Evaluation of Thesis – Referee's Report

Referee: Igor C. Almeida

Overal evaluation and questions

In this PhD thesis, the student Piya Changmai has carried out detailed studies on the biogenesis of iron-sulfur protein clusters in the mitochondrion of *Trypanosoma brucei*. In addition, the student performed some preliminary studies on a putative *T. brucei* heme transporter. The thesis is divided into six sections, of which the first five are on Fe-S clusters and the last one on the heme transporter. This is a very strong and well-developed project, particularly regarding the first five chapters on Fe-S clusters in *T. brucei*. On these, the main contributions and findings are:

i) the biochemical and functional analysis of *T. brucei* Isd11, a binding partner of the cysteine desulfurase (Nfs). Here, it is demonstrated that the Nfs is critical for Fe-S cluster formation and tRNA thiolation in the cytosol and mitochondrion of the parasite;
ii) the biochemical and functional characterization of *T. brucei* homologues (*Tb*Isa1 and *Tb*Isa2) of prokaryotic Isa proteins, which are essential for maturation of mitochondrial Fe-S proteins. Here, it is shown that both *Tb*Isa1 and *Tb*Isa2 are essential for parasite growth and assembly of mitochondrial but not cytosolic Fe-S proteins.
iii) the identification and functional analysis of two *T. brucei* homologues (*Tb*FdxA and *Tb*FdxB) of ferredoxin, a protein that is involved in electron transfer during assembly of Fe-S cluster. Depletion of *Tb*FdxA, but not *Tb*FdxB, is lethal for the parasite. Interestingly, when human ferredoxins, Fdx1 and Fx2, are expressed in *Tb*FdxA-knocked-down parasites, they are able to complement *Tb*FdxA function and restore Fe-S cluster pathway and parasite growth.
iv) vital Fe-S cluster assembly proteins are active in *T. brucei* bloodstream stage mitochondrion, although the rudimentary nature of organelle in that stage. An intriguing observation is the dual localization of cysteine desulfurase (Nfs) in the nucleus and mitochondrion.
v) the identification and functional analysis of the putative *T. brucei* ABC transporters, *Tb*Atm1 and *Tb*Mdl. In other cells, Atm1might be involved in the Fe-S protein maturation in the cytosol, whereas Mdl has no assigned function. Intriguingly, it is shown here that dual depletion of *Tb*Atm1 and *Tb*Mdl did not result in altered growth phenotype, although single knock-downs of each gene led to growth defect.

The last chapter of the thesis on the characterization of a putative heme transporter of *T. brucei* (TbHrg) is very interesting and great potential, because not much is known regarding heme uptake by *T. brucei*. Recent data have indicated an important role of the *L. amazonensis* Hrg homologue, LHR1, in the uptake of heme in that parasite. The preliminary findings presented here is that knockdown of *Tb*Hrg in procyclic forms leads to a decreased cell growth only if the parasites are kept in medium containing low heme concentration. Overall, the data, discussion, and conclusions for this chapter are still very preliminary and much development on the topic is needed.

Questions to the student:

1) Does Fe-S cluster play any direct or indirect role in parasite virulence? Or this pathway is just involved in basic metabolic functions for the parasite?

2) Considering that ISC machinery is practically vital for all organisms, including higher eukaryotes, can Fe-S clusters be specifically targeted for development of new chemotherapeutics against *T. brucei*? If so, in your opinion what are the most attractive protein targets in the Fe-S cluster pathway in *T. brucei*? Are there any specific inhibitors for ISC machinery components are already available?

3) What is known about the ISC machinery in *T. brucei* subspecies (i.e., *T. brucei gambiense* and *T. brucei rhodesiense*) that cause African sleeping sickness in humans? Do you envisage any important difference in ISC in these parasites as compared to *T. brucei brucei*?

