Prof. RNDr. Jan Černý, PhD
Chairperson of the thesis committee
Department of Cell Biology
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Charles University
Viničná 7, Prague

5th September, 2022

Dear Professor Černý,

It was with a great interest that I read and evaluated the thesis: "The role of Nuclear Phosphatidylinositol 4,5-bisphosphate in RNA Polymerase II Transcription" by Can Balaban performed under the supervision of Prof. Pavel Hozák at the Laboratory of Biology of the Cell Nucleus, Institute of Molecular Genetics, AS CR, v.v.i.

In the presented thesis the author describe a comprehensive and extensive research project, spanning three manuscripts (two published, one in form of a yet to be published manuscript), concerning the role of nuclear phosphatidylinositol 4,5-bisphosphate (PIP2) in the transcription mediated by RNA polymerase II (RNAPII). The project started with the identification of the interactome of PIP2, using a novel mass-spectrometric approach that enable the team to identify Myosin Phosphatase Rho-Interacting Protein (MPRIP) as an PIP2 effector. It followed by a thorough investigation of the properties of MPRIP with regard to its subcellular localisation and its ability to phase-separate into condensates *in vivo*. Lastly, they investigated the role of MPRIP in mediating the association of PIP2 with RNAPII.
Overall, these results provide an important contribution into our understanding of the principles of self-organisation of the cell with particular emphasis on the role of membrane-less organelles into the process. I believe that this is one of the key biological question that we are yet to fully answer and comprehend, and the achievements presented in the thesis should be highly commended.

The thesis itself is logically organised into introduction, aims, results and discussion, summary and conclusions, and future prospects. Overall, the thesis is written in good English, but a one more round of revision of the text would help, with particular emphasis on grammar.

In the introduction section, the author provides a thorough review of the role of the different types of modifications of phosphatidylinositols that exist in the cells, followed by a rather brief description of the transcription, principles of liquid-liquid phase-separation (LLPS), nuclear actin, its polymerisation, and the role of actin and myosins in the RNAPII-mediated transcription.
Generally, in this section, I had the feeling that the author rather than guiding the reader and providing the reader with the necessary information, felt the need to meet certain range criteria. I would recommend shortening the sections concerning the role of different phosphatidylinositols that exist in the cell and expand the sections concerning LLPS, its mechanisms, and general principles. The same applies for the sections regarding transcription. This, in my opinion, would improve the understanding of the impact of

author's research in the latter sections of the thesis, particularly for those who are not familiar with the rapidly growing field of LLPS.

The aims are clearly stated and were met. Here I would suggest the author to link their research with the broader interest of the research group and broader scientific field.

In the results and discussion section, the results are well summarised. However, I would suggest splitting off the discussion into a separate section and delve more in depth with place the author's results within the broader scientific field.

I commend that the author included section concerning future prospects, as it clearly shows that the student thinks about their research.

Overall, this thesis provide an important insight into the organisation of the nucleus, with particular emphasis on the role of phosphatidylinosintols and how PIP2 is linked with transcription. It also shows that the author has familiarised themselve with scientific research worthy of the PhD level research. Therefore, I recommend this thesis to be accepted by the committee.

I have the following questions for the author:

1) Could you identify, based on your knowledge, what is the most important unanswered scientific question in your field of study? What experimental strategy would you pursue to answer the aforementioned scientific question?
2) In the result section, you describe association of MPRIP with various forms of the C-terminal domain (CTD) of the catalytic subunit of RNAPII. Do you envisage a direct, physical interaction between MPRIP and the CTD? If so, what domain do you think would be responsible for the interaction?
3) You performed the *in vivo* assays assessing LLPS of MPRIP using an overexpression system. As this is inherently prone to produce artefacts, due to artificially increasing the levels of the studied protein, do you plan to assess the LLPS of MPRIP using endogenous levels of expression?
4) For the LLPS of the CTD of the catalytic subunit of RNAPII, it is proposed that the aromatic systems of the tyrosine residues are crucial. It is likely that phosphorylation of tyrosine impinges upon the LLPS of the CTD. How would you reconcile your observation of LLPS of MPRIP, its preferential interaction with Y1P-modified CTD and possibility that MPRIP may promote LLPS of the RNAPII?

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