Review for Julie Kovářová's master's thesis titled: "Localization of the Fe-S cluster biosynthesis in the bloodstream stage of *Trypanosoma brucei*"

Dear Ms Kovářová,

Below you will find my comments, questions and suggestions for your Master's thesis. I would like to point out that the thesis was overall well written and you have demonstrated some interesting results. I have decided my review into two sections: a general section which includes important questions that should be tackled and a specific section which I have included typos and grammar mistakes.

Good luck with your defense.

Sincerely,

Dr. Anastasios Tsaousis

Review:

General comments/questions:

In both the introduction and the discussion, you have dedicate a large portion on the biochemistry (ATP production) of both the bloodstream and procyclic stages, without mentioning at all its relation with the Fe-S cluster assembly. Why you have chosen to do that?

Why didn't you use a figure to show the life-cycle of the trypanosomids and it will be easier for the reader to understand the physiology of each life-stage of the organism?

In your discussion, you have provided with several reasons why the digitonin procedure is not the best strategy to get pure mitochondrial fractions. Can you suggest any alternative strategies?

In page 6, 2nd paragraph you have wrote: "It is also worth mentioning that another form of RNA editing, although mechanistically different from the uridine insertion/deletion type, was described in trypanosome tRNAs (Wolgamuth-Benedum *et al.*, 2009)." Why is this worth mentioning?

In page 6, 3rd paragraph: you have mentioned in the first sentece that there are three different ways to produce ATP and you have dedicate the whole paragraph describing the first one, but you don't even descibe the 2nd and 3rd method at the end, instead you are breifly mentioning it. How is the ATP produced in the glycosomes and how th ATP is produced by acetate:succinate CoA transferase and where?

A figure demonstrating and comparing the alternative ATP pathways in both BS and PS would be useful.

In page 8, 3rd paragraph: It is not clear which enzyme is present in which stage and what are the results. For non-biochemist readers, mentioning the "three last steps of glycolysis" it is confusing; you will need to be more specific or remove this section.

In page 8, last paragraph: There is no connection of this paragraph with anything above. I would rephrase the first sentence: e.g. "Even though in *T. brucei* there are alternative sources for ATP production, in most eukaryotic cells the source of enegy is the mitochondrion …".

Why have you chosen to use the yeast nomeculture for your proteins (Nfs & Isu) instead the general nomeculture that has been used in other protists (IscS & IscU)?

Page 9, 2nd paragraph: Is CIA machinery not a biosynthetic pathway? Why is not mentioned? Also, the NIF pathway is present in *Entamoeba* and *Mastigamoeba* in addition to the nitorogen fixing bacteria and archaea.

Page 10, last paragraph: "The ISC assembly pathway is followed by a transport system exporting the newly formed Fe-S clusters outside of the organelle." Even though you are mentioning later in the paragraph that it is not clear what is the substrate exported, this sentence is WRONG!

Page 21, 1st paragraph, last sentence: "I also intended to compare the cysteine desulfurase activity of Nfs in separate cell fractions, but because of difficulties with the activity assay and unreliability of preliminary results, this aim was eventually abandoned." Do you really need to mention this? Why don't you remove the aim and focus on your actual results?

Figure legends: You should descibe briefly what the reader should see in your figure. What are your results?

Page 21, section 4.1: You are not describing your results. You should mention what other proteins are localized in the same fraction as the Nfs and Isu proteins. From figure 2, I don't see any difference in the enolase concentration, suggesting that you have cytosolic contamination in all of your fragments?

Page 23, section 4.2: "The anti-v5 antibody recognizes a single specific band … " In the 8th and 9th fractions I can see 3 bands and not a single one! Can you explain this? Also, if the Nfs and IsU antibody were specific in fraction 9 (figure 2) along with trCOXIV and VDAC, why didn't you use the same antibodies in figure 3 as well?

Figure 4, page 24: Can you provide the DIC image to be able to see the cell?

Page 25, Section 4.3: "Although the controls display the same pattern as in the previous experiment, the Isu-v5 protein is most abundant in the 7th fraction and the signal disappears in later fractions. " The controls do not display the same pattern as in the previous experiment. If you check the enolase pattern, you will see that in Figure 5 (8th and 9th fractions) the concentration of enolase is less than the previous experiment. So we can't compare the two.

In both of your experiments (PS & BS), have you used the pT7v5 vector by itself as a control and subsequently use the anti-V5 antibody to see if there is any unspecific binding? Also, you could transfect a typical mitochondrial protein (e.g. mtHsp70) to see if there is a consistency to your results (additional control).

Page 34, 3rd paragraph: "One possible explanation is that this unexpected localization is an artifact caused due to the added tag. While Nfs is a large protein (48 kDa), Isu is more than half the size (19 kDa) and it is therefore possible that even a small 5 kDa tag influences its localization within the organelle. It may have interacted with the mitochondrial targeting sequence, disabled proper import and resulted in the miss-localization in the intermembrane space of the mitochondrion." As I have mentioned previously, this could be tackled with proper controls.

Specific comments:

- Contents: 1.1 and 3.1: *Trypanosoma brucei* should be italiced.
- References: all the genus names should be italiced.
- western blot is without a capital letter.
- Page 6, 3rd paragraph: Besides NADH dehydrogenase and succinate dehydrogenase (complex II) … "Besides" should be change to "In addition to"
- Page 6, 3rd paragraph: From ubiquinone, electrons pass into two directions. I will use ":" instead of "." since you are describing the directions in the next sentence.