4) In your results on section 3.5 (page 101 onwards), you observed that "To our surprise, the simultaneous depletion of TbAtm1 and TbMdl did not cause any growth phenotype, even though single knock-downs of each gene resulted in an altered growth." Besides the two hypotheses –i) compensation for the lack of bot transporters, and ii) opposing functions of the two proteins) you raised on 3rd paragraph, page 111, do you have any other explanation(s) for this intriguing phenomenon? What is the phenotype in other organism(s) (e.g., *S. cerevisiae*, etc.) when both proteins are knocked-out or knocked-down?

We ask the referee to kindly answer the following points:

1. Is an original intellectual imput of the student aparent from the thesis?

Yes. It is very clear that the student had a major original intellectual input on the thesis. He is first author in a recent paper (Changmai et al., Mol Microbiol 2013, 89: 135-151), second author in two others (Paris et al., J Biol Chem 2010, 285: 22394–22402; Long et al., Mol Microbiol 2010, 81: 1403–1418), and third author in a manuscript submitted to Eukariot Cell. In the Mol Microbiol 2013 paper, for instance, he did most of the experiments and analyzed the data and wrote the manuscript. We could not expect a better original intellectual input from a PhD student. In the other two papers (J Biol Chem 2010 and Mol Microbiol 2010) and in the manuscript submitted to Eukaryot Cell, in addition to being involved in several experiments, he analyzed the data and revised the manuscript. However, it is not clear in these papers what his intellectual input in design experiments was. However, it is evident the student was highly involved intellectually in all studies, considering the knowledgeable and fluid manner he wrote the thesis. In my opinion, altogether, this is an excellent original intellectual input of a PhD student.

2. In the referee,s opinion, did the student work independently in his/her experiments as well as in their interpretation and writing?
   Yes, this is very evident in the thesis.

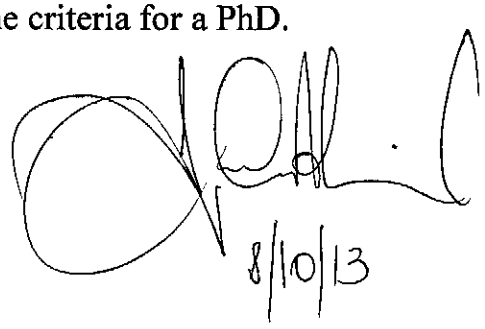
3. Is the project original and what is its main outcome?
   Yes, this is a highly original project. The student carried out several studies on the iron-sulfur (Fe-S) clusters (ISC) machinery in Trypanosoma brucei which was mostly unknown, and made several important discoveries. The main outcome was the publication of at least three excellent papers, of which one in J. Biol Chemistry and two in Mol Microbiology. In addition, the student has one manuscript submitted to Euk Cell. This is an excellent outcome for a PhD project. Also, he performed some preliminary studies on a putative heme transporter in T. brucei. Taken together, the studies carried out in the project have provided a much better understanding of generation of Fe-S clusters in Trypanosoma brucei and their importance for the parasite biology. In addition, this thesis has opened numerous new avenues to be explored by the group of Dr Lukes and other groups in the field.

4. Would this thesis earn PhD at the referee,s own Institution -- please answer Yes or No.
   Yes.

5. How would the referee evaluate this student relative to all PhD candidates he/she has previously known: among top 10%, among top 25%, within the top 50%, among the worst 20%.

Among top 10%.

6. Please do not grade the thesis, only state whether or not it meets criteria for PhD.
   This thesis undoubtedly meets the criteria for a PhD.



Brussels, 05.10.2013

Evaluation of the PhD thesis of Piya Changmai entitled: "Formation of Fe-S clusters in the mitochondrion of Trypanosoma brucei" performed under the supervision of Prof. Julius Lukes at the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czeck Republic

By Benoit Vanhollebeke, PhD, Assistant Professor, Universite Libre de Bruxelles, Belgium

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General evaluation :

The PhD thesis manuscript written by Piya Changmai reports several investigations related to the biogenesis of Fe-S clusters of Trypanosoma brucei. The presented work has made important contribution to the field and did result in three peer-reviewed publications including one as first author. In addition, another collaborative work is currently under review and two other investigations should lead to first-author publications in the near future. Overall, this reviewer acknowledges both the importance of the scientific investigation and the quality of the PhD thesis manuscript and would like to congratulate the candidate on a productive and successful PhD.

