In Nový Jáchymov 11th of June, 2018

The opponent's review of the PhD thesis of Mgr. Michaela Procházková: Mitochondrial gene expression in trypanosomatids.

The thesis consists several parts in the following order: Introduction, Aims and course of research and four (I to IV) parts corresponding to one publication and three unpublished areas of research, Summary and References.
The introduction describes lifecycles and possible therapy of medically important kinetoplastid species (*T. brucei, T. cruzi* and *Leishmania*). It is followed by a section on peculiar molecular and cellular characteristics of *T. brucei,* specifically trans-splicing, antigenic variation, the presence of glycosomes and the overall metabolic changes between mitochondria of procyclic or bloodstream form. Last part of the introduction is dedicated to the actual topic of the thesis – Mitochondrial gene expression involving RNA editing and the translation on mitoribosomes. In general, the introduction is well written, my main criticism is about the chosen content of the introduction. I would be much better to read more details about the mitochondrial translation, which is the main focus of the work and less about the very general biology of the parasitic species. Thus I find the introduction not very useful for the rest of the thesis.

Part I – Mitochondrial release factors in bloodstream from trypanosome.

The core of the part I is the published article in Scientific reports with PhD candidate as the first author. The story already went through the rigorous review process. In total, it is very well and carefully performed research on the function of Mrf1 in the bloodstream form of *T. brucei*. The paper analyzes the phenotype of the complete knock-out of TbMrf1 – evidenced by the instability of ATP synthase complex and ribosomal SSU. The phenotype is more profound when the expression of codon-independent release factor Pth4 is also decreased. Surprisingly, the phenotype of TbMrf1 knock-out strain partially reverts after prolonged cultivation. It is very nice and interesting biochemical paper that shows the connection between the mitochondrial translation to metabolic adaptability of *T. brucei* mitochondria. It also demonstrates the high quality of the supervisor's laboratory.

1. *Could the candidate comment on what is the reason behind the difference in need of release factor methylation in bacteria and eukaryotic cytoplasm vs the mitochondrial system?*

2. *What is the functional/structural difference between Mrf1 and Pth4 enzymes?*

Part I is extended by three directions of research that are linked to the published paper. These sections indicate other exciting directions of the research, unfortunately they were burdened by some crucial technical limitations and, if I understood well, also by lack of time.
Firstly, the effect of deficiency in translation release factors on mRNA levels. Intriguingly, while the absence of TbMrf1 results in decreased (edited or non-edited) transcript amounts, the concomitant knock-down of TbPth4 has the opposite effect. The logical question would be, why such interesting direction has not been followed further.

3. *Could the candidate comment more about the interesting observation? Also how does the transcription sense the inefficient translation?*

Secondly, in addition to mitochondrial release factor, putative cytosolic orthologue was identified and partially characterized. However, comparison of the methylation status of both orthologues in the delta MTQ background could not be assessed due to low protein expression of the mitochondrial proteins.

4. *Could this, however, be done for the cytosolic protein? Also, in regard to the sensitivity of current mass spectrometers one would expect, that such modification is possible to detect even in complex protein samples.*

Thirdly, the function of mitochondrial TbMTQ1 methylase has been investigated, specifically the KO cell line was generated and possible triggered phenotype was examined. However, only mild changes have been detected when compared to the parental line, indicating that trypanosomes, in contrast to other organisms can cope with the lack of the methylase.

5. *Given the initial failure to obtain material for the in vitro methylation assay, has the candidate considered to use in vitro protein expression systems (such as PUREXPRESS), which usually generates amounts of proteins enough to measure the enzymatic activity?*

Part II – Optimization of the safranine O assay for the measurement of of FoF1 ATPase activity.
In this rather methodological part, the PhD candidate describes successful introduction and optimization of safranine O as substrate, which can be used to measure mitochondrial membrane potential.

6. *Can you use JC1 compound to measure membrane potential of trypanosome mitochondria as it used in mammalian cells?*

Part III – Destabilization domain technique in mitochondrion.
This part of the thesis describes expression of DD in *Leishmania* with the aim to introduce regulatable protein expression in this organism. DD is a renowned engineered domain of FK506 binding protein 12 from Wandless laboratory, which can be stabilized by the addition of small ligand. The overall principle has been shown to work in *Leishmania* for the cytosolic version of the protein. However, in case of the constructs carrying the mitochondrial targeting sequence, the stabilization resulted by the mistargeting of the protein to the cytosol. The PhD candidate then correctly concludes the protein folding may interfere with the post-translational protein import to mitochondria, which requires only unfolded substrates. Negative or inconclusive results were also obtained when DD was fused to T7RNA polymerase, a heterologous RNA polymerase routinely used in trypanosomes.

7. *Can you comment on the actual effect of non-stabilized DD domain, what is the fate of the protein of interest in such case?*
8. *Can you compare structure and activity FK506, rapamycin or Shield-1 molecule in DD stabilization.*

Part IV – Respiratory complex IV – associated protein MIX in bloodstream form trypanosome.
MIX is a mysterious protein with more less unknown role in mitochondrial biology. While MIX knock-down has been previously shown to slow the growth of the procyclic form no such phenotype has been observed for the bloodstream form trypanosomes. However, the inability to detect protein by raised polyclonal antibody in the latter stage hampered further characterization of the protein.

9. *Have you considered in situ tagging of MIX when antibody was not sensitive enough?*
10. *Can you find MIX orthologues outside kinetoplastids?*

The thesis ends with a short summary and the list of literatures
Some references (at least one found by the reviewer, reference 156 on page 53) are not correctly numbered,

Finally one more general question:

11. *In the thesis, there is an expression "T. brucei is an ancient organism", what does the PhD candidate mean about it and is it correct at all?*

To sum up, the thesis of Mgr. Michaela Procházková is a very solid piece of science, loaded with huge amount of the experimental work, which unfortunately in some cases did not lead to positive outcomes.
Despite my several comments mainly to the concept of the introduction section, I fully re-commend the thesis for its defense next week.

Pavel Doležal, Ph.D.

The thesis of Michaela Prochazkova deals with several projects with regard to analysis of mitochondrial translation in unicellular parasites. With two mitochondrially encoded genes necessary in the bloodstream (mammalian host) life cycle stage, *Trypanosoma brucei* provides a highly simplified model in which to study mitochondrial translation termination.

The main project uses *T. brucei* to examine the mitochondrial translation termination factor TbMrf1, using a gene knockout. It seems that the peptidyl-tRNA hydrolase TbPth4 is able to soften the TbMrf1 knockout phenotype by its ability to rescue mitoribosomes from becoming stalled in the absence of TbMrf1.

Additionally a lot of extra experimental approaches to study trypanosomatids were being implemented or attempts were made to develop them (e.g. modifying methyltransferase of TbMrf1, the TbMTQ1, the development of protein expression regulation methods in Leishmania parasites, protocols for measuring the mitochondrial membrane potential in kinetoplastids and improving purification protocols for hydrophobic recombinant proteins to be used in the study of these organisms).

Overall, the thesis represents an impressive amount of technically challenging work, which, all in all, is well presented.

I gladly state the work to be deserving of a PhD degree, in my opinion.

Amsterdam/Ceske, 19-06-2018,

Dave Speijer