Kraków, 3.03.2022

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**EVALUATION**

**of the Doctoral Dissertation prepared by Agata Sekrecka, MSc,**

**a PhD student at the Institute of Molecular Biology and Biotechnology,**

**Faculty of Biology, Adam Mickiewicz University in Poznań**

**Title of thesis:**

**"The role of GAF, ISGF3 and IRF1 complexes in time-dependent IFNα- and IFNγ-activated transcriptional responses and functional overlap"**

Interferons (IFNs) were first identified almost fifty years ago through their antiviral properties, as cytokines of the first lines of defense against invading pathogens. For more than 25 years they have been used to treat malignant, autoimmune and viral disorders. IFNs are produced in response to various challenges, such as viruses, bacteria, and tumor or damaged cells. Their effects are pleiotropic – they activate the adaptive immune system, have immunomodulatory and anti-angiogenic functions, and directly affect malignant cells through regulation of cell cycle, differentiation and apoptosis. Thus, the topic of the dissertation chosen by Ms. Agata Sekrecka concerned one of the most important signal transduction pathways in cell biology, which gave a chance to obtain the relevant data.

On the other hand, IFNs have been extensively studied over many years, which has resulted in fundamental insights into their cellular signaling mechanisms. Conducting research in such a competitive field is somewhat risky and often could only generate confirmatory results, which do not bring a new understanding of the topic. However, this was not the case here. The results obtained by Ms. Agata Sekrecka in her doctoral dissertation are novel and significantly enrich our knowledge on the regulation of the interferon response.

Two main types of interferons (type I and type II) use distinct although similar receptor systems and partially overlapping intracellular mediators, responsible for the regulation of hundreds of target genes. Analysis of the

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promoters of genes that were induced by IFNs, identified the conserved DNA elements. Proteins bound to these elements were purified and characterized as STATs and IRFs. Within this complex signal cascade lie the combinatorial differences through which IFNs foster their pleiotropic responses. The underlying mechanisms of such a crosstalk remain, however, still poorly understood.

Generally, despite years of studies, direct unbiased comparisons of global responses to IFN I and IFN II are scarce, whereas time-course analyses of the activation of transcription factors are missing. Obtaining such data sets was the main aim of the doctoral dissertation prepared by Ms. Agata Sekrecka.

Her research focused on the organization of GAS and ISRE binding sites and their occupation by ISGF3, GAF, and IRF1 in response to IFNα and IFNγ. She investigated how IFNs initiate phosphorylation of STAT1 and STAT2 proteins, which subsequently form homo- and heterodimers that bind to the promoters of target genes. She considered also the role of IRF9 and IRF1. In her doctoral thesis Ms. Agata Sekrecka described a repertoire of transcription factors and the time-course of stimulation of GAS and ISRE binding sites. Her work, performed under supervision of prof. Johannes Bluyssen, is a direct continuation and logical follow-up of previous studies carried out and published by the team. Experience of the Supervisor and his support was undoubtedly important for the success of the performed research.

The main goal of Ms. Agata Secrecka's doctoral dissertation was to characterize the genome-wide IFNα and IFNγ signaling pathways in Huh7.5 human hepatoma cell line. She investigated the profile of phosphorylation and abundance of signaling proteins, RNA expression, and chromatin interactions in response to IFNα or IFNγ at different time points, starting from untreated cells till 72 h post stimulation.

In my opinion, the most interesting results obtained by the PhD Candidate are: i) Characterization of three groups of interferon-regulated targets: GAS only, ISRE only and GAS+ISRE (composite) genes. All three groups of genes are activated by both types of interferons and recruit a different set of transcription factors. ii) Demonstration that GAS-containing genes, bound

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by phosphorylated STATs, are the first and rapidly induced gene group, activated in response to both IFNs. On the contrary, the number of activated ISRE sites increases at later time points. iii) Recognition of a group of composite genes and identification of sequences and distance between their GAS and ISRE elements. Because such genes can switch between regulatory elements to preserve the expression when one of the sites is mutated, they could potentially play a role in immune response if pathogens evade host response by sequestrating some components of a signaling pathway.

