antiviral response in different cell types and under different conditions. These models address the issues presented as the objectives of this dissertation. Each model is very well illustrated. The only element, which I miss in this Discussion, is a summary paragraph, in which the Author could comment on the potential impact of her findings for the development of the research field, in a broader context, perhaps also the potential applications in the development of strategies against viral infections.

4. Specific comments and questions

Some parts of Materials and methods could be more elaborate, e.g.:

1. There is no information on the assessment of RNA integrity before qPCR. Was RNA integrity only checked for the samples used for RNA-seq?
2. What were the parameters of RNA-seq: was it single-end (SE) or paired-end (PE)? What was the length of the reads? From the description of the sequencing chemistry, I assume these were single-end reads of 75bp in length, but this should be described in the text. What was the read depth of coverage - how many reads per sample were aimed for? The same remarks apply to the description of ChIP-seq.
3. Different parameters were used for different cell lines to define differentially expressed genes: page 56: „Genes with adjusted p-values (padj) less than 0.05 and log2FC > 0,5 (for ST2-U3C) or log2FC > 1 (for the rest of the cell lines) were considered as DEGs." It should be explained, why these different parameters were applied.
4. There is a factual error on page 32, Introduction. The Author states: „Viruses are mutating all the time to develop ways to bypass the host's immune response, so the host immune system has a variety of ISGF3-independent signalling pathways that provide protection for viruses (...). The mechanisms that provide the antiviral abilities of the host cells are.:...". And here, the Author enumerates the mechanisms, which however are not antiviral mechanisms of the host cell, as previously stated, but are viral mechanisms of immune evasion. It seems that a part of the text has been confused or deleted. Perhaps both types of mechanisms were originally meant to be described.
5. There are editing errors:

   Page 82, Results, Figure 4.10: „To estimate the protein levels and their phosphorylation profiles Western blot analysis of STAT1, STAT2, IRF9 and IRF1, as well as α-tubulin, were done (...)" while there are no results shown for IRF1 in this figure. The same refers to Figures 4.17, 4.20, and 4.33, no results are presented for IRF1. Perhaps these results were originally planned but finally not included.

   Page 99, Results, Figure 4.18 legend: „The expression profiles for 2fTGH treated with JII are indicated in orange colour, while untreated in green." The colours of the plots are pink and violet; orange and green are in figure 4.19.

   Page 132, Discussion, Figure 5.1: The legend is incomplete, the last part of the text is missing („The level of U-STATs is not high enough to provide....").
6. There are several spelling errors, e.g.:

   Page 15, Introduction: „As IFN Type-I receptor does not have kinase activity itself indispensable phosphorylation event is provided by attached proteins JAK1 and TYK2 which together with JAK1 and JAK3 are members of JAK family." Should be: „which together with JAK2 and JAK3".

   Page 31: Introduction: „It the beginning... in was believed" Should be: „In the beginning... it was believed."

   Page 66: „seeded into 96-well plates at an appropriate amount per welThe nextext day, cells were pre-treated with..."

   Page161, Abstract in Polish: „...aby zapewnić skuteczną ochronę wirusową". Should be: „aby zapewnić skuteczną ochronę przeciwwirusową".
7. The Abbreviation list is incomplete. There are several abbreviations used in the text or in figures, which were not explained in the text at their first use, nor explained in the figure legends and not placed in the list of Abbreviations, e.g. the names of various IFN receptors in the Introduction or the names of several genes belonging to ISGs, in the Results section. These might be obvious for the researchers in

this field, but all abbreviations used in the dissertation should consequently be included in the Abbreviations' list.

8. In the whole text, the more generous use of commas, to separate complex sentences, would increase the clarity of the text even more.

5. Final conclusions

This is a valuable dissertation focused on a valid research problem. It applies a wide range of complementary research methods, provides original findings and broadens the knowledge in the field of the basic mechanisms of IFN-dependent antiviral response. Difficult issues are presented in an accessible way, indicating an in-depth understanding of the topic by the PhD Candidate. This thesis shows that the Candidate has extensive theoretical knowledge in the field, uses a wide range of research methods, can plan and conduct experiments, and efficiently present and discuss the results. She developed abilities needed in scientific work, including objectivity and well understood criticisms of her own findings. All these demonstrate not only the effort and scientific development of the PhD Candidate but also the effort and commitment of her Mentor.

The doctoral dissertation prepared by Hanna Nowicka, entitled "The role of phosphorylation of ISGF3 components in the regulation of ISG expression and viral protection" meets the requirements for candidates for the doctoral degree (art. 187 section 1-2, Act of 20 July 2018 Law on Higher Education and Science; Journal of Laws 2022 item 574). Therefore, I recommend the admission of Hanna Nowicka to the next stages of the PhD award process. Due to the high scientific value, I recommend this doctoral dissertation to be distinguished.

Małgorzata Dawidowska