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Review of doctoral dissertation

'The role of ISRE and GAS composite-containing genes in long-term IFN-I and IFN-II responsiveness'

by **Sanaz Hassani**

prepared under supervision of

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1. Importance of the research

Interferons are produced by inflammatory cells in response to viral invasion. They are essential in triggering and orchestrating the antiviral response of the organism by initiating signaling cascades leading to the transcription of interferon-stimulated genes (ISGs). Despite the discovery in 1997 a class of ISGs containing ISRE and GAS elements (so called composite genes), their role in the long-term response to IFNs has still not been fully elucidated. This justifies the undertaken research and the objectives of this thesis, which focus on the elucidation of the mechanisms governing the transcriptional regulation of ISRE and GAS composite genes.

2. Formal evaluation of the dissertation

The thesis has a form of a monograph, consisting of 190 pages and is written in English. The structure is typical for this kind of dissertations and includes: Introduction (comprising of 25 well-structured sections, 44 pages), Hypothesis And Objectives (8 objectives), Material and Methods (16 sections, 14 pages), Results (15 sections, 64 pages), Discussion (4 sections, 21 pages), References (almost 300), a List of 45 Figures (and 2 supplementary figures), a List of 13 Tables (and 1 supplementary table), Abbreviations, and a List of 3 Publications and 1 manuscript in preparation co-authored by the PhD Candidate, information about funding, Acknowledgements and a Summary in Polish and in English.

In terms of the editorial preparation, this dissertation in very diligent, aesthetic and practically flawless - I haven't noticed any editorial mistakes. The language is appropriate. The Figures are of good quality and accompanied by very informative legends.

3. Content-based evaluation

Introduction

The PhD Candidate provides a broad overview of information necessary to further present her research. She explains the mechanisms and factors inducing the production of IFNs by different cell types and presents the pathways activated by each of the IFN types, highlighting the roles of JAK kinases, homo

and heterodimers formed by STATs alone or by STATs and IRF9. Finally, she introduces the role of ISRE and GAS elements in the initiation of expression of the interferon stimulated genes (ISGs). She highlights the synergistic roles of IFN-I and IFN-II in the activation of some of the ISGs and the similarities between IFN-I and INF-III activation pathways.

The Introduction presents the mechanism of IFN production and the roles of external factors (pathogen-associated molecular patterns, PAMS) as well as the internal stimuli (damage-associated molecular patterns, DAMPs) in the production of interferons. The mechanisms of the recognition of DAMPs and PAMPs by macrophages, fibroblasts and dendritic cells are also described, including the role of TLRs and other receptors and interacting proteins, finally leading to the activation of interferon regulatory factors (IRFs) and their entry to the nucleus to stimulate the expression in ISGs.

Separate sections of the Introduction are devoted to a more in-depth presentation of signaling cascades triggered by IFN-I and IFN-II, to the description of the roles of JAK kinases in the phosphorylation of STATs and to the description of STATs itself. The structures, functions and interactions of STAT1, STAT2, IRF9 and IRF1 proteins are described in details and well-illustrated. The roles of STAT1, STAT2 and IRF9 in the formation of ISGF3 complex; STAT1 and STAT2 in the formation of GAF-like complex and STAT1 dimerization to form GAF complex are also described and illustrated. These transcription activator complexes as well as the IRF1 transcription activator are further presented in more details, with the focus on their specific roles in the activation of the interferon stimulated genes containing different elements in their promoters: ISGF3 complex and IRF1 target the ISRE element (interferon-stimulated response element), while GAF and GAF-like complexes target the GAS element (γ-activated sequence). Genes stimulated by IFNs might be activated via the binding of transcription activators to ISRE element, to GAS element or to both. Considering that the main focus of the thesis is transcriptional regulation of ISGs containing a composite sequence, comprising both ISRE and GAS elements, Figure 2 is instrumental in summarizing the signaling pathways triggered by IFNαand IFNγ and leading to the activation of the genes containing ISRE, GAS or both elements.

I appreciate that the PhD Candidate not only describes physiological conditions but pays attention to the dark side of the IFNs and interferon-regulatory factors (IRFs). Their dysregulated functions underly the susceptibility to autoimmune diseases like multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis.

