Referee's comments for PhD thesis "Functional assessment of Bcl-2 family proteins in mitochondrial metabolism and beyond"

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The main focus of the presented thesis is the role played by Bcl-2 family members in the regulation of mitochondrial metabolism. Primarily, the thesis explores how they influence mitochondria independently on their role in apoptosis. However, papers from the collaboration with the group of Prof. Klener explore both apoptosis-related as well as independent actions.

The thesis is based on five published primary papers (one first author) and one review. One of the papers listed as under revision in the thesis has, in the meantime, been accepted for publication in Blood Advances. All are in respectable journals with Ifs spanning from 5 to almost 9. In its form, the thesis follows the "short" format with introduction, description of used methodology, short summaries of individual papers, and finally, general discussion.

Overall, I really liked the introduction, which nicely covers all aspects of the studied topic. We learn about various modalities of regulated cell death, Bcl-2 protein family and its function, mitochondrial metabolism as well as non-metabolic functions of mitochondria. On 23 pages, it provides just enough detail to provide background for the following sections without digging too deep and losing the attention of the reader. I appreciated the possibility to learn something new about Bcl-2 family and considered this part really well written. Rather unsurprisingly, once I got to the area of my expertise (mitochondrial metabolism), I started to disagree with some of the postulated facts. On the other hand, apoptosis experts would surely see it the other way round. Anyway, those three points may be found in the literature, but that does not mean they are correct:

- Respiratory complexes I, III, and IV do transport protons across the inner mitochondrial membrane, but that does not necessarily mean, that they are proton pumps (p. 28 and 29). C III is definitely **NOT** a pump and C IV is **NOT ONLY** the pump.
- Supercomplexes of the respiratory chain clearly do exist, but their function remains uncertain and the hypothesis regarding improved effectivity is not widely accepted.
- I might be the fight of Don Quijote de Innsbruck, but C II does not oxidize $FADH_2$. It may be part of the cartoon in Nature Reviews in Endocrinology, but it is wrong. Neither do you claim that C I oxidizes FMN.

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While the thesis also contains the Materials and Methods section, I find this relatively rudimentary. It pointedly states that detailed methodologies are parts of the published papers, and this is just a brief summary, likely to conform to the regulations of the board. Anyway, the outlines of the methods are correct and in line with what is stated in the accompanying papers.

Results section is represented by commented summaries of individual published papers. As with other parts of this thesis, the main focus is on the first author paper, which describes phenotypic presentation of BAX/BAK double knockout in cell lines representing a diverse spectrum of cancer types. Authors identified high variability in metabolic response towards BAX/BAK absence and pinpointed it to the posttranscriptional regulation of Tefm levels. In this section, additional data which are not present in the original paper are presented as well. Published data demonstrated decreased respiration in BAX/BAK KO HBL-2 B lymphoma cells and the additional data demonstrate analogous drop in UPF1G and UPF1H B lymphoma cell lines. This observation further verifies the uniformity of response towards BAX/BAK absence in cells of hematopoietic lineage. Overall, for all papers, the author's contribution is discussed thoroughly. Most often it includes preparation of cell models with modified gene expression and characterisation of mitochondrial metabolism, clearly documenting the author's expertise in this area.

Formal aspects are kept under control, with well-prepared figures and generally good standard of English scientific writing. Still, there are a few formatting issues (e.g. FADH2 instead of $FADH_2$ and NAD+ instead of NAD^+ on p. 33, $O2/O_2$ p.28 and I would also bet, that I saw F_0 "zero" instead F_o (letter "o") on p. 29), typos (softer vs. software p. 40, Beckton Dickenson vs. Beckton Dickinson p. 41) or inconsistent nomenclature (MPTP p.24 vs. PTP p.27), scattered throughout the text. Given the length of the text, such issues are quite rare, and as I already said before, the text is engaging and easy to read, yet still of sound scientific quality.

I would like to ask several questions regarding the current thesis:

1. On p. 15 you mention that the loss of cytochrome c during apoptosis is accompanied by attenuation of mitochondrial membrane potential during apoptosis, which is the standard storyline. But has this really been shown? One can imagine that upon such conditions, ATP synthase may reverse and start building membrane potential independently of the respiratory chain, which naturally requires the presence of cytochrome c.
2. On p. 27 you discuss the role of mitochondrial permeability transition pore (MPTP) in calcium-mediated cell death. You propose, that MPTP opening subsequently leads to the release of cytochrome c. How can this be achieved, since MPTP forms pores spanning both outer and inner mitochondrial membranes and thus does not allow the release of molecules from the intermembrane space?

3. Apoptosis paper Fig. 4 and thesis p. 55 – why do you report NAD^+/NADH ratios from LC-MS data where you most likely do not have a direct calibration curve and not from NAD^+/NADH glo kit data, which do have calibration for NAD^+ as well as NADH? It should be said that your reported NAD^+/NADH data are a bit counter intuitive. Upon dysfunction of mitochondrial respiration, we often see a significant reduction of NAD^+/NADH accompanied by a **decrease** in respiration. Do you have any explanation for why you observe a reverse relationship?
4. Do you have any interaction data supporting either BAX/BAK interaction with TOM complex or with MPP? Some sort of interaction would probably be required for the regulation of TEFM import/processing.
5. Do your proteomic data suggest dysregulated import/processing also for some other mitoproteins targeted to matrix? Or do you have any suggestion why BAX/BAK presence specifically affects import/processing of TEFM only?

In conclusion, the presented thesis fulfils all requirements, and I fully recommend it for defence.

In Prague, 20.6.2024

Signature:

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