Review of

"Erv1 associated mitochondrial import-export pathway and the cytosolic iron-sulfur protein assembly machinery in *Trypanosoma brucei* "

a PhD thesis of **Somsuvro Basu**

by Prof Terry K Smith (University of St Andrews)

General comments

Overall the thesis is well written and presented well, however as always with written pieces of work, there are always typos and grammatical errors but no more or less for a thesis from my own group/university.

The thesis consists of a general introduction to Trypanosoma brucei and some of its peculiarities that make it such a good model organism to work with. This is followed by a brief description some aspects of mitochondrial protein trafficking and the biochemistry involving iron-sulfur cluster formation and usage. The iron sulfur cluster biogenesis and processing is the main topic of this and is well introduced in terms of its importance in disease and essential biochemical processes, as well as its formation via the mitochordrial machinery and the downstream cytosolic machinery, namely SUF, ISC and CIA. Most of the information delivered seems to be form other model organisms, i.e. yeast. It would have been good to see more literature where they described other unusual aspects of iron-sulfur cluster formation or use from other organisms in other branches of life.

The thesis is then broken up into three sections, **Review articles, Published results and Unpublished results.**

I will know go through each section in tern with comments and a couple of questions followed by more general questions at the end, relevant to all of the work presented in this thesis.

***Trypanosoma brucei* and African trypanosomiasis**

Page 12-cytosolic protein synthesis is mentioned, please explain what is meant by this.
Please clarify what you mean by CX3C CX_3C.
Figure legends for some of the figures should have been done, for example Figure 3 p14 and figure 5 p18.
Page 13- No Tb Mia40 homologue, please explain how you determined this, and how can you be sure that there is a non-homologous protein doing the same function. How could you experimentally prove this?
How does EPR work, and how can you tell difference between [2Fe-2S] and [4Fe-4S]?

Review article # 1. Fe/S protein biogenesis in trypanosomes – a review

I found the introduction very hard going (verbose) and far to long winded for my liking. The following lines could have been written/constructed better and more concisely, Ln31, 39, 62.
Ln 121 is this Fe^{2+} or Fe^{3+} ?
L 102 explain what you mean by "rescue experiment?
So how is the aconitase targeted to different parts/organelles within the parasite?
Why does the puruvate increase in the absence of aconitase activity?
What normally metabolically happens to pyruvate in a eukaryotic cell?
Ln 381 "cues"?
So ERV1 is multifunctional, replacing the function of a Tb Mia40 homolog, so from an evolutionary

point of view what is your opinion, "Has Mia40 evolved after Tryps branched off and thus never had Mia40, but higher eukaryotes then evolved Mia40, or have Tryps lost Mia40 and if so why do you think that might be?
Where do you think the cytosolic source of "S" is?

Review article # 2. The malleable mitochondrion of *Trypanosoma brucei*

Several points mt is used, I assume as abbreviation for mitochondria, but never define, but also "mitochoindria" full name is used in same sentence.
How does lipoic acid involve "S" metabolism in cells?
What well-known protein is lipodated in cells?

Study # 1. Divergence of Erv1-associated mitochondrial import and export pathways in trypanosomes and anaerobic protists

Fig 2: Explain how do you show FAD rather than FMN binding, and how do you know it is 100% occupancy?
Table 1 enzyme activity of refolded ERV1, very lucky to get good refolding and activity, what do you understand by Km, Vmax and what does Kcat tell you.
Explain the subcellular fractionation of mitochondria and what controls are used.
Why are there 2 pairs of primers for the RNAi construct?
You explain the –tet (no induced) growth affect due to leakiness in inducible system, please explain?
An observed phenotype was a highly enlarged mitochondria to as depicted by EM, what other technique might have been good to visualize the size and state of the mitochondria.
TbErv1 you suggest that TCEP or DTT could act as e donors, why did you not try GSH or trypanathione as more natural sources?
So is TbERV1 a good drug target?

Study # 2. Cytosolic iron-sulphur protein assembly is functionally conserved and essential in procyclic and bloodstream *Trypanosoma brucei*

Please comment on Figure 4, bloodstream growth phenotype
If there is no export of to cytosolic Fe-S in the ERv1 knock-down, what further consequence could there be for the mitochondria what happens to the Fe-S destined to be export out of the mito by Atm1?
Why is enolase a good control for your knock-down experiments.
Please explain briefly the yeast complementation and how the different promoter used for both the overexpression and the knock-down of yeast genes work/driven.
What are HMS174 cells?
Why do you have pLew 100 constructs for Cfd1 and Nbp35 from Andre Schneider in experimental, but never used?

