Summary

This PhD thesis by Karolina Šubrtová describes four studies that, taken together, represent a substantial advance in our understanding of mitochondrial biology in trypanosomes. The thesis is generally well written, contains an up-to-date and comprehensive introduction that demonstrates a very good grasp of the field and its literature, and presents and discusses its findings in a way that shows critical judgment with regard to the candidate's own work. The experimental work is state of the art and generally of high standard. Detailed comments on the individual sections are below, including questions that will be explored in the candidate's defense.

Detailed comments

Chapter 1 succinctly introduces the aims of the thesis. **Chapter 2** contains an introduction to the field that is a pleasure to read, up-to-date, and supported by – as far as I can tell – all references that are critical for the questions investigated. The introduction was particularly strong in its discussion of the ATP synthase complex. I noticed only a few factual inaccuracies (for example, it inaccurately states that "*T. b. evansi* and *T. b. equiperdum* became independent from the insect vector by locking themselves in the bloodstream form of the parasite"), an acceptable number of typographical errors, and only a minor issue with out-of-order citations.

Questions that can be explored during the defense include:
What is the molecular basis for the difference between petite positive and petite negative yeasts? Are there correlates for these groups in trypanosomes?
Why are some F_OF_1 subunits highly conserved among eukaryotes, while others are very divergent or even appear to be confined to particular groups of organisms?
What is the evidence that subunit *a* (or 6) is kDNA encoded in trypanosomatids?

Chapter 3 lists the results, **beginning with a paper published in *PLoS Pathogens*** (first author Šubrtová , 100% contribution) that investigates the function of F_OF_1 subunit Tb2 (the protein is described as trypanosome-specific, although a recent study suggests it may also be present in *Diplonema*). The study is of a high experimental standard and its most significant finding is that Tb2 is required for normal growth of a laboratory strain of *T. b. evansi*. The study presents evidence that Tb2 is a part of the peripheral stalk that is involved in anchoring the F_1 headpiece to the inner mitochondrial membrane, presumably in the vicinity of the AAC. Thus, Tb2-anchored F_1 parts appear to contribute significantly to the F_O-independent generation of a mitochondrial membrane potential in this subspecies.

Questions that can be explored during the defense include:
Fig. 4: how do you know reduction of $\Delta\Psi m$ is a primary effect, considering the cells are already 'sick' by day 2 when $\Delta\Psi m$ is analyzed?
How do you reconcile your proposed function of Tb2 and the model for F_OF_1 assembly you favour with the fact that the protein is substantially less abundant in the two dk lines you investigated?
How would you rate the relative importance in dk cells of F_1 headpieces that are associated with Tb2 vs. those that do not contain Tb2? How could this be investigated?

The second study, published in *Eukaryotic Cell* (first author Gnipova; Šubrtová 30% contribution), explores the potential association in procyclic *T. brucei* of the AAC with the respiratory chain, the F_OF_1 ATP synthase, and other transporters in the inner mitochondrial membrane. Despite considerable effort, the study does not find convincing evidence for such associations and concludes that they probably do not exist, but is careful to point out the limitations of the absence of evidence.

Questions that can be explored during the defense include:
You and your co-authors propose the existence of an additional ATP transporter in the inner mitochondrial membrane. This proposal rests on the observation that ATP hydrolytic activity in mitochondria isolated after AAC RNAi is only reduced by 50%. Have you considered alternative explanations for this observation?

The third, unpublished study (first author Gahura; second author Šubrtová but % contribution not specified) describes biochemical purification of the F_1 moiety from procyclic *T. brucei*, identification of its components and their characteristics, and functional analysis of the p18 subunit *in vivo*. This is the only part of the thesis with serious problems, not because of the technical standard, which is very high, but because it is not made sufficiently clear which findings are novel and which are confirmatory and have been reported before. Two key papers that have described purification of F_1 from *T. brucei* before (Williams & Frank, 1990; Nelson *et al.*, 2004) are not cited or discussed, which is a very serious omission. Fortunately this is to some extent corrected in the concluding discussion of the thesis (Chapter 4). The text contains a number of grammatical and factual errors (e.g. cleavage of the alpha subunit and presence of p18 are described as unique features of the *T. brucei* enzyme) and the data shown don't always clearly support what is stated in the text (e.g. phylogenetic relatedness of various alpha and beta subunits; specific activity of F_1 is not shown).