As such, the reviewer believes the present work fully meets the criteria for Ph.D.

Comments on the manuscript:

Major comment:

- The manuscript is well written and the data clearly presented .The thesis manuscript starts with a concise and clear introduction. It is followed by a result section that represents a succession of the publications linked to this work and ends with a very concise conclusion paragraph. This reviewer believes that the thesis manuscript could have been strengthened by the addition of a section in the introduction that exposes the main objectives of the PhD. Similarly and importantly, a global discussion section is lacking and the conclusion section is too brief (half a page) at the end of the manuscript. Although the reviewer welcomes PhD theses that consist of a succession of publications (which is a hallmark of a productive PhD) he believes that this should not alleviate the need to globally discuss the key findings of the PhD work. The reviewer encourages the candidate to do so during the oral defence of the PhD.

Minor comments:

- It would have been useful to expand the page numbering of the thesis to all sections, including the publications in the result section.
- Illustrations are limited and the legends are minimal. They should describe all terms highlighted in the illustration (for example in Fig.1, metacyclic, long-slender,...)
- All supplementary information and methodology sections should be included in the thesis.

Questions for the defence:

- Page 47: Could the candidate comment on why, in his views, only HsFdx2 is able to complement Yah-1-depleted yeast cells? And why such a scenario does not happen in T.brucei, where both HsFxd1 and 2 complement endogenous Fdx deficiencies?
- Page 50: "a total elimination of the TbFdxA protein occurred". Does the methodology used in this experiment support such a statement?
- Page 50: "...TbFdxA, but not TbFdxB, is essential for the viability of PC T.brucei". Does the methodology used in this experiment support such a statement?
- Page 51: Why was 18S rRNA used as standard in Q-RT-PCR experiments? Could the candidate discuss this choice?
- Page 51: "Comparative Western blot analysis" How was it performed?
- Page 52: How is aconitase distributed in mitochondria and cytosol? Is there any traffic of the protein in and out of the organelle? Could the candidate find support in his answer from other results of the manuscript?
- Page 52: How can Threonine deshydrogenase activity be unaffected by TbFdxA RNAi despite overall disruption of mitochondrial metabolism?
- Page 52-3: How could one explain the modest (30%) decrease of cytochrome c reductase in contrast to the severe effect on aconitase and succinate dehydrogenase (70%).
- Page 53: Why does TbFdxA RNAi result in heme b decrease? Could the candidate comment on the proposed feedback mechanism between heme a and b, given the data presented on page 131?
- Page 54: Could the impact on heme levels be indirect?
- Page 46 and 54: "lowered the level of intracellular haem": Is such a statement supported by the statistical analysis?
- Page 50 and 55: the mitotracker staining looks very different (reticulate versus puncta structure) why?
- Page 56: "Since ablation of TbFdxB in PC". Does the methodology used in this experiment support such a statement?
- Page 57: "The highly efficient import of both human proteins...is unexpected" Could the candidate illustrate the current knowledge about mitochondrial import sequences across the different domains of life? What is the import machinery composed of in mammals? What is the MitoprotII algorithm based upon.

- Page 101: Could the candidate speculate on the nature of compound X? On which basis can ALA be excluded ("which is, however, not ALA)? What determines the substrate specificities of ABC transporters?
- Page 119: How were the phylogenetic analyses conducted?
- Page 123: How was the Atm-1 Ab generated?
- Page 123: The finding of the absence of phenotype in TbAtm1+Mdl double KD is very interesting. The double mutation strategy in yeast looks promising. Have any results been obtained so far? Has there been any attempt to obtain RNAi-independent evidence for the genetic and/or metabolic interaction in T.brucei?