A separate section of the Introduction is devoted to the description of interferon-stimulated genes (ISGs). The definition of this group of genes is broad – the genes that undergo activation upon IFN response. These include protein-coding ISGs and non-coding ISGs, which produce long non-coding RNAs in response to IFNs or viral infection. Collectively, these gene products have multiple roles in antiviral response (e.g. inhibition of viral invasion, suppression of viral mRNA and protein synthesis, degradation of viral genome etc.) but they also contribute to basic cell functions like apoptosis or cell growth. Importantly, STAT1, STAT2, IRF9 and IRF1 all belong to the group of ISGs and their expression is regulated in a positive feedback loop with the involvement of ISRE+GAS composite sequence (in case of STARTs and IRF9) or GAS sequence (in case of IRF1). The existence of this positive feedback loop is crucial for the long-term response to IFNs.

Finally in the section entitled *Comprehensive Exploration Of STAT1, STAT2, IRF9, And IRF1 Binding Across The Genome: Impact On ISRE And GAS-Dependent Transcription Regulation*, a historical sketch of the most important developments in the field, as well as the state-of-the-art knowledge relevant to the topic of the thesis are presented. Despite the discovery of the ISGs containing both ISRE and GAS elements in 1997, their role in the long-term response to IFNs has still not been fully elucidated. The introduction ends with several questions showing research gaps that still exist in this field. These questions provide a smooth link to the following section.

Hypothesis and Objectives

The Hypothesis is clearly stated and it assumes that IFN-I and IFN-II dependent transcription of composite genes depends on ISRE and GAS composition and differential binding of ISGF3, IRF1, STAT1/IRF9, GAF and GAF-like complexes.

The PhD Candidate defined 8 objectives of the dissertation focusing on composite genes' identification, their transcriptional regulation through the binding of ISGF3, IRF1, GAF and GAF-like complexes, and the biological function of composite genes in antiviral activity.

These objectives are:

1. *To generate a **complete list of IFNα- and IFNγ-induced ISRE and GAS composite site-containing genes** with their ISGF3, IRF1, GAF and GAF-like **binding profile** and putative biological function.*
2. *To further characterize the **genome-wide** role of IFN-I and IFN-II-activated ISGF3, IRF1, GAF and GAF-like **complexes in time-dependent ISG expression** through ISRE and GAS composites.*
3. *To further characterize the role of IFN-I and IFN-II-activated ISGF3, IRF1, GAF and GAF-like complexes in **binding to distal ISRE and GAS-containing elements** and in transcriptional regulation.*
4. *To further characterize the IFN-I and IFN-II induced transcription of ISRE-GAS composite genes **in WT, STAT1, STAT2, IRF9, IRF1 and IRF1.9dKO cell lines.***
5. *To further understand the role of **ISRE and GAS distance and organization in transcriptional regulation** of ISRE+GAS composite genes in response to IFN-I and IFN-II.*
6. *To further understand the ability of the ISRE+GAS composite site to act as a **molecular switch** in response to IFN-I and IFN-II.*
7. *To further characterize the role of the GAS and ISRE sites in **transcription of different classes of ISRE+GAS composite genes** in response to IFN-I and IFN-II.*
8. *To further investigate the role of ISRE+GAS composite genes in IFN-I and IFN-II **mediated anti-viral activity.***

Material and methods

This section is well-structured and easy to follow, accompanied by 9 tables and 2 Figures. The choice and the description of the methods leave no doubts. It shows that the PhD Candidate has mastered a wide range of research methods and techniques. Part of this work was performed in collaboration with other researchers from Adam Mickiewicz University in Poznan (RNA-seq experiments, ChIP-Seq experiments, and bioinformatics analysis), in collaboration with the group of Dr Sada from University of Fukui in Japan (obtaining the Huh7.5 and KO cell lines), while antiviral experiments were performed during the PhD Candidate stay at Professor Chien-Kuo Lee's laboratory in the Graduate Institute of Immunology, College of Medicine, National Taiwan University in Taipei. The exposition of the PhD Candidate to a number of collaborations is a valuable experience for a young scientist, expanding the knowledge and expertise.

Results

This part consists of 7 major sections and several subsections, presenting the outcomes of the experiments and corresponding to the objectives of this dissertation.