Unpublished results

There was no methods section for this part of the thesis, which is a shame as there were several aspects I wanted to check in terms of methodology and controls.

 # 1
Page 114: By what criteria can say that Cia 2a and b are essential in BSF?
Page 116: Explain cryogenic entrapment, why use GFP?
How would you confirm hits that you suspected of being in a complex with Cia 2A or B?

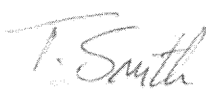
2
Giardia have mitosomes, do you think these are evolutionary more advanced that mitochondria?
Please explain what the pADpuro vector is, is it tet inducible?
Are you surprised by the cytosolic location of both GiTah18.1 and 18.2, considering GiTah18.1 is normally mitosomal?
Was there any phenotype of overexpression in WT background?

3
How do you assess aggregation of Hsp70, what biochemical methods could be used?
You stated t5hat you had done the recombinant expression of Mge1, but not shown or discussed.

General Questions

1. Why study iron-sulfur cluster formation and its subsequent use in trypansomatids?
2. Where does *T, brucei* get its iron from and explain how it is initially processed by the parasite?
3. Can you explain the processes involved in constructing a RNAi vector and getting the vector inside a trypanosome?
4. In general terms how does RNAi work?
5. What are the advantages / disadvantages of using RNAi as opposed to forming a conditional knockout?
6. Can you think of an alternative way to the method you used for assessing if the RNAi is working?
7. What other types of phenotyping could have be done? Include in your answer what information these other techniques could they have told you?
8. By what criteria do you determine if a gene is validated as a drug target?
9. Why did you not make any conditional knockouts, especially of single RNAi that should no phenotype?
10. What biotransformation does aconitase do?
11. Please explain the aconitase assay used in your studies.
12. Please describe in detail the aconitase mechanism and highlight how its iron-sulfur cluster is important for its activity.
13. Is the activity of cytosolic aconitase within the parasite essential and if so why, what are the downstream affects of not having it?
14. Where does the source of "S" come from
15. What characteristic does "X-S" have to have and what is your best guess for its identity?
16. How would you go about identifying all of the Fe-S cluster dependent proteins/ marker activities?
17. Explain if and why any of the genes you have studied would make a good drug target?
18. Explain how you go about identifying a chemical entity (compound) that could inhibit Fe-S cluster formation or usage.
19. Explain what you understand by chemical validation?
20. Given the slightly different life styles of the closely related *T.cruzi* and *Leishamania* do you think they will have similar SUF, CIA and ISC machinery and explain you reasoning?
21. Given more time for your studies what would you do next in this area?

In summary, it is obvious that Somsuvro has worked hard and has successfully published a good portion of this work. This body of works fulfills the criteria of this part of obtaining a PhD.



Prof TK Smith 14th Sept 2014

In Prague 15th of September 2014

Opponent´s Review of the PhD thesis of Somsuvro Basu.

The submitted PhD thesis of Somsuvro Basu is a compact summary of his experimental and intellectual activity during his PhD study in the laboratory of Julius Lukeš at the University of South Bohemia. The title of the thesis: "*Erv1 associated mitochondrial import-export pathway and the cytosolic iron-sulfur protein assembly machinery in* Trypanosoma brucei." very well refers to its actual content. The author presents his interests in the biogenesis of the FeS proteins occurring outside the mitochondrial matrix within the cells of the unicellular eukaryote *Trypanosoma brucei*, which is an important human pathogen.

This metabolic pathway is of great interest around the international scientific community, as it represents one of the ancient and essential processes occurring in living organisms. Many unknowns concerning the mitochondrial export and the cytosolic synthesis of FeS groups remain until today, which makes it an excellent and exciting topic for an early-career researcher such as Somsuvro Basu.

T. brucei, the experimental organism of the supervisor´s lab poses additional challenge to the task. Two quite distinct life stages of the parasite demand for parallel experiments to be done on procyclic (insect) stage and blood stream (mammalian) stage. This, however, is also a great beauty of the system, where overall metabolic changes between the stages may relate to the different functions (or the impact) of the studied proteins and subsequently to the manifestations of the experimentally triggered phenotype.

The thesis is divided into Summary, Overview, Review and Research articles, Unpublished results and Conclusions.

