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Substrate specificity, mechanism and activity regulation of the rhomboid family intramembrane proteases

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Submitted for the degrees of Doctor of the Charles University, Faculty of Science

Degree Programme: Biochemistry

"Examiners' Detailed Comments"

As the title indicates the submitted thesis is focused on mechanism and specificity of intramembrane proteases from the rhomboid-like superfamily. Basically the thesis can be divided in two parts: 1) Investigation of substrate binding, specificity and reaction mechanism of the main rhomboid model GlpG from the bacterium E. coli leading to papers I to III, and 2) study of dimerization properties of one of the four human secretase rhomboids, RHBDL2 in membranes of giant plasma membrane derived vesicles (GPMVs) leading to paper IV. The thesis is written in the "shortened version", which means that first a) in 18 pages the reader is introduced to "intramembrane proteolysis" and rhomboids and on half a page the aims of the studies are defined. This is followed by b) the four published manuscripts and finalized by c) 5 pages' discussion and conclusions. The key paper of the first part of the thesis which describes the substrate binding, specificity and reaction mechanism of GlpG has been published already in 2014, while the two follow up papers published in 2017 are introducing a fluorogenic transmembrane peptide substrates based on the "EDANS-DABCYL fluorophore-quencher pair or TAMRA / dark quencher pair" and oligopeptides acting as inhibitors for rhomboid intramembrane proteases, respectively. Due to the complex nature of these contributions all three papers do have many (i.e. 8, 16, and 16, respectively) authors and Mgr. Jan Škerle's contributions to each publication are quantified to be 15%. While the first part of the thesis is certainly a lot of "Biochemistry", the second part and by that publication IV falls clearly the field of Molecular Biophysics. Here the contribution of Mgr. Jan Škerle is quantified

to be 50%. Thus this recently published paper can be considered as the main paper of his thesis. It has been discussed in the literature whether rhomboid proteases dimerizes in biomembranes. Mgr. Jan Škerle has applied cutting edge fluorescence approaches to search for such dimers. Specifically, he used Foerster resonance energy transfer (FRET) combined with Monte Carlo (MC) simulations (FCS was here elegantly used for determining the D/A acceptor concertation's) and fluorescence correlation cross spectroscopy (FCCS) to address dimerization of human rhomboid protease RHBDL2 in biomembranes. In this impressively thorough contribution no evidence for stable dimers was found, which ultimately suggest that the rhomboid transmembrane core is monomeric.

Although, I find that the thesis could have been written with more care, I conclude that Mgr. Jan Škerle proved his scientific competence during his PhD studies. He serves as the first author of one article. Moreover, he is a co-author of 3 more contributions. Overall the scientific content of the thesis fulfills the standards for obtaining a PhD on the Czech level. The overall scientific content of the four papers can be rated also internationally as outstanding. I conclude, that the author of the thesis proved to have the ability to perform research and to achieve scientific results. I do recommend the thesis for presentation with the aim of receiving the Degree of Ph.D.

Questions to be answered at the defense:

- On page 30 is stated that "RHBDL2 was studied ***in vivo*** with emphasis on its behavior in a natural biomembrane". In the corresponding manuscript I learn that the experiments were done on GPMVs. This brings me to a question which I would like to get answered for some years: Where is the borderline between *in vivo* and *in vitro* experiments?

- I was quite disappointed not to find neither a chemical structure of the "EDANS-DABCYL fluorophore-quencher pair" nor a more elaborated explanation of the photophysical background. The same counts for the TAMRA / dark quencher pair. I would like to ask Mgr. Jan Škerle to present a comprehensive overview on the photophysics of these dyes in his PhD defence.

- In this context I read on page 49 "red-shifted fluorophores such as carboxytetramethylrhodamine (TAMRA)"; Could it be that "shifted" is not an appropriate wording?

- Page 83: "We used Foester resonance energy transfer (FRET) and fluorescence correlation spectroscopy (FCCS) combined with Monte Carlo (MC) simulation to address dimerization

of human rhomboid protease RHBDL2 in its native biomembrane." Please find one spelling mistake, one wrong description of an abbreviation, one non-logical assignment, and a possible overstatement. I find that a key sentence in the summary for the main paper should be written without mistakes and clearly!

- I like the way the concentrations of donor and acceptor molecules used in the FRET experiments in the GPMVs were determined (Paper IV). As this is a key pre-requisite for the MC-FRET approach, I would like Mgr. Jan Škerle to explain that during his defense.

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Prague, 1.9.2020 Prof. Dr. Martin Hof, DSc.