- Page 7, 4th paragraph: "The mitochondrion of the BS is characteristic …" Instead I would use: "The mitochondrion of the BS is characterized by …"
- Page 10, 1st line: Why do you use "Nilsson et al., 2010" as the genome reference and not the genome paper?
- Page 10, 5th line: add"the" in front of Isd11
- Page 11, last paragraph: I will start the paragraph differently: e.g. "For the purpose of this project, I have focused …".
- Page 25, line 7: Change the position of "Fig. 6" : Using immunofluorescent assay, the result from the Isu-v5 cells (Fig. 6) corresponds to Nfs-v5 cells …
- Page 27, line 6: Don't use the word "failed" – instead you can say that the your efforts were unsuccessful.
- Page 33, 1st paragraph, 2nd sentence: I will recommend to rephrase it to: "This outcome is in agreement with previous studies on *T. brucei* (Šmíd *et al.*, 2006), yeast (Mühlenhoff *et al.*, 2004) and mammalian cells (Land and Rouault, 1998)."
- Page 33, 2nd paragraph, 4th line: although typo

Sincerely,

Dr. Anastasios Tsaousis

In Prague 24.5.2012

REVIEW OF THE MASTER THESIS OF JULIE KOVÁŘOVÁ

LOCALISATION OF FE-S CLUSTER BIOSYNTHESIS IN THE BLOODSTREAM STAGE OF *TRYPANOSOMA BRUCEI*

The master thesis of Julie Kovářová is relatively brief but well written text supplied with high quality figures from fluorescence microscopy and western blots. The thesis is focused at proving that iron sulphur assembly pathway is present and functional in blood stream form of *Trypanosoma brucei*. The text shows clearly that Julie Kovářová has sufficient theoretical background, understands the methodology, is able to logically describe her results and discuss them in the context and finally, that she is equipped with good written English with minimum formal mistakes (listed at the end of my review).

The thesis has typical structure. In the **Introduction** Julia introduces the topic to sufficient details. I have several questions to this part, partly because I think some statements are not entirely correct and partly because I am curious in some facts.

Page 1. Julie cites our paper (Hampl 2009) in the context that kinetoplastids are one of earliest branches of eukaryotic tree. I doubt we have stated anything like this in the paper, because we have not analysed the position of the eukaryotic root. Cavalier-Smith (2010, Biology letters) would be more appropriate citation.

Page 4. From the text I got an impression that acidocalcisomes are organelles specific to trypanosomes, perhaps adaptation to parasitism. This is definitely not true as acidocalcisomes were reported from many other groups of eukaryotes and might be present even in the last eukaryotic common ancestor (see Docampo et al 2005).

Page 5. I do not think that compartmentalisation of glycolysis into glycosomes is adaptation to anaerobiosis as glycosomes are present in free living *Diplonema*, the sister group of kinetoplastids.

Page 8. Julie states that the ATP production in glycosomes of BS is balanced due to the production of 3-phosphoglycerate. Does it mean that the ATP production is imbalanced in PS trypanosomes. I thought that in PS the balance is maintained by ATP production from PEP?

The part **Methods** describes sufficiently the experimental procedures. I have just two comments.

Table I. The final digitonin concentrations are in my opinion not calculated correctly. The final concentration in sample 8 should be 0,8 mM (double dilution of 1,6 mM) and the concentration in other samples should be lowered accordingly.

Table III. Hsp70 should be changed to mtHSP70 as only mtHSP70 is mitochondrial marker.

The parts **Results** and **Discussion** are well written, though I would prefer to move the passage on the mitochondrial functions in BS (pages 36 and 37) to the introduction. Here I come to my major concern about the thesis. The results convinced me that in the transformants the

majority of over-expressed tagged Nfs and Isu are localised within mitochondrion. The question that worries me is if the situation is the same in WT trypanosomes. Could you actually expect some other results if you over-express in BS the same protein that in PS targets to mitochondrion, I assume that the protein contains functional targeting sequence? How could it NOT go to mitochondrion? What if in wild type BS the Nfs and Isu is actually not present in mitochondrion from one of the following reasons?

1. BS does not express this gene and expresses other copy, whose product is not targeted to mitochondrion? How many copies of these genes are present in the genome?
2. The transcripts of these genes are alternatively spliced in BS and loose the targeting sequence. The splicing machinery may not handle the very high amount of over-expressed proteins in transformants and most of them will not be processed and so non-physiologically targeted to mitochondrion.
3. What if the targeting sequence of these proteins is processed by MPP in the cytosol and the protein is not targeted to mitochondrion. There are indices that MPP may be present in cytosol of BS, aren't they. Again, MPP could not process all over-expressed proteins in transformats, which would result in their non-physiological targeting to mitochondrion.

Can you exclude all of these theoretical concerns? Could northern blots help to understand how it is with the expression of these proteins? My concern that natural Nfs behaves differently from the over-expressed tagged Nfs is supported by western blots. If you compare the blots of WT Nfs (page 27) and over-expressed Nfs (page 28) you see different pattern in digitonin fractions. How could you explain it?

I wonder if the iron-sulphur assembly pathway is really necessary in BS mitochondrion. Are there any proteins in BS mitochondrion that contain FeS-clusters?

In summary and despite concerns that can be and should be raised in any research project, I would like to state that the thesis presents a significant piece of work that is well presented and certainly fulfils the demands for master thesis.

Hampl

Vladimir Hampl

Formal mistakes:

Page 5 The sentence is not well worded: Maxicircles encode typical mitochondrial genes, however, some of them present are in an encrypted form.

Page 36 The following sentence is not well worded: To the best of my knowledge, the biosynthesis of Fe-S clusters is most likely an important function of the mitochondrion in BS.

I wonder why the nomenclature Nfs, Isu is used in the thesis. I am familiar more with IscS, IscU.

I would prefer to include list of abbreviations used in the text.