0) Overview

The Overview section describes on 17 pages the key cellular and molecular features of *T. brucei* and its medical importance. The more emphasis is then put on the cytoplasmic FeS clusters assembly in the following parts of the Overview. This summary draws predominantly from the experiments performed on *Saccharomyces cerevisiae* as it represents the key cellular system for the characterization of the eukaryotic FeS cluster assembly machinery. Several connections to the biochemistry human cells and connected diseases are mentioned. Using illustrative images adopted from key review article the author presents individual components of the cytoplasmic biosynthetic pathway as well as their target FeS cluster carrying proteins. The summary is writer in great English, contains only small number of grammatical errors. The overall reader´s feeling is that author understands the topic very well and he proves that even such a complicated biochemical matter can be presented in a short and interesting form.

Questions and comments related to the Overview (please answer questions highlighted in italics during the defence, no need to reply to the comments not highlighted)

1. *Could T7 RNA pol driven transcription system be used in other organisms like giardia?*

2. In addition to the transport and oxidative folding of several IMS proteins, MIA pathway has recently been shown to assist during the import of Tim22 (Wrobel et al., 2013).
3. *Figure 3 in the Overview section indicates that the steps of substrate release from Mia40 and the reoxidation of Mia40 are separated processes mediated by a single enzyme Erv1? Is it really like that?*
4. *The presence of X-S compound and the cytosolic iron insertion suggest that it is rather the sulphur metabolism, which is the crucial mitochondrial function? Can you comment on that?*

Included articles and submitted manuscripts.

1) Lukeš J., Basu S. **Fe/S protein biogenesis in trypanosomes**. Invited review - BBA Molecular Cell Research- revision submitted

S. Basu wrote 45% of the MS.

The manuscript summarizes number of recent papers coming predominantly from the supervisor's laboratory on the functional characterization of the proteins involved in the FeS cluster biosynthesis and related biochemical processes in *T. brucei*. The review is well written and represents quite a mature piece of scientific writing. Most of the data mentioned by the authors point to overall conservation of the FeS cluster biosynthesis among different eukaryotic lineages but also to the differences between the procyclic and the blood stream stages of the parasite.

Comments and Questions:

1. *P.37 Are Mrs3/Mrs4 they true iron importers or are there any doubts about that? Can you comment on that?*
2. P. 42 "signal peptide" refers rather to secretion pathway, should not be used in relation to mitochondria
3. P.43 and following "the disease-causing BS" – why do use the disease-causing if just BS is used earlier in the text?
4. *How do you know that TbHsp70 is Ssc1 and not Ssq1 or Sse1? Labelling the protein TbSsc1 may be too narrow.*
5. *P.46 How do you think the absence of MIA pathway is compensated in T. vaginalis , G. intestinalis and others, in respect to the protein import and "X-S substance" export? Can you hypothesize on the reasons for the loss of Mia40 in kinetoplastids?*
6. *P. 47 If TbDre2 lacks the entire N-terminal SAM domain, is it still a Dre2 homologue or cannot it be something else?*
7. *P. 48 Is there another proof that TbNfs-TbIscU are present in the nucleolus except the EM data? (more experimental evidence would be needed to say so with peace of mind)*
8. *How would you search for trypanosoma-specific FeS proteins?*

2) Zdeněk Verner, Somsuvro Basu, Corinna Benz, Sameer Dixit, Eva Dobáková, Drahomíra Faktorová, Hassan Hashimi, Eva Horáková, Zhenqiu Huang, Zdeněk Paris, Priscila Peña-Diaz, Lucie Ridlon, Jiří Týč, David Wildridge, Alena Zíková and Julius Lukeš . **The malleable mitochondrion of *Trypanosoma brucei*.**

Only the 'Fe-S cluster and protein biogenesis' section of the review is included in the thesis.

Understandably, this part of the review manuscript highly overlaps with more-specialized review article currently under review in BBA Molecular Cell.

3) Somsuvro Basu, Joanne C. Leonard, Nishal Desai, Despoina A. I.Mavridou, Kong Ho Tang, Alan D. Goddard, Michael L. Ginger, Julius Lukeš and James W. A. Allen **Divergence of Erv1-associated mitochondrial import and export pathways in trypanosomes and anaerobic protists.** Eukaryotic Cell 2013.

The paper describes the characterization of Erv1 homologue in T. brucei (TbErv1). Here, the authors successfully expressed recombinant protein in *E. coli*, purified and reconstituted TbErv1 *in vitro* towards the enzymatic characterization. Interestingly, the sulhydryl oxidase of *T. brucei* is shown to simultaneously transfer electrons to both cytochrome C and oxygen, the feature not reported before for this type of enzyme. By site-directed mutagenesis of TbErv1 authors also question the necessity of the 0_2 channel inside the enzyme for the efficient 0_2 reduction. The silencing of TbErv1 expression results in the mitochondrial swelling phenotype, known from the analogous experiments performed on *S.cerevisiae*. This phenotype authors consider as an evidence of the involvement of the TbErv1 in the mitochondrial protein import. The presence/absence of the components involved in the IMS and cytosolic pathway of FeS cluster assembly in selected eukaryotes is shown. The discussion then focuses on the biochemical properties of TbErv1 and the evolution of the MIA and CIA pathway.