ChIP-seq experiments were performed in WT-Huh7.5 cells in several time points (0, 0.5h, 2h, 8h, 24h, and 72h) upon IFNα-treatment, using antibodies against pSTAT1, pSTAT2, IRF9 and IRF1 and in IFNγ-treated WT-Huh7.5 cells using antibodies against pSTAT1, IRF9 and IRF1 at set time points (0, 0.5h, 4h, 24h, and 72h). To identify composite genes induced by IFNα and IFNγ, a strategy for gene selection was developed, based on ChIP-seq and RNA-seq data. The workflow of this strategy is nicely presented in Figure 15. A list of ISRE+GAS-composite genes was prepared based on ChIP-seq peak scores, focusing only

on protein-coding genes, then investigating expression levels of these genes in RNA-seq data upon IFN stimulation. This resulted in the **list of 89 ISRE+GAS composite genes**, including IFNα- induced genes, IFNγ-induced genes and composite genes induced by both IFNs. This was followed by the selection of **30 genes, commonly stimulated by both IFNs with random ISRE+GAS orientation and varying distances.** The binding patterns of relevant transcription activators was investigated in WT cells in response to IFNα and IFNγ. Then, for 13 pre-selected genes (belonging to different classes of composite genes in terms of the distance of regulatory elements), the binding patterns were studied in WT and in a set of KO cells to investigate if **ISRE/GAS distances and orientation affect the regulation of composite genes** upon IFN treatment. Subsequently, promoter-luciferase reporter assays were performed in WT cells upon IFNα and IFNγ- treatment for 8 composite genes (*PARP14, APOL6, DDX60, NLRC5, DDX58, NMI, STAT1, IRF1*) to investigate the role of ISRE and GAS elements. Finally, **promoter-luciferase reporter assays** upon IFNα and IFNγ-treatment were performed in WT and in a set of KO cells for 5 composite genes (*PARP14, APOL6, DDX60, NMI, STAT1*) to **investigate the roles of ISGF3, GAF, GAF-like, IRF1, STAT2/IRF9 and STAT1/IRF9 complexes** in the regulation of these genes. A comprehensive overview of **different transcriptional mechanisms of ISRE+GAS composite genes** dependent on the **composition of ISRE and GAS** in response to IFNα and IFNγis elegantly presented in Figure 45. Finally, antiviral response triggered by IFNα and IFNγ stimulation was investigated using antiviral assays.

In my opinion, the most important outcomes of the research include:

1. Creation of the **list of 89 ISRE+GAS composite genes and the identification of their putative biological functions** by enrichment analysis, using terms from Gene Ontology and Kyoto Encyclopedia of Genes and Genomes. This revealed, as expected, the involvement of the studied genes in the processes and signaling pathways linked **to viral defense and immune system activity**.
2. The finding of various **time-dependent expression patterns of composite genes** (early, intermediate and late in response to IFNα and intermediate and late in response to IFNγ) and conclusion that the distance and arrangement of ISRE/GAS elements seem not to influence these time-dependent patterns of expression. The majority of composite genes displayed maximum expression at 8h in response to both IFNs.
3. The finding that the ability to **switch between GAS and ISRE binding sites is dependent on the availability of transcription activators** and act as an important mechanism in transcriptional activation of ISRE+GAS composite genes in response to IFNs.
4. The finding that there is **no correlation between ISRE/GAS distances or orientation** and **binding** patterns of **ISGF3, GAF, GAF-Like, and IRF1 complexes.**
5. The finding that ISRE+GAS composite genes are regulated by **various transcriptional mechanisms depending on the composition of ISRE and GAS** (different mechanisms in case of single vs. multiple ISRE and GAS elements in gene promoter and in genes with distal ISRE/GAS organization).
6. The discovery that in some of the composite genes (e.g. NMI gene) with two ISRE elements in their promoter, **one of these ISRE elements might be inactive** and that **GAS might have inhibitory effect** on ISRE.
7. Characterizing the **involvement of ISRE+GAS composite genes in antiviral response**, including the observation that IRF1.9dKO cells do not show any antiviral response to IFNs at either time point, which highlights **GAS-dependent mechanism is not potent enough** to lead to an effective antiviral response.
8. The finding that **ISRE-only and ISRE+GAS composite-dependent mechanisms**, involving ISGF3, IRF1, STAT2/IRF9 and possibly STAT1/IRF9 complexes are **crucial in antiviral response**, while GAS-

only mechanism plays a minor role. This suggests that GAS-only containing genes are also involved in other functions than antiviral response.

The Results section is well-structured and supported by 29 figures and 3 tables. The content, the quality of these graphics and informative legends nicely guide the reader through this quite extensive section. The description of the results is well balanced with comments on the meaning of the results.

