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REPORT ON THE DOCTORAL THESIS OF MSC KAMIL FILIPEK

PhD thesis title: **Functional plasticity of the GTPase associated center as a response of the translation machinery to environmental changes**

PhD thesis supervisor: **prof. dr hab. Marek Tchórzewski**

PhD thesis assistant supervisor: **dr Barbara Michalec-Wawiórka**

The PhD thesis presented by Kamil Filipek is a continuation of studies carried out in the Department of Molecular Biology at Maria Curie-Skłodowska University in Lublin (headed by professor Marek Tchórzewski), concerning the function of the translational machinery in eukaryotic cells. The general aim of this PhD project was to expand the knowledge on phosphorylation of the ribosomal pentameric complex termed the P-stalk. The ribosome, the central protein synthesis machinery of the cell, is composed of two structurally and functionally distinct subunits: large ribosomal subunit and small ribosomal subunit. The small subunit contains a decoding center that is involved in the deciphering of genetic information by enabling the base pairing of the codon on mRNA with anti-codon on a cognate tRNA loaded with an amino acid. The large subunit comprises the peptidyl transferase center, where the peptide bond between two amino acids is formed. On the large subunit there is also the GTPase-associated center (GAC) composed of an rRNA fragment named sarcin-ricin loop and a specific pentameric structure of proteins termed the P-stalk. The P-stalk is a critical element of the GTPase-associated center, and its removal impairs the ribosome actions. The mammalian ribosomal P-stalk complex is composed of one uL10 protein and two P1/P2 protein heterodimers (uL10-$(P1/P2)_2$). The most prominent feature of P-stalk proteins is their highly conserved C-terminal region, with a specific sequence motif composed of glutamic and aspartic acid residues and two serine residues. It has been already demonstrated that all P-stalk proteins (uL10, P1, and P2) undergo phosphorylation within these two serine residues; however their phosphorylation status (so-called phosphostatus) and the biological role of this modification remain unclear. To address these issues, Kamil Filipek undertook two research tasks: (i) characterization of the *in vivo* phosphostatus of the ribosomal P-stalk proteins in mammals and (ii) investigation of the role of the phosphorylation of P-stalk proteins in the function of the translational machinery in mammalian cells in changing environmental conditions. Thus, the PhD thesis of Kamil Filipek relates to important and still unsolved or poorly understood issues in the area of the regulation of the functioning of eukaryotic ribosomes.

The doctoral dissertation has a standard layout, and it includes the following sections: Introduction, Objectives of the work, Materials and methods, Results, Discussion, Summary and Bibliography. The list of abbreviations placed at the beginning of the dissertation is really helpful and makes the manuscript easier to follow. The Introduction sets up the topic of the research and provides basic information on the composition and function of the ribosome – a large ribonucleoprotein complex that is centrally placed in the translational machinery. The PhD candidate nicely presented information on heterogeneity of the ribosome, both concerning the trans-acting factors

(such as translation factors and ribosome-associated proteins) and the cis-acting factors (ribosomal RNAs and ribosomal proteins). From this section, we learn that phosphorylation is an important factor contributing to ribosome heterogeneity and consequently, its functioning. A special attention is given to phosphorylation of P-stalk proteins, which raises questions about the biological consequences of this post-translational modification. Regarding the Introduction, I would like to emphasize that I appreciate figures that nicely summarize the content of this section. I have also a general comment regarding the description of the polarity of RNA or DNA strands: the "prime" symbol should be used, instead of the "apostrophe"; e.g. 5′-terminus (not: 5ʾ-terminus).