1. *P. 79. A technical question: why was the recombinant TbErv1 lyophilized and then dissolved in the 8M guanidine buffer instead of being re-buffered directly with the guanidine buffer during the purification procedure?*
2. While oligonucleotides are shown for the generation of the RNAi contruct, no information is provided on the oligoes used for the other constructs (bacterial expression vectors, mutagenesis), also the source of the antibody is not mentioned.
3. *P. 80 Figure 2B legend says: "SimplyBlue-stained SDS-PAGE gel documenting in vitro folding of recombinant TbERV1." But the gel by itself doesn't document any in vitro folding as essentially the same pattern of denatured proteins can be observed with no insight into protein folding.*
4. *It is written in the paper that the evidence of the involvement of TbErv1 in the mitochondrial protein import is provided. I understand the argument that the observed phenotype of mitochondrial swelling is not seen in the knocked-down lines of FeS cluster machinery components but this bring rather an indication and not the evidence of the import pathway to be affected. Can you think of an experiment directly proving that TbErv1 RNAi line has a defect in the mitochondrial protein import?*

5. *Do you think that the dual function of Erv1 (protein import, FeS cluster assembly) could be explained by a single target protein partner of Erv1?*

4) Somsuvro Basu, Daili J. Netz, Alexander C. Haindrich, Nils Herlerth, Thibaut J. Lagny, Antonio J. Pierik, Roland Lill and Julius Lukeš. **Cytosolic iron-sulphur protein assembly is functionally conserved and essential in procyclic and bloodstream *Trypanosoma brucei*.** In Press, Molecular Microbiology.

The paper describes the analysis of the CIA pathway components in *T. brucei* using two different approaches (i) the transcription silencing in T. brucei by RNAi and (ii) the complementation assays of the *S. cerevisiae* mutants by *T. brucei* genes. In addition spectroscopic analysis of recombinant TbTah18 and TbDre2 are performed. The paper embraces straightforward experimental approaches, which come up with some interesting finding on the dispensable function of most of the components except TbCfd1 and TbNbp35. More profound effect of RNAi is achieved when double lines are generated indicating their genetic interaction. On the other hand the complementation experiments showed that only TbCia1 and the pair of TbDre2 and TbTah18 can complement the growth defect of the yeast mutant.

1. *How can you explain that only when both TbDre2 and TbTah18 are absent or introduced for the complementation assay a phenotype can be observed? They carry entirely different activities determined by their domain structure and the prosthetic group, yet a single knock-down doesn't produce clear phenotype.*

2. P.107 The following explanation of the decreased activity of the mitochondrial aconitase seems a little bit odd to mee: "In TbTah18/TbDre2 and TbCia1/TbNar1 double RNAi knock-down cells, the mitochondrial aconitase activity also drops, although much less when compared to the cytosolic aconitase (Fig. 5Aand C). Possible reason of this effect could be the strongly disarrayed cytosolic Fe/S protein biogenesis affecting genome stability and translation efficiency. The oxidative damage resulting from the severe phenotypes caused by the RNAi double silencing could make the oxidative stress-sensitive mitochondrial aconitase less active, leading to the above-mentioned observation."

   Have you measured some other mitochondrial enzyme or check for the oxidative stress in the cell to support such claim?

3. *P.108 The laboratory of André Schneider is acknowledged in the paper for providing RNAi cell lines for Cfd1 and Nbp35. As these were shown to be the only genes with the observable phenotype upon RNAi induction, could it be that different genetic background or different kind of RNAi construct was used?*

Additional unpublished and preliminary data are presented as three separate projects:

5) Somsuvro Basu, Daili J. Netz, Alexander C. Haindrich, Antonio J. Pierik, Roland Lill and Julius Lukeš. Functional analysis of Trypanosoma brucei cytosolic iron-sulfur protein assembly target complex.

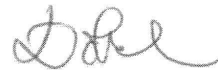
6) Jan Pyrih, Martin Kolísko, Eva Martincová, Somsuvro Basu, Julius Lukeš, Andrew Roger and Jan Tachezy. Cytosolic iron-sulphur protein assembly machinery in Giardia intestinalis.

