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Review of the thesis entitled: "Functional plasticity of the GTPase-associated center as a response of the translation machinery to environmental changes" by MSc. Kamil Filipek.
This work was supervised by prof. dr hab. Marek Tchorzewski and co-supervised by dr Barbara Michalec-Wawiorka.

Thesis evaluation

The dissertation is written in English. It is presented following a classical scheme including all the chapters required to evaluate the work efficiently. A list of the abbreviations used in the dissertation is given followed by an abstract in Polish and in English. Then a detailed introduction is presented containing all the information, including data from the recent literature, required to understand the rational of the work and the biological context. The rational and the objectives of the work are presented before the Materials and Methods and Results sections. The result section is followed by a Discussion and a Summary which recapitulates the main outcomes of the work and their interest in biological research. Finally the bibliography and a scientific CV are provided.

The introduction part starts with a presentation of ribosome heterogeneity. A very interesting historical view of the ribosome is presented. This part shows elegantly how the plethora of researches dedicated to elucidate the structure-composition relationships allowed to unravel that ribosome could exhibit different compositions and finally be specialized elements of the translational machinery. As a molecular platform the ribosome heterogeneity encompasses not only cis-acting or intrinsic elements of the ribosomes but also trans-acting factors interacting directly with the ribosomes. Among these latter there is the translation factors and the ribosome-associated proteins. The translation factors play key roles to control the unidirectionality of the translation process and the translational efficiency. This part is described with highly relevant molecular examples illustrating the global message. Ribosome-associated proteins are transiently associated with either pre-ribosomes or mature ribosomes. This part is exposed following a ribosome evolution point of view starting with procaryote ribosomes up to higher eucaryote ribosomes. This point of view is impressive and is even illustrated by original figures constructed by MSc. Filipek (Figures 2-4).

Among the cis-acting factors there is the ribosomal RNA (rRNA) and the ribosomal Proteins (RP). Again, to illustrate the heterogeneity of rRNA MSc. Filipek has chosen highly relevant examples from prokaryotic and eucaryotic organisms as well as from archae. This paragraph demonstrates very well how the diversity of genes coding for rRNA could account for ribosome heterogeneity. This diversity could even be increased by chemical modifications of these rRNA. The biochemical pathways underlying these chemical modifications are highly complex and differs from organisms to organisms. This part has not been exposed, instead relevant examples demonstrating how these chemical modifications could tune the ribosome activity have been described. Then numerous data, many from the very recent literature, have been presented to expose the variations of RP composition,

modifications and stoichiometry, including paralog RP, and again, how these variations in RP composition could affect ribosome functioning.

Among the modifications of cis and trans-acting factors, phosphorylation appears one them for which there is many validated experimental evidence. Therefore, an important part of the dissertation was focused on this modification with many details and examples. However, a major effort has been made to synthesized all this data into an original figure (Figure 7) that is very informative for the readers.

Finally the last paragraph of the introduction is dedicated to the GTPase associated center (GAC) which represents the heart of the thesis. The structure of the ribosome is presented with an enlargement of the region containing the GAC. The GAC is composed of several elements containing the P-stalk made of uL11 and uL10 interacting with two dimmers of P1-P2 proteins. The composition of the stalk varies depending on the domains of life. Then the functions of the P-stalk proteins are presented. One of the crucial aspects of P-stalk function is to allow GTP hydrolysis. Interestingly, although it is known that the P-stalk is responsible for anchoring the trGTPase on the ribosomes and stimulating trGTPases to GTP hydrolysis the molecular mechanism is not known. Several high-resolution structural studies highlighted the importance of the CTD domains of the P-stalk proteins for this function.

The last paragraph of the introduction points out the several studies that have been performed in many species to understand the role of the phosphorylation of P-stalk proteins. From these studies encompassing many different experimental procedures such as phospho-proteomics it can be concluded that the status of phosphorylation of P-stalk proteins and their functional role remains to be determined. This is indeed the objective of the experimental work presented in this dissertation thesis.

More precisely, the objective of the work was first to determine the phosphorylation status of P-stalk proteins *in vivo* and second to determine the role of these phosphorylation in the ribosome activity according to environmental changes.

To reach these objectives an adequate experimental strategy has been set up and described clearly. The Materials and Methods section is written with a great precision and contains all the data necessary to reproduce the experiments in another laboratory.

The Result section is organized into 3 parts.

Parts 1 and 2. Functional analysis of uL10 N-terminal phosphorylation and phospho-status of C-termini of mammalian P-stalk proteins. In this part biochemical data such as 2DE and western blot analyses as well as search within mass spectrometry data bases has been used to investigate the phosphorylation status of uL10 in regions that were not identified and/or well characterized previously, notably the N-terminal part. These analyses allowed to determined that uL10 could be phosphorylated at several residues located at the N-terminus, notably residues Y24 and T59. Biochemical and dynamic imaging analyses (FRAP) using recombinant GFP proteins wild type and phosphor-/dephospho-mimetics allowed to determine that the phosphorylation of Y24 residue act as a regulatory element controlling association of the protein to the ribosome and therefore translational activity of the ribosome whereas that of T59 is probably involved in the control of 60S maturation and therefore to the control of ribosome amount.