Discussion

The PhD Candidate elegantly summarizes and interprets the most important outcomes of her research, presenting them in a broad perspective of the current knowledge in the field. Many of the references are the positions published by the group of prof. Bluyssen, which shows that this PhD project was performed in the scientific environment of the experts in the field. The way in which the PhD Candidate discusses her results, the fact that she refers not only to most recent works but also to those published years ago, demonstrates insightfulness and broad orientation in the research topic.

The Discussion starts with the description of a **strategy leading to the identification of the list of 89** ISRE-GAS composite genes. Then it describes a step-wise strategy of a more in-depth analyses in selected numbers of genes to explain the role of ISRE and GAS elements and their distance and arrangement in transcriptional regulation of these genes by IFNs in a time-dependent manner and with the role of different activator complexes in this regulation. The focus is also on a very intriguing characteristics, which is the **flexibility of the mechanisms of transcriptional activation** in case of the composite genes as compared genes containing ISRE-only or GAS-only elements. A **model of dynamic switching between utilizing ISRE and GAS element is proposed** and nicely illustrated (Figure 44). This dynamic switching, dependent on the availability of transcription factors, might serve as an adaptive mechanism ensuring effective antiviral response in various conditions. **Different mechanism of transcriptional regulation in response to IFNs were proposed in case of composite genes with a single ISRE+GAS motif**, in case of genes with **multiple ISRE+GAS** and in case of **distal ISRE+GAS orientation**. These mechanisms were elegantly illustrated (Figure 45). Finally, the roles of composite genes in the antiviral response were discussed, with the major conclusion that **ISRE-only and ISRE+GAS dependent mechanisms of ISGs regulation are key in antiviral response**, while ISGs regulated by GAS-only mechanism play a minor role in antiviral response.

Altogether, the Discussion shows solid knowledge of the PhD Candidate in the field and the ability to present and comment her results in the perspective of the world literature, including the discussion with the works of her Mentor and previous works of her colleagues. In my opinion, the **Discussion should end with a summary paragraph**. In this last section the PhD Candidate could comment on the limitations of her study (there are always some) and present future perspectives for this research and potential practical applications of her findings. The ability to identify limitations and to sketch further perspectives is one of the elements of scientific maturity.

4. Specific comments and questions

1. In the Introduction the PhD Candidate only briefly mentions (in one sentence) the utility of therapeutic IFNs. In my opinion a broader view of their application in the treatment of immune-related disorders could be provided.
2. Generally, what I miss a bit in this dissertation is the presentation of the possible applicational or translational aspects of this research. I fully understand that this is basic research, but even in such cases, I highly appreciate when a PhD Candidate can identify and present the potential impact of the conducted research for the development of the discipline, or even more broadly for the society. I would gladly hear the opinion of the PhD Candidate in these matters during the defence.

3. Generally, the language of the dissertation is very good. The only fragment which I would re-edit is the first paragraph of section 5.4 of the Discussion (page 146): 'ISGs employ a variety of strategies and mechanisms to combat viral infections. These include the degradation of viral RNA, inhibition of protein translation, and targeting multiple stages of the viral life cycle. By using these different approaches, ISGs can effectively disrupt viral replication and propagation'. I suggest not using this personification style in relation to the genes and express this differently: 'ISGs are involved in different strategies (...) and in different approaches of how the body effectively disrupt viral replication and propagation'. But this is really the pickiness of the reviewer, since generally the dissertation is flawless in terms of the language and editing.

4. Final conclusions

In my opinion this is a valuable dissertation dealing with original solutions to a scientific problem, which is the understanding the mechanisms of the transcriptional regulation of ISRE and GAS composite genes in response to IFNs. To this aim a wide range of complementary research methods was used. This thesis shows that the Candidate has extensive theoretical knowledge in the field, has mastered a variety of techniques and methods, can plan and conduct experiments, and efficiently present and discuss the results. Apart from the scientific value, the thesis is very solid and diligent in terms of the editorial preparation.

The doctoral dissertation prepared by Sanaz Hassani, entitled 'The role of ISRE and GAS composite-containing genes in long-term IFN-I and IFN-II responsiveness' meets the requirements for doctoral dissertations specified in art. 187 of the Act 'Law on Higher Education and Science' (art. 187 ust.1-2 Ustawy z 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce; Dz.U. 2024 poz. 1571). Therefore, I recommend the admission of Sanaz Hassani to the next stages of the PhD award process. Due to the high scientific value, I recommend this doctoral dissertation to be distinguished.

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