Conducting of the project required from the Candidate application of unbiased genome-wide analyses of gene regulation and expression profiles in the *in-vitro* cultured wild-type and genetically modified hepatoma cell lines. The most important and challenging part was the comprehensive and integrative analyses of the obtained RNA-seq or ChIP-seq data sets. The Candidate applied among others DESeq2 package, DAVID and Revigo resources for gene ontology analysis and visualization, DEGreport package for comparison of gene expression patterns or IGV, HOMER and BETA software tools for the ChIP-seq data interpretation and integration. Help offered by the collaborators, namely Dr. Katarzyna Kluzek and Dr. Mahdi Eskandarian Boriujeni (Adam Mickiewicz University in Poznań) is clearly indicated in the thesis. Importantly, the methods used were mutually complementing, and supported by additional analyses, such as evaluation of gene expression at mRNA levels using quantitative RT-PCRs, ChIP-PCRs, co-immunoprecipitation assays, western blottings, and luciferase-based reporter assays supplemented by site-directed mutagenesis in tested regulatory sequences. Thus, research methodology was diverse and correctly chosen to achieve the aims of the project. It is worth emphasizing that the research carried out on modified lines with switched off genes allowed for a mechanistic approach and experimental verification of the observed correlations.

Experiments were carried out by the Candidate using wild type cells or modified cell lines. Some modification were done earlier (as clearly indicated in the thesis), others were performed by the Candidate using the CRISPR/Cas9 technology.

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Dissertation by Agata Sekrecka consists of 172 pages, with 51 figures or supplemental figures and 13 tables or supplemental tables. The list of references contains 226 items. The thesis is classically composed and consists of Summary and Polish Streszczenie, Introduction with Hypothesis and objectives as a separate chapter, Materials and methods, Results, and Discussion with separated Conclusions and Future ideas.

Whole text is preceded by Contents, while ended by References, Supplementary materials, List of figures, List of tables, List of abbreviations, List of publications coauthored by the Candidate, Acknowledgments and Funding information. List of abbreviations is short and indeed consists of the most important ones. Lists of figures and tables are well prepared and facilitate reading of the thesis.

The Summary introduces the topic well, provides sufficient information on the methodology, and clearly summarizes the most important results. The way the hypothesis is formulated is a slightly weaker element. The Summary would be more intriguing if it clearly indicated the knowledge gap that the research is to fill. Streszczenie is correctly written, although the syntax of sentences is not typical of the classical literary Polish language.

The Introduction (consisting of 40 pages) is a very well written broad overview, describing interferons as pleiotropic cytokines and characterizing interferon-responsive signal transduction pathways. The text is interesting, easy to follow and excellently introduces the readers to the dissertation topic. It reflects a comprehensive knowledge of the Author, and good understanding of signal transduction mechanisms, including relation between protein structure and function. The description is detailed and illustrated with very good schemes. Particular subchapters create a cohesive and logical content. Underlying the controversies resulting from discrepant results published by different teams serves as a good rationale for performing further studies. It should be appreciated that the text refers not only the newest publications but also the older ones. I would not just use, however, the term "recent" for the paper published in 2000 (page 22). Overall, the Introduction is clear, orderly picture of a very complex story and can be easily prepared for publication as independent review paper.

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Materials and Methods used in the study are sufficiently described (on 22 pages), allowing repetition of the experiments. All primer sequences, maps of plasmids, composition of buffers, as well as concentrations, incubation conditions, catalog numbers and producers of antibodies are provided, what can be helpful. The methods of bioinformatics analyses, including integration of RNA-seq and ChIP-seq data, are also sufficiently explained. Description of quality controls of the prepared libraries confirms the high value of the performed analyses.

My only suggestion is to provide a more clear rationale for choosing a cell line - why hepatoma? Was it because a set of modified lines was already prepared or was there any additional reason? In some experiments cells were modified using CRISPR/Cas9 approach. Did this method trigger any background interferon response?