Questions that can be explored during the defense include:
What is novel in this study and what has been reported before?
Examine evidence for/against p18 being the equivalent of subunit b.
The negative effect on cell growth of knocking down F_1 subunits is generally much less pronounced than described in Zikova *et al.* (2009). How can you explain this?

The fourth study (first author Šubrtová but % contribution not specified) presents a preliminary investigation into the function of F_OF_1 subunit Tb1. The candidate acknowledges that a number of experiments need to be repeated before definitive conclusions can be drawn, but taken at face value the results are very interesting indeed. The protein is only found in the complete (or nearly complete) F_OF_1 complex or its dimer, and ablation of Tb1 by RNAi in PF and BF cells appears to have quite different outcomes. Knockdown in PF cells results in disappearance of F_OF_1 complexes, followed by a slow growth phenotype beginning between days 3 and 4. In contrast, ablation in BF cells does not appear to have an immediate effect on F_OF_1 abundance, but it does have a very rapid effect on cell growth. The candidate proposes that ATPaseTb1 may function as an assembly factor of F_O, or that it might be responsible for the stability of the highly hydrophobic subunit *a*.

Questions that can be explored during the defense include:
Fig. 4 shows a tricky experiment. Why would you expect to see a change in EC_{50}?
Fig. 5. Why do you think higher ROS is a more likely cause of the growth phenotype than decreased ATP production?
You propose that ATPaseTb1 may function as an assembly factor of F_O, or that it might be responsible for the stability of the highly hydrophobic subunit *a*. Why would this result in a proton leak? Why would the growth phenotype be quicker than for F_1 knockdown?

The concluding discussion (**Chapter 4**) is concise, factually accurate, and shows critical judgment Considering the substantial advance in knowledge represented by this body of work I think it might have deserved a more thorough summary of its contribution to trypanosome research and to ATP synthase research in general; this is not meant as criticism, but rather as encouragement to consider writing a review on the topic in the near future. I think this would be of considerable interest to the field.

(Achim Schnaufer)

Review of the Ph.D. Thesis of Karolína Šubratová
"F_0F_1-ATP synthase/ATPase in the parasitic protest, *Trypanosoma brucei*"
Reviewer: Dr. Noreen Williams

Overall review: This is an outstanding thesis focused on the unusual ATP synthase/ATPase in *T. brucei* as well as the functionally related adenine nucleotide (ATP/ADP) carrier protein. For the ATP synthase, the majority of the work is focused on characterization of the structure and function of several novel subunits of the complex, Tb2 (published work) and Tb1 as well as subunit p18. An additional chapter (published work) describes the structure and function of one of two putative adenine nucleotide carriers and its unusual relationship to the components of the oxidative phosphorylation system. The thesis is well written overall and the introduction is an excellent and comprehensive review of the literature. The research presented in the thesis is sufficient for a doctoral dissertation. This thesis would certainly pass at my home institution and be ranked highly.

Chapter 2: Overview

The overview provides a solid basis for the rest of the thesis and is an excellent and comprehensive review of the relevant literature for the thesis topics.

Question: Based on your discussion of the TCA cycle (2.6.3) and later studies of your own, what do you think are the limitations of using only cultured procyclic and "bloodstream" cells? Are the bloodstream cells we use a good model for actual bloodstream parasites? What experiments might require an animal (or insect) infection?

Question: Based on your understanding of the enzyme complex, can you speculate on why the *T. brucei* ATP synthase is not 100% oligomycin sensitive as it is in beef heart mitochondria?

Chapter 3: Results

3.1.1 ATPase Tb2, a unique membrane bound F_0F_1-ATPase component, is essential in bloodstream and dyskinetoplastid trypanosomes.