In his doctoral project, the PhD candidate used different mammalian cell lines, such as: HeLa (human cervical cancer cells), HCT116 (human colorectal carcinoma cells) and MEF (mouse embryonic fibroblasts), and also mouse liver tissue. The first approach defined in the PhD dissertation was to clarify the *in vivo* P-stalk protein phosphostatus under basic conditions, i.e. in the absence of stress. It is already known that the uL10 protein (i.e. the protein constituting the structural backbone of the P-stalk complex) can be phosphorylated at its C-terminus presumably by the CK2 kinase, which is a Ser/Thr protein kinase controlling several signaling pathways. Moreover, the proteomic data has revealed that the uL10 protein may as well undergo phosphorylation at its N-terminus. Using HeLa cells as an experimental model and the 2-dimensional electrophoresis followed by Western blotting, Kamil Filipek demonstrated that the uL10 protein can be phosphorylated at four different amino acid residues. These results were further proved by the *in silico* analysis of the available proteomic data, which revealed two phosphorylation sites located at its N-terminus and two phosphorylation sites located at its C-terminus. The structural data revealed that the two N-terminally located residues, that may undergo phosphorylation, are important for interactions between uL10 and rRNA (precisely, 28S rRNA). Further investigations carried out by Kamil Filipek indicated that uL10 N-terminal phosphorylation may have a regulatory potential governing the uL10 interaction with the ribosome and may control the activity of the GTPase-associated center. Then, by using Pro-Q staining approach (the Pro-Q dye detects the phosphate groups) and Phos-tag-SDS-PAGE analysis coupled with immunodetection, the PhD candidate revealed that in MEF cells, under physiological conditions, the P-stalk proteins (represented by uL10 and P1) are phosphorylated. In addition, in MEF and mouse liver, the P-stalk proteins were found in the fully phosphorylated form on actively translating ribosomes (i.e. on polysomes).

The next issue that was tackled, concerned the possible P-stalk protein phosphostate changes. Consequently, the PhD candidate asked the question of whether P-stalk proteins phosphorylation is a permanent state or P-stalk proteins may be dephosphorylated under different stress conditions? To address this question, MEF cells were exposed to multiple stress conditions, such as: (i) amino acids starvation, (ii) oxidative stress, (iii) endoplasmic reticulum stress (ER stress) and (iv) ribotoxic stress. Collected results revealed that amino acids starvation and oxidative stress (induced by H_2O_2) did not cause the changes in P-stalk proteins phosphorylation state (uL10 and P1 remained fully phosphorylated). In the case of ER and ribotoxic stress, 24 hours after exposure to the stress factor, the small fraction of non-phosphorylated or dephosphorylated uL10 and P1 proteins was observed. The observed changes in the P-stalk proteins phosphorylation state were found not to be connected with the CK2 kinase inhibition (the kinase involved in P-protein phosphorylation). In my opinion, for this set of reactions (the results of which are shown in Figure 21), a control reaction under non-stress conditions should be included. This control would allow to monitor the phosphostate of uL10 and P1 proteins over the observation time, under basic conditions. I have also a question regarding the diminished signals observed in lanes corresponding to the experiment carried out for MEF (-AA), 8-24 hrs, for P1 (Figure 21A) or for MEF (+H_2O_2), 2-4 hrs., for uL10 (Figure 21B)? How this decrease in the signal can be explained? Whether a loading control was used?

Next, Kamil Filipek investigated CK2 kinase responsibility in the P-stalk protein phosphorylation. These studies involved two cell lines: MEF and HCT116, and the highly specific CK2 inhibitor named CX-4945, also known as

Silmitasertib. The initial studies showed that after 1 hour of treatment of MEF cells with CX-4945, the CK2 kinase activity was almost fully blocked. Further studies demonstrated that there was a positive correlation between the CK2 inhibition and the changes in the P-stalk protein phosphorylation status. CX-4945 caused the appearance of a non-phosphorylated fraction of ribosomal P-stalk proteins, both in the cytoplasmic fraction and in ribosomes, including translationally active polysomal fraction. The results of these studies underscore the fact that the CK2 kinase is involved *in vivo* in the phosphorylation of the ribosomal P-stalk proteins. Investigation of the CX-4945 inhibitor influence on the P-stalk proteins demonstrated also a decline in the polysomal fraction (incubation of MEF and HCT116 cells with CX-4945 reduced the quantity of translationally active ribosomes), which pointed to the emergence of stress. Consequently, Kamil Filipek examined the metabolic status of MEF and HCT116 cells upon CX-4945 treatment, and he found that CX-4945 induced the eIF2α phosphorylation in both used cell lines. eIF2α is an α-subunit of the initiation factor eIF2 that is required for most forms of eukaryotic translation initiation. The eIF2α phosphorylation pattern (i.e., two-wave pattern) was the same in MEF and HCT116 cells treated with CX-4945. The first wave of eIF2α phosphorylation correlated with the ATF4 synthesis (ATF4 regulates the transcription of survival genes), and the second wave of eIF2α phosphorylation correlated presumably with the changes in the P-stalk phosphostatus. I believe that the set of control reactions involving MEF and HCT116 cells non-treated with CX-4945 (matching Figure 25) would be beneficial to monitor the level of the selected proteins (phosphorylated and non-phosphorylated) over the observation time, in non-stressed cells.