The Results (47 pages supported by 17 pages of supplemental materials) are described in details and illustrated with very well prepared, good-quality graphs. Figure legends are understandable and detailed. Presentation of the data in the way which underlies the logical connections between subsequent stages of work should be appreciated. Analyses are complementary and mutually supporting, technical quality of the result is very good, as illustrated *inter alia* by low standard deviations in experimental repetitions. Step-by-step description is clear and convincing. It is not the first example from this research group how relatively simple experiments followed by extensive analyses may provide a general information on fundamental biological process, not limited to the specific experimental setting.

The strength of the presented studies is the use of appropriate controls and checking the quality of research models, primarily at the functional level. In one fragment of the text (page 65), however, information that clones were analyzed by RT-PCR at the DNA level is a bit confusing.

Importantly, the Author points out and warns the reader that the technical quality of some of the results was substandard (due to the poor quality of the available anti-IRF9 antibodies) and avoids drawing too far-reaching conclusions from data that may be distorted for technical reasons. Generally, despite the application of mainly unbiased, genome-wide analysis,

the research is not limited to correlative inference. An elegant supplementation of the core experiments is the use of knockout and double knockout cell lines to directly confirm the significance of the transcription factors studied. Such an approach is valuable and significantly enhances the relevance of the results.

The Discussion, set out on the 23 pages, is cautious and well balanced. The Author clearly indicates which suppositions have been confirmed and which still require a direct experimental verification. In my opinion such inference indicates the Candidate's maturity. First part of the Discussion is, to some extent just a summary of the results that repeats the descriptions presented already in the Result section. But then, the Discussion develops into a well-narrated story that shows the Author's commitment and explains the reasoning. Ms. Agata Sekrecka succeeded in the difficult art of telling a very complex story in a relatively simple way, when listing the most important conclusions of her research. Nevertheless the final graphical summary (upper part of Fig. 39) is not straightforward.

The results are discussed mainly in the context of the time-course of classical and non-classical interferon response pathways at cellular level. Less attention is paid to regulation at the level of the whole organism, although the Author refers to viral diseases. These are the pathways whose regulations have been studied for a long time. The Author refers not recent papers but also reports published in the early period of research. In some places the citations of the source papers are missing, like in case of sentence (page 113) "This event was in agreement with the results of the different studies conducted on HeLa cells". These "different studies" should be cited. There are, however, only isolated oversights.

The Discussion summarizes that GAF and GAF-like binding appear earlier than ISGF3 and follow the phosphorylation pattern of STAT proteins, while ISGF3-driven response appeared later and is dependent on IRF1/IRF9. On the other hand, integrative analysis of commonly regulated genes by IFNα and IFNγ shows the GAS, ISRE and composite-containing genes are activated by both stimuli and recruit a different sets of transcription factors, identified by the PhD Candidate. It is very interesting and convincing to draw attention

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to possible general biological consequences: composite genes, predisposed to switch between GAS and ISRE sites can support more universal immune response.

The Discussion ends with final conclusions, followed by a description of future ideas – framed as direct, important questions. Due to my personal interest I would add one more question - what can be a role of the described mechanisms in stem cells and how they evolve with stem cell differentiation? Stem cells are rather refractory to interferon and viral infections, but intrinsically express a subset of interferon stimulated genes. The results obtained by Ms. Agata Sekrecka may facilitate the understanding of the mechanism of such regulation.

In editorial terms the Dissertation is prepared very carefully, and contains only a few typos (e.g. side-mutagenesis instead of site-mutagenesis) or minor editorial errors (e.g. *E. Coli* instead of *E. coli*). The thesis is written in English, in a fluent, communicative way. All paragraphs are coherent and well-formed, without non-necessary repetitions.

To sum-up, research described by the PhD Candidate is a valuable study, based on comprehensive, manifold and unbiased analyses. In my opinion, the Candidate, Ms. Agata Sekrecka, MSc, has achieved the aims of the planned research and her Dissertation meets all criteria of doctoral thesis. Therefore, I recommend the Dissertation for acceptance. Due to the high quality of analyzes and the ability to integrate data in search of answers to important biological questions, I propose to distinguish the Dissertation with a proper award.

Yours sincerely

Alicja Jakowicz

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