Dr hab. Sebastian Glatt
Max Planck Research Group Leader
Deputy Director Science MCB
Head of the National Cryo-EM Facility
Malopolska Centre of Biotechnology (MCB)
Jagiellonian University Krakow
Gronostajowa 7a str
30-387 Krakow, Poland
Ph: +48 12 664 6321
Fax: +48 12 664 6902
E: sebastian.glatt@uj.edu.pl
www: mcb.uj.edu.pl/en/mpl

Krakow, 22th July 2023

REVIEW OF THE DOCTORAL THESIS of Kamil Filipek entitled "Functional plasticity of the GTPase-associated center as a response of the translation machinery to environmental changes".

The Ph.D. thesis by Kamil Filipek was performed within the Ph.D. program of the "Institute of Biological Sciences" at the University of Maria Curie-Skłodowska in Lublin under the supervision of prof. dr hab. Marek Tchórzewski and under the co-supervision of dr Barbara Michalec-Wawiórka. In the presented thesis Kamil Filipek aims to identify regulatory mechanisms of the human translation machinery, which is responsible for producing all of our cellular proteins. The topic of the thesis is also related to the rather novel concept of heterogenous ribosomes that regulate protein synthesis beyond the simply translation of mRNA codons into nascent polypeptide chains. This idea has attracted the attention of many researchers in the field over the last decade.

The original thesis is presented in a form broadly accepted by the Polish research system, with a structural organization that contains the following features - title page, index, abbreviations, abstract, introduction, objectives of the work, materials and methods, results, discussion, summary, bibliography, and the current CV of the student listing all publications that arose from this thesis. The arrangement of thesis is very clear and well-structured, which makes it easy to follow and to comprehend the main concepts. Of note, I have genuinely enjoyed reading the thesis and despite working on ribosomes myself, I also gained a few new insights.

The introduction of the thesis covers a broad range of topics that are highly relevant for the presented results. The chapters include comprehensive summaries of current knowledge in the field and include the following chapters – (i) a general preface, (ii) heterogeneity of the ribosome, (iii) phosphorylation as a factor contributing to ribosome heterogeneity and (iv) GTPase-associated center (GAC). In detail, the doctoral student describes the various factors that can lead to ribosome heterogeneity. The factors include various translation factors, different ribosome-associated proteins, rRNA variants, dynamic rRNA modifications, variations in the composition of ribosomal proteins, post-translational modifications of ribosomal proteins and variation in the stoichiometry of ribosomal proteins. Each category is supported by examples and nicely illustrated by dedicated figures. In the next chapter, the doctoral student goes into greater detail about the role of specific phosphorylation events in various key factors, including translation initiation, elongation and termination factors. Subsequently, the phosphorylation of ribosomal proteins is described and commented. As parts of the thesis focus on the GAC, the doctoral students dedicates a individual chapter to introduce the architecture of the GAC, P-stalk proteins and the recruitment of regulatory GTPases. Finally, the introduction is completed by a state-of-the-art summary about the phosphorylation sites in the GAC and their suspected role in translation regulation.

The doctoral students cited an astonishing number of 406 individual literature references, which shows that he has broad overview of the field, which is crucial to interpret the findings and embed the obtained results in the available knowledge. The introduction is followed by a detailed description of the used methodology, protocols and materials that have been used during the experimental parts of the doctoral thesis. The student uses a combination of cellular biology, molecular biology, biophysics, biochemistry, enzymology, structural modeling, biochemistry and metabolomics to answer the clearly defined research questions. The results section is logically organized and it is easy to follow the scientific rational that guided the experiments. In the discussion section, the author summarizes the main findings and interprets these findings in bigger context. In addition, he speculates about future directions and concluding remarks.

The main topic of the doctoral dissertation concerns the molecular characterization of P-stalk proteins and their regulation by specific phosphorylation events. P-stalk proteins constitute the GTPase-associated center (GAC), which is located on the large subunit of the 60S ribosome. The ribosomal P-stalk proteins form a pentameric complex, harboring 1 copy of uL10 and 2 copies of the heterodimeric P1/P2 complex. The pentameric complex binds to the ribosomal RNA via its uL10 protein. All five proteins are known to get phosphorylated, but the specific phosphorylation sites, their dynamic exchange and their functional consequences remained elusive.