The other part of this experimental section was to determine in cell line (*in vivo*) which forms of P-stalk proteins, phosphorylated or non-phosphorylate, was present within mature cytoplasmic ribosomes. For this, several complementary biochemical techniques were developed to confirm the results using Pro-Q dye, Phos-tag-SDS-PAGE, western blot approaches and CK2 kinase assay with purified ribosomes from crude cytoplasmic extracts as well as from polysomes. This allowed to determine that the fully phosphorylated forms of P-stalk proteins are present in the translationally active ribosomes.

Part 3. P-stalk proteins phosphorylation and ribosome-dependent stress response.

The results obtained in the previous part of the study (Part 1 and 2) showed that P-stalk proteins are fully phosphorylated within actively translating ribosomes at the steady-state level. As exposed in the

introduction of the thesis, one of the major outcomes in this emerging field of specialized ribosome and regulatory ribosome would be to determine whether the phosphorylation status of these proteins and as a consequence their functional activity could vary according to environmental conditions. This would place these proteins and the ribosome at the center of regulatory signaling pathway that have not yet been explored. And therefore, these findings could unravel potential actionable targets for particular disease such as cancer.

Analyses of P-stalk proteins phosphorylation status using the Phos-tag electrophoresis system, immunodetection after western blotting, specific inhibitors of CK2, purification of ribosomes from total cytoplasmic extract and polysome fractionation allowed to determine that ER and ribotoxic stress induces a decrease of phosphorylation status whereas oxidative and amino acid deprivation did not induced this effect and that a correlation could be established between these phosphorylation status and the activity of CK2. The effect on major translational regulatory pathways following inhibition of P-stalk proteins by specific CK2 inhibitors was then determined. These analyses showed that eIF2a phosphorylation is dependent on GCN2 activation when CK2 is inhibited. A finding that is not dependent on amino acid starvation.

Interestingly, because GCN2 is a major regulator of the integrated stress response which is reflected by an activation of autophagy and proteasome activity, it has been evaluated whether P-stalk proteins could be degraded in stress condition through these well-known pathways. This analysis showed that these two pathways are at work for the degradation of P-stalk proteins but also for ribosomes in stress conditions.

Finally, the last part of the experimental program was performed to determine whether GCN2 kinase was associated with polysomes during inhibition of CK2. Polysome profiling performed in cells treated or not with the CK2 inhibitor and detection of GCN2 on purified ribosomes allowed to clearly show that this major protein kinase is found associated with ribosomes concomitantly to the appearance of a pool of unphosphorylated P-stalk proteins.

Final conclusion

The document is very well written in good English and very well organized. All the information required to understand the work and the interest of the work for the scientific community working in the field are provided.

The work performed by MSc. Filipek is part of a field of research in full renewal and which is experiencing exceptional growth. Both in terms of fundamental research and in that of applied research. Indeed, the ribosome was identified as the actor of protein synthesis more than forty years ago. However, over the past ten years, laboratories including the one headed by Dr. Tchorzewski have provided new data showing that the ribosome exhibit a still underestimated plasticity through different compositions and chemical modifications of their RNA and protein components driving their direct contribution to protein synthesis regulation.

In this context, MSc. Filipek carried out a major piece of work for the field because it provides very precise data on molecular mechanisms which support this paradigm shift. Importantly it shows how the ribosome, and therefore the translational apparatus could adapt to respond to deleterious environmental changes such as oxidative stress or amino acid starvations. This adaptation is crucial for cell survival.

The rational of the work is very well explained and is undoubtedly fully relevant in the field of “modern” ribosome biology. The general objectives of the work are first exposed and then each objective representing an experimental task is exposed very clearly.

The Materials and Methods section reflect the high quality of the experimental work that has been performed. This section is informative, it contains enough details and data allowing to another laboratory to reproduce the experiments.
The experimental part is of high quality, the amount of work is impressive. Many techniques have been developed to confirm the same result using several methodological approaches. The way the experimental part is presented and discussed reflects a great scientific maturity of MSc. Filipek.
The discussion is relevant and all the aspects of the work are discussed, either all along the result section or more specifically in the discussion section. Particularly in the discussion section the results are integrated very interestingly within the state of the art of this moving scientific field of research.
Given the very high quality of the research and the new data provided by this work for the research field that are undoubtedly beyond the current state of the art, I recommend with no reserve that the research effort made by the doctoral student should be awarded appropriately.
In conclusion to my point of view the doctoral dissertation meets all the conditions required for a PhD degree. Therefore, I recommend that the Scientific Council of the Institute of Biological Sciences of the Maria Curie-Skłodowska University in Lublin allows MSc. Kamil Filipek to fulfill the next steps of the PhD degree.

Yours sincerely,

Jean-Jacques Diaz