Finally, characterization of the GCN2 kinase involvement in the response to CK2 kinase inhibition was performed. GCN2 is a Ser/Thr protein kinase, and its activity is presumably dependent on the P-stalk proteins. Conducted studies involving MEF_{wt} cells and the MEF knockout cell line that does not produce GCN2 kinase (MEF_{GSN2KO}) revealed that treatment with CX-4945 induced the GCN2-dependent phosphorylation of eIF2α in the MEF_{wt} cell line. Studies on the effect of CX-4945 on cell metabolism have also revealed the activation of the integrated stress response pathway dependent on the GSN2 kinase, which led to degradation of P-stalk proteins. The collected results indicated that there is a correlation between the emerging fraction of non-phosphorylated P-stalk proteins and the activation of the stress response pathway, in which kinase GCN2 might perform a dual function: as a receptor sensing the changes in the phosphorylation state of P-proteins and as a stress-signal transmitter that switches on the integrated stress response.

In summary, the findings presented by Kamil Filipek in his doctoral dissertation allowed to propose that the phosphostatus of the P-stalk proteins represents the optimal form for translational machinery. Phosphorylation of P-stalk proteins, by lowering their affinity to the trGTPases (and decreasing the GTP hydrolysis), might provide the ribosome enough time to ensure that the selected amino acid-loaded tRNA is correct. I have no doubt that these new data will lead to a better comprehension of the regulation of the functioning of eukaryotic ribosomes.

I have some general questions to the PhD candidate:

- What is a half-life of the P-stalk proteins under physiological conditions?
- Are there known naturally occurring mutations in human P-stalk proteins that may affect their functioning or cause disease?
- Whether the knowledge gained during his PhD studies can be used in applied biochemistry and biotechnology, for example in the designing of new selective inhibitors against CK2 and GSN2 kinases?

To summarize, I would like to underline that the results presented by Kamil Filipek in the PhD thesis might be of general interest for the researchers working in the field of molecular biology and biochemistry. The PhD candidate used a variety of advanced techniques from the area of molecular biology and biochemistry, including advanced imaging and protein immunoblotting. Various approaches have been used to confirm that the observed

results are reliable. The experiments were adequately designed and clearly presented, though I believe that some additional controls would be beneficial. The interpretation and discussion of the results were adequate and proved the expertise of the PhD candidate in the field of ribosomal studies. I appreciate the novelty of the presented research, especially concerning the hypothesis that the phosphorylation of the P-stalk proteins can be considered as important factor that improves the correctness of decoding genetic information. I also would like to emphasize that Kamil Filipek is the first author on the manuscript *Phosphorylation of the N-terminal domain of ribosomal P-stalk protein uL10 governs its association with the ribosome*, FEBS Lett. 2020 (data presented in this manuscript make up a substantial part of the PhD thesis).

Taking all the above into account, I state that the PhD thesis presented by Kamil Filipek certainly meets the requirements laid down for the degree of PhD in biology by the status in the Journal of Laws of the Republic of Poland (Dz. U. 2018 poz. 1668. Z późń. zm.). I recommend that the Scientific Council of Institute of Biological Sciences of the University of Maria Curie-Skłodowska would proceed with further procedural steps to confer the PhD degree in biology on mgr Kamil Filipek.

Z pełnym przekonaniem stwierdzam, że przedstawiona mi do oceny rozprawa doktorska spełnia warunki określone w ustawie Dz. U. 2018 poz. 1668. USTAWA z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce z późniejszymi zmianami i wnioskuję do Rady Naukowej Instytutu Nauk Biologicznych Uniwersytetu Marii Curie-Skłodowskiej o dopuszczenie mgr Kamila Filipka do dalszych etapów przewodu doktorskiego.

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