Opponent's Review on the Master's Thesis: "Molecular Mechanisms of MICAL Activation in Axon Guidance" by Bc. Jonáš Vlasák

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The thesis focuses on the biochemical and biophysical characterization of *drosophila* and human MICAL proteins and their cognate receptors from the Plexin family. These proteins are crucial factors in axon guidance and serve as key regulators in neural development. Understanding the molecular basis of their interaction is expected to provide valuable insights not only into this signaling axis but also into broader aspects of neural development.

In the Introduction, the author provides an extensive description of the structure of the axonal growth cone and its regulation through various signaling pathways. Although the chapter is highly detailed and certain parts may be challenging to follow, the inclusion of several diagrams significantly aids in clarifying the complex interactions within neuronal cells.

In the Aims section, the author outlines the primary objectives of the research, which include designing and producing the *drosophila* MICAL CC domain and the cytoplasmic part of PlexinA using an *E. coli* recombinant system. The study aims to define the binding constant for the interaction between these two proteins using surface plasmon resonance (SPR). Over the course of the study, the scope was broadened to include human variants of the proteins, which were produced in insect cells.

The Methods section demonstrates that the author has acquired substantial experience with various techniques. Utilizing AlphaFold, Mr. Vlasák conducted structural predictions of the studied proteins and used PCR to design constructs suitable for dimerization. He produced the two binding partners – *drosophila* MICAL CC domain and PlexinA cytoplasmic domain – in *E. coli*, followed by a two-step purification process via nickel chelating and size exclusion chromatography, and aimed to characterize their interaction using SPR. Additionally, he produced human forms of the studied proteins in *Spodoptera frugiperda* cells. Through this work, Mr. Vlasák gained considerable expertise in molecular genetics, recombinant protein purification across different expression systems, and biophysical characterization methods.

In the Results section, the author demonstrates successful expression and purification of both monomeric and dimeric versions of Plexin proteins, as illustrated by the attached chromatograms and SDS-PAGE gels. The dimeric versions were first modeled using AlphaFold to optimally position the leucine zipper helper sequence relative to the Plexin protein, as depicted in the provided images. Although the SPR measurements of MICAL binding to Plexin did not yield conclusive results, the binding of MICAL to its known binder, Rab8, produced a representative curve, which is shown along with the fitting statistics.

Potential drawbacks of methods used to study presented interactions are discussed in the Discussion section. It is suggested that the interaction between MICAL and Plexin proteins reported in the literature could be misinterpreted, possibly due to the absence of positive controls, unknown cellular cofactors, or modifications. The author effectively highlights which experiments could be further used to investigate this interaction. Overall, the author expresses himself clearly and demonstrates critical thinking that is necessary in his subsequent academic career.

Finally, everything is summarized in the Conclusions, and the used sources are properly cited in the References chapter.

Questions for further discussion:

1. MICAL proteins:
   - For production of Plexin proteins you show results from size exclusion chromatography and SDS-PAGE. How did you characterize your MICAL samples? You expressed the *drosophila* MICAL CC domain in E coli. What was the source of the full length human MICAL-1? I understand that working with protein of this size in full length version must be very challenging.

   - What is the actual size of the MICAL CC domain? You mention residues 4529-4723 for your construct. Can you provide a scheme of the (maybe full length) protein with highlighted domains and your construct?

   - The 3D structure of MICAL protein is not known as it is very complex and huge protein. Is the putative interaction site for Plexin known/validated to be exclusively the CC domain?

2. Your experiments to characterize the interaction between MICAL and Plenix were not yet successful. Did you attempt to validate the interaction described in the literature using alternative techniques, such as SEC or NMR?

3. You mention the reason for not observing the direct interaction of your proteins in SPR can be the lack of post translational modifications and you mention phosphorylation as an option to look into. Did you try to model the interaction of your proteins with AlphaFold to see if there are residues to be phosphorylated? Or this approach does not yield a conclusive model?

4. Did you consider working with endogenous samples? If so, from which source and why?

In conclusion, Mr. Jonáš Vlasák has successfully met all the stated objectives of his thesis and has demonstrated the ability to conduct independent scientific research. Submitted thesis meets all the necessary requirements, and I fully recommend it for defense.

In Prague, on 30th of August 2024 Mgr. Rozálie Hexnerová, Ph.D.

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