Review of Markéta Petrů’s doctoral thesis entitled “Bacterial proteins in the biogenesis of mitochondria of unicellular eukaryotes”.

Evaluation of thesis:

The doctoral thesis of Markéta Petrů deals with mitochondrial biogenesis and fission as mediated by proteins of bacterial origin, presumably inherited directly from the endosymbiont that gave rise to the organelle. The work is comprised of a thorough introduction written by the candidate and three chapters. The first two are articles published in the prestigious journals PNAS and BMC Biology, in which the candidate is second author and first author, respectively. The third chapter is a manuscript that is currently under consideration in yet another prestigious journal, Nature Communications. Although she is listed in the middle of the author list, she made a critical contribution in generating antibodies that were vital for facilitating study of a currently non-model organism, the ameboflagellate *Naegleria gruberi*.

I duly note that Markéta was directly involved in writing the accepted articles. This does not surprise me as I found the introduction to be remarkably well written. The candidate clearly has an excellent command of the English language. Nevertheless, the incorrect use of definite and indefinite articles does give away this was not written by a native speaker. But this is a common and understandable mistake among Czech writers in English, and in fact I found this reassuring as it is clear Markéta really wrote this text herself. I am confident that Markéta will be able to deal with this aspect of English writing with more practice and once again stress that this is the only real weakness in terms of language in an otherwise excellently written text. Occasionally I did catch some awkward phrases, *e.g.* on page 35 she writes, “Mosaic occurrence of Tat and almost disappeared Sec in mitochondria outlines a question...”. “Almost disappeared Sec” is not so elegant, “outlines a question” does not make much sense, and last AND least, there should be a definite article in front of “mosaic” as it modifies the noun “occurrence”. But these occur toward the end, when I imagine the candidate was rushing to make her deadline and again such cases occur rarely in the text.

More importantly, the introduction represents a nice synthesis on the current state of knowledge of protein translocases of prokaryotes as well as the topic of the thesis, translocases of bacterial origin
in eukaryotes. In this section, Markéta convincingly demonstrates that she is able to synthesize information from various literature sources into a cohesive whole and clearly convey this information to the reader. This also demonstrates her ability to be a truly independent scientist, who can write her own publications and grants. It clearly shows that she truly understands her topic as well.

The three subsequent chapters are very interesting articles and manuscripts that represent a truly impressive body of work. The first chapter convincingly shows that the ancestral bacterial division system is more widely implemented in mitochondrial division than originally thought. The article clearly demonstrates the value of looking outside of typical model organisms, mostly belonging to opisthokonts, to truly understand mitochondrial biology. The second chapter in which Markéta is first author deals with vestigial TAT translocases distributed throughout eukaryotes. Because the more complete TatAC system is found in non-genetically-tractable eukaryotes such as jakobids, the study employs a stunning range of exogenous systems such as *Escherichia coli* and *Saccharomyces cerevisiae*, and even delves into *N. gruberi* directly! The work convincingly and elegantly shows that TatAC is a minimal eukaryotic TAT translocase and pinpoints modifications to TatC that allowed it to be transferred to the nucleus of chloroplast-containing eukaryotes. The third manuscript is an impressive work demonstrating that a vestigial bacterial T2SS secretion system is retained in malawimonads, jakobids and heteroloboseans, with exciting evidence that it may be retained in the last lineage to mediate export of proteins from the mitochondrial IMS after some kind of modification that can only occur in this space.

I really enjoyed reading the thesis. I recognize the high quality of the work presented in the chapters and the excellent synthesis in the introduction produced directly by the author. I also learned a lot from reading about this fascinating topic. I think this is the highest praise I can give any doctoral thesis.

However, no thesis is perfect and Markéta’s excellent work is no exception. I note three major weaknesses of the thesis:

1) I did not think the title, “Bacterial proteins in the biogenesis of mitochondria of unicellular eukaryotes” did much justice to the impressive content of the thesis. Something like “Mitochondrial biogenesis of unicellular eukaryotes relies on ancestral bacterial proteins”
would pack a better punch. I believe that Markéta could come up with a better title given her facility with English.

2) The introduction does not deal with the bacterial division system at all. This is a puzzling omission given her significant contribution to the article that deals with this topic in the first chapter. However, I do admit the author covers protein translocases with enough thoroughness that I almost overlooked this omission.

3) Supplemental materials are missing in the article and manuscript chapters. In the case of the articles, these are accessible on the internet. Still, I would have appreciated not having to go through the effort of doing so. There is no excuse for its exclusion in the manuscript chapter as such material for an article pending acceptance is inaccessible. Given there are 13 supplemental figures and 5 supplemental tables, this is a glaring omission indeed. I get that some excel tables may be too large to print out, but the figures should have been provided to the reviewer for their evaluation in the very least!

Despite these 3 major weaknesses, I do enthusiastically recommend Markéta for the title Ph.D. based on the vast positives of her thesis: the excellent introduction and great publications that she has co-authored. This interesting work has stimulated several questions that I look forward for Maréta to answer at her thesis defense. These are appended at the end of this report and are dividing into major and minor questions, the latter of which can be addressed if there is time.