- Page 128: "conserved histidine in TM2". The reviewer could not find the conserved residue in the C.elegans and Leishmania orthologes.
- Page 129: What is the expression level of TbHrg at the different stage of the T.brucei life cycle?
- Page 131: After how many days of tetracycline was heme measured?
- Page 131: Heme measurements: can one exclude that some of the measured heme results from exogenous heme sticking to the membrane coat? How?
- Page 131, Figure 1C. Wrong label. Percentage heme B/heme A.
- Page 132: Where is TbHrg localized? Can the localization be anticipated given the modest homology with the orthologes?
- How does heme cross the intracellular biological membranes? Could TbHrg play a role in this transport? Can one visualize heme distribution in the cell?
- Could the candidate comment on the heme requirements of T.brucei at the different stages of its life cycle?

Other questions will be asked during the PhD defence.

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Ph.D. Thesis

University of South Bohemia in České Budějovice
Fakulty of Science

Formation of Fe,S clusters in the mitochondrion of *Trypanosoma brucei*

Piya Changmai

Supervisor: Prof. RNDr. Julius Lukeš, CSc.
Co-supervisor: RNDr. Eva Horáková, Ph.D.

Institute of Parasitology
Biology Centre of the Academy of Science of the Czech Republic

Evaluation of Thesis – Referee's Report

The mechanism by which iron-sulfur (Fe-S) cluster biogenesis occurs in eukaryotic pathogens is incompletely understood. This dissertation has used the protist *Trypanosoma brucei* as a model system to address this important topic using a combination of genetics, molecular biology, and biochemistry. The work described here has already resulted in three publications (one in which Mr. Piya Changmai is the first author and two where he is a second author). In addition, at least two other manuscripts are in the works based on the material elaborated here. In the grand scheme of things, the results outlined here will broadly impact our understanding of several cellular processes, including iron and heme metabolism, tRNA modification, and energy production steps.

The major findings resulting from this dissertation are:

(a) Link between Fe-S cluster metabolism and protein biosynthesis. Specifically, Mr. Changmai and colleagues have shown that tRNA thiolation and Fe-S cluster assembly are coupled.

(b) Unlike other eukaryotic system, the two mitochondrial Tblsa proteins are essential for *T. brucei*, directly functioning in Fe-S cluster assembly.

(c) Heterologous expression of human ferredoxins in *T. brucei* is necessary and sufficient to complement the functionalities of several proteins that require Fe-S clusters to

work. This observation highlights the evolutionary conservation of ferredoxin function.

(d) *T. brucei* at the bloodstream stage lacks a fully functional mitochondrion, yet is able to mediate Fe-S cluster biogenesis.

(e) Discovery of TbHrg, which may function in heme transport.

Questions:

1) Given the role of radical SAM enzymes in tRNA thiolation, what is the likelihood that the link between the latter and protein biosynthesis is mediated by enzyme activation?
2) To what extent does compartmentalization of the ISC system benefit the pathogen?
3) Ox-phos and ATP generation are not found in all stages of *T. brucei* life cycle. Is there a disproportionate use of Fe-S proteins and enzymes during particular stages of pathogenesis?
4) How does this work relate to the results of Paiva et al who demonstrated that antioxidant pathways inhibit *T. cruzi* proliferation via a macrophage-dependent pathway. Does *T. brucei* exhibit similar behavior?
5) What are the mechanisms by which *T. brucei* acquires iron and does its pathogenesis have parallels with innate immune activation by bacteria?

In summary, this is an excellent, exemplary, and very original scientific study/thesis. The thesis represents state-of-the-art approaches, methodologies, applications, results, discussion. The thesis also reflects the enormous work and dedication of the author.

Without any reservation I fully recommend Mr. Piya Changmai to be awarded a Ph.D. based on the present thesis.

In Prague, Setember 30, 2013

Pavel Martásek, MD, PhD
Professor of Medicine