Previous work identified a number of trypanosome-specific subunits of the F_0F_1-ATP synthase/ATPase. In this paper ATPase Tb2 is characterized as a component of the complex in both its monomeric and multimeric forms. RNA interference was directed against the subunit to allow characterization of the effect of loss of the protein. Loss of membrane potential and decrease in the growth rate was seen. In a dyskinetoplastid strain lacking the a subunit, both F_1-ATPase and monomeric and multimeric F_0F_1-ATP synthase were found and Tb2 was a part of all complexes. Loss of Tb2 in this dk strain also caused a loss of growth and membrane potential.

Question: How do you envision the association of the complex in the absence of the a subunit? What role do you think Tb2 is playing?

Question: In Figure 5B and C, it looks like the F_0F_1 is present at day 2 but activity is gone- please explain?

3.1.2 The ADP/ATP carrier and its relationship to oxidative phosphorylation I ancestral protist *Trypanosoma brucei*

The adenine nucleotide carrier is an extremely important component of the cell since it allows communication between the mitochondrion and the cytosol via the exchange of ATP for ADP depending on the bioenergetics conditions of the cell. In this work the findings suggest that the AAC is not a component of a supercomplex (Complexes III plus IV or the ATP synthasome) as in many other cells. RNA interference directed against the

AAC causes a growth defect and a decrease in ATP synthesis (from both substrate level phosphorylation and oxidative phosphorylation). An increase was seen in cytosolic ATP, the membrane potential, and reactive oxygen species. Results also suggest the presence of a second ATP carrier (perhaps an ATP/Mg-Pi carrier).

Question: Why do you think your results are different than previous results on the association of the AAC with supercomplexes? Do you think growth conditions or life cycle stages might have an effect on this association (that it might be dynamic rather than static) ?

Question: Please expand on what you think it means that the *T. brucei* oxphos system has lost supercomplexes? Why would this be of benefit to the parasite? Is there an advantage to having the AAC as a monomer? What is the advantage of having an ATP synthasome in other organisms but not in trypanosomes? You later mention you think Tb2, as part of the peripheral stalk, may help anchor the F_1 close to the AAC suggesting this is important (Ch. 4).

Question: How would you identify a second ATP carrier protein?

3.2. Unpublished results – preliminary manuscripts

3.2.1. The functionality of *Trypanosoma brucei* F_1-ATPase requires the additional subunit p18.

In this manuscript the F_1-ATPase is purified by chloroform extraction and two different cleavage sites are identified for the α subunit. P18 is also shown to copurify with the F_1-ATPase. RNA interference directed against p18 alters both PF and BS growth and a decrease is seen in both F_1-ATPase and F_0F_1-ATP synthase. The current results suggest that p18 is part of the F_1 component of the ATP synthase.

Question: What further work could be done to determine if p18 is part of the F_1 or part of the complete ATP synthase ? What does it mean to be part of F_1 and why would this be important?

Question: Why is it important that the α subunit is cleaved? How do you think (or do you think) this might change the function of the complex?

Question: Loss of p18 (or α) leads to loss of β. You suggest this may be due to the loss of stability of the protein when other members of the complex are absent. How else might this occur and how would you test it?

3.2.2. Functional analysis of ATPase Tb1, a novel and essential subunit of the F_0F_1-ATP synthase in *T. brucei*.

In this manuscript ATPase Tb1 is shown to be part of the monomeric and oligomeric ATP synthase complex but not the F_1-ATPase. Loss of Tb1 causes a loss of F_0F_1-ATP synthase complex but an increase in F_1-ATPase, then two days later a decreased growth phenotype is seen. Interestingly, oligomycin sensitivity does not appear to be altered. Membrane potential appears to be increased.

Question: Does the mild decrease in Tb1 associated with a strong phenotype support the idea that Tb1 might be an assembly factor? Would you expect such a factor to remain associated with the complex?

Chapter 4: Conclusions

Question: How do you think your studies might provide information that would lead to a specific inhibitor to chemotherapeutically target trypanosomes?

Noreen Williams
5/10/15