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In České Budějovice
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Questions for discussion:

1. Introduction
   a. Major questions:

      1) On page 18, the author writes “…TatA from [the] TatAC-only system of *Bacillus subtilis* can substitute for the function of both TatA or TatB in *E.coli*.” However, it was written earlier that TatA and B have distinct functions. TatB remains in contact with TatC until a twin arginine substrate binds to this complex, causing TatB to dissociate. This in turn allows TatA oligomerization to form a pore adjacent to TatC-substrate adduct. How can a single *B. subtitis* TatA serve both functions. Does it have the C-terminal extension present in *E. Coli* TatB?

      2) The author writes on page 27 that there is a “distant homolog” of YidC present on ER membranes. This is consistent with the previously stated role of YidC in insertion of co-translated proteins via the SecYEG pathway in prokaryotes. What is the presumed origin of this ER YidC homolog? Is it essential for protein insertion into ER? Do archaea have a YidC homolog?

      3) The author states on page 30, “category I contains proteins with [a] strong relationship to α-proteobacteria, which is surprisingly only 10-20% of the whole mitochondrial proteome”. Why is this surprising? How does this compare to other organellar proteomes?

      4) The author writes on page 36 that protein export from mitochondria is no longer necessary. However, the manuscript about the retention of a rudimentary T2SS secretion system in certain eukaryotes goes against this statement. I can also recall some other examples from extant mitochondria of proteins exiting the mitochondria. What are some examples of this? Does Markéta not consider these to be “export”?

   b. Minor questions:

      1) On page 18 the author writes, “gram-positive bacteria with low G+C content rely only on TatA and TatC proteins, which are sufficient to mediate [omit the] translocation.” Why would the G+C content of the bacterial genome influence the configuration of the TAT system?

      2) Does TatC have higher affinity for TatB than TatA? Is TatA more numerous in the cytoplasmic membrane than TatB? I am trying to understand more about the mechanism of regulation of TAT translocation and why TatB seems to be more dispensable than TatA. This question is related to major question 1.
3) On page 16, the Markéta writes YidC association with SecYEG enhances co-translational translocation. What is the evidence for this statement? Does YidC deletion also affect proteins translocated via Sec? This question is related with major question 2.

4) This is a rather didactic question, but given the rather cursory discussion in the discussion may be warranted. On page 31, the author mentions the well-studied translocase of the inner membrane TIM22 and TIM23. Which IM-embedded proteins are inserted by TIM22 and which by TIM23?

2. Ancestral bacterial system chapter

   a. Major questions:

      1) In Figure 3, the author convincingly shows that expression of amoebozoan and jakobid Min orthologs are targeted to the budding yeast mitochondria. Given these are presumably involved in mitochondrial fission, and they were exogenously expressed in an organism with the dynamin-like protein mediated system, did the author investigate any effect on the morphology of the yeast mitochondria? What about yeast growth? If no, why not?

3. TAT translocases chapter

   a. Major questions:

      1) On page 6 of the article, the author speculates that the lack of a key enzymatic glutamine at position 170 and phenylalanine at 94 may underlie N. gruberi TatC inability to complement the deletion of E. coli TatC. How can the author directly test this hypothesis? Can the same thing be done to address the heterologobean-specific insertion in TatC?

      2) In Figure 4, the degenerate twin arginine motif of the Rieske homologs in Andalucia godoyi and N. gruberi exogenously expressed in E. coli were exported to the periplasm, even in the absence of s functional TAT translocase. This is quite puzzling. However, when I look at the colonies shown in Figure 4A, it seems to me the colony in the TAT mutant is less dense than the one from the train with intact TAT, suggesting TAT is the main translocase for such substrates. Is it possible to address relative fitness of the strains in question by limiting dilution, as is done routinely to examine the fitness of S. cerevisiae mutants (e.g. Figure 4F of the Nat. Comm. Manuscript in the third chapter)?
b. **Minor questions:**

1) I really liked the assay to test complementation of *E.coli* TatC deletion mutants by growth in the presence of SDS to see if periplasm export is restored (Figure 2). The assay is convincing in showing that the TAT substrate AmiA, a cell wall hydrolase needed to mitigate growth in presence of the detergent, is indeed targeted to the periplasm. Is there another way to directly assay if a protein is imported into the periplasm?

2) Are there any chemical inhibitors of TAT? If yes, can they be used to treat the eukaryotes retaining the vestigial TAT system?

4. **Mitochondrial protein secretion system chapter**

a. **Major questions:**

1) Given that Markéta generated the antibodies used in Figure 2 and Figure 5D, did she ever consider using them to probe Native gel resolved complexes from *N. gruberi* to see whether the containing complexes overlap, particularly OM GspD with IM GspF and IMS GspG? What about using them for immunoprecipitation since Figure 2 shows that the antibody recognizes native complexes and Figure 4 shows the GspG antibody is pretty clean?

2) Given that *N. gruberi* can live in microaerobic environments, did the author consider looking at the proteome of *N. gruberi* grown under hypoxic conditions to see how the Gsp co-occurring proteins are affected. Perhaps some of these proteins are imported into the IMS for oxidative folding, after which they are exported into the cytosol by the T2SS system.

3) In a related question, can the author speculate on why the Gsp co-occurring proteins need to be somehow processed in the IMS? What are potential co-factors that are represented by the circle in Figure 7?