The results presented in this theses define and validate the phosphorylation status of ribosomal P proteins in human cell lines. Furthermore, the phosphorylation patterns of the basic cellular state are compared to patterns obtained under different stress conditions. The student uses structural modeling to interpret the functional role of the specific sites and employs a mutational scanning analyses to challenge the mechanistic models that could indeed explain the observed effects. The human CK2 kinase was suspected to phosphorylate the respective sites and the presented results nicely corroborate this view. In addition, the doctoral student uses available CK2 inhibitors to analyze the role of CK2 on the specific phosphorylation events and translation elongation under normal and stress conditions. The use of inhibitors in cells can lead to a multitude of consequences (including the induction of unknown off-target effects). The student checks the status of various cellular pathways in different cellular models and carefully interprets his results - also mentioning the limitations of the approach, which shows a highly professional attitude. In summary, the thesis addresses a very timely topic, uses state-of-the-art technologies and provides several interesting insights into the regulation of human protein synthesis.

I have a few general and specific questions that I would like to the student to clarify during the thesis defense.

1. I am not sure why the students has not performed phospho-proteomics on the available samples directly. The combination of 2D gel electrophoresis and database search is reasonable, but highly indirect. The samples could have been enriched for phospho-peptides and analysed by high-res mass spec to provide an experimental validation of available data and discover novel sites as well.
2. Did the student perform antibody staining of the untagged protein to check whether the GFP-fusion has any effect on the localization of the wild-type uL10 protein?
3. Did the student perform cellular fraction assays coupled to western blots to support the results from the fluorescence microscopy approach and to check the localization of the endogenous proteins?
4. Fig. 19 - Does the elevated sucrose level in the gradient have any effect on the migration behaviour of the samples in Phos-tag gel and how was this controlled for?
5. Fig. 19 + 20 – In the thesis it is stated that the *"…P-stalk proteins are in the phosphorylated form on translationally active ribosomes."* 80S (also called monosomes) contain a variety of ribosomes, but most of them are translationally inactive (e.g. ICs, inhibited/hibernating ribosomes). How did the author conclude that only translationally active ribosomes carry phosphorylated P-stalk proteins?
6. Fig. 21 – could the lower band result from induced apoptosis – did the author check proliferation rates and induction of apoptosis for the treated cells after 24 h?

7. Fig. 21E – was a statistical significance analyses performed for the different time points? In my opinion the conclusion by the author that the CK2 activity is down is not fully clear to me and would require a more thorough analysis.
8. Fig.23 – are there any evidences of other kinases that work on P1? There are two options to interpret the result - (i) either the experiment was performed too short to see a full drop of P1-P or (ii) the remaining phospho-sites are not dependent on CK2. Did the student test any other more broad kinase inhibitors to see the full disappearance of P1-P?
9. Fig.24 – as mentioned above, the monosome fractions seems to be as affected as the polysome fraction? What would the mechanism be responsible for in the monosomes and what would that mean for the model?
10. The statement – "However, it should be noted that the observed kinetics of the appearance of non-phosphorylated P-stalk is slow, even if CK2 kinase is almost completely inhibited, showing that there is no specific phosphatase or process for the stalk dephosphorylation." – I am not sure, if I follow the logic here fully. It could also be that the turnover of production and phosphorylation is so high that the responsible phosphatases simply can't catch up, but they would still work on it. Maybe the candidate can comment on this and the identity of possible phosphatases?
11. Page 89 – "I don't fully understand the following sentence – "Importantly, recently it was shown that the GCN2 kinase activation is the P-stalk proteins dependent".
12. Fig.27 – would the student expect that the cellular levels of amino acids are changing already after 2h or 6h – are there any known systems that affect the aa levels that fast – and could they be used as positive controls?
13. Fig.29 - was a statistical significance analyses performed for the different populations?

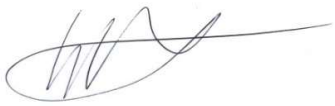
Final conclusion

The thesis presents several lines of high quality research, where the scientific question was clearly defined and successfully resolved. The doctoral student investigated a machinery that is known for decades and still manages to describes several important details about its regulation that have previously remained elusive. The thesis is written in a clear language and the results are embedded in the available literature. Hence, the author has shown high scientific capabilities and well-developed technical skills.

The doctoral dissertation meets the conditions specified in Art. 187 of the Act of July 20, 2018 on academic degrees and titles in science and arts (Journal of Laws 2018, item 1668 as amended). Therefore, I recommend that the Biological Sciences Discipline Council of the University of Maria Curie-Skłodowska in Lublin admit Kamil Filipek for the subsequent stages of the doctoral proceedings.

Concurrently, given the high quality of the research and generated knowledge taking us beyond the current state-of-the-art and providing a new view on mechanisms of the basic translational machinery, I recommend that the research effort made by the doctoral student should be awarded with distinction.

Sincerely yours,



Dr hab. Sebastian Glatt
Max Planck Research Group Leader
Deputy Director Science MCB
Head of the National Cryo-EM Facility