7) Jiří Týč, Alexander Haindrich, Tomáš Skalický, Somsuvro Basu, and Julius Lukeš, Chaperone needs chaperone.

Undoubtedly, these additional data present the great experimental as well as intellectual efforts of the author towards to understanding of the cytosolic cluster assembly and his co-operation with the lab mates and other colleagues. However, the incompleteness of the experiments as wells a patchy presentation of the data makes it impossible for the reviewer to critically evaluate this piece of the work. Therefore, in my opinion these studies should have not been included in the submitted thesis.

8) The Conclusions chapter highlights in short the key findings of the thesis.

To conclude, the presented thesis of Somsuvro Basu proves very well that the author (i) possesses excellent understanding of this dynamic field of cell biology, (ii) has the expertise in the molecular and biochemical approaches to test his hypothesis experimentally and finally, (iii) uses his capability to discuss the obtained findings appropriately. The high quality of the written English is also another author´s value. While I may express particular criticism to couple of things in the thesis, as mentioned above, I believe that the thesis deserves to be defended by Som this Thursday.

Pavel Doležal 

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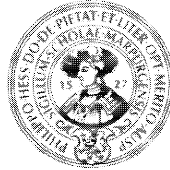

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Ph.D. Thesis "Erv1 associated mitochondrial import-export pathway and the cytosolic iron-sulfur protein assembly machinery in Trypanosoma brucei."

by Somsuvro Basu

Department of Molecular and Cell Biology and Genetics, Faculty of Science, University of South Bohemia (USB).

Review

Dear Prof. Oborník,

Mitochondria, commonly known as the "powerhouses of the cell" are indispensable cell organelles in all forms of eukaryotes known so far, including those that do not produce ATP by oxidative respiration, for instance because they are facultative or obligate anaerobes. This also includes the human parasite *Trypanosoma brucei* that does not perform respiration during certain stages of its life cycle, but nevertheless cannot survive without its large single mitochondrion during any stage of its life. So far the only known essential cellular functions performed in mitochondria are related to cellular iron metabolism. These are the biosynthesis of heme and of iron-sulfur (Fe/S) clusters, two important co-factors for a variety of proteins that participate in essential biosynthetic pathways of the eukaryotic cell. While heme is stable and can frequently be taken up, for instance by parasites from the blood of their hosts, Fe/S co-factors are intrinsically instable without a protein shell and thus have to be synthesized within the cell. Since iron plays a crucial role in infection by the *T. brucei,* the study of its cellular

machinery for the maturation of Fe/S proteins is central to understand the physiology of this parasite and may - with a little luck - be of therapeutic relevance. Consequently, the group of Dr. J. Lukes is investigating the mechanism of cellular Fe/S cluster formation in *T. brucei* for several years with remarkable success. In the context of these ongoing studies, the thesis by Somsuvro Basu deals with the analysis of two central aspects of the maturation of extra-mitochondrial Fe/S proteins, the mitochondrial Fe/S cluster (ISC) export pathway and the cytosolic iron-sulfur protein assembly (CIA) machinery.

In the first part of his thesis, (published in Eukaryotic Cell) Mr. Basu studies the *T. brucei* homolog of the sufhydryl oxidase Erv1, an essential inter-membrane space protein with a dual function in ISC export and protein import into the mitochondrial inter-membrane space by the MIA pathway. The work combines a comprehensive study of the purified enzyme *in vitro* with a reverse genetic study of *T. brucei* depleted for Erv1 by RNA interference. On the bottom line, this work shows that *T. brucei* Erv1 is a common suflhydryl oxidase that catalyses disulfide bond formation, a catalytic function that is required for a role in protein import by the MIA pathway. This observation is compatible with the observation that Erv1-depleted *T. brucei* display a similar altered mitochondrial ultra-structure as seen in strains lacking components imported by the aid of Erv1. More direct evidence, for instance an *in vitro* import assay into isolated mitochondria, is missing, likely due to technical limitations. What remains puzzling is that *T. brucei* and apparently several related protists lack Mia40, the enzyme that directly co-operates with Erv1 during protein import by the MIA pathway. For an explanation, Mr. Basu argues that Erv1 may work in *T. brucei* without Mia40, despite the fact that his own experiments with the isolated protein suggest that Erv1 is not able to do so. The equally likely conclusion that protist lacking Mia40 may utilize an alternative enzyme was not followed. Furthermore, the study fails to convincingly proof or disproof a role of Erv1 in cytosolic Fe/S protein maturation in *T. brucei*. This is most likely due to a lack of tools for accessing activities of cytosolic Fe/S proteins in *T. brucei* at the time of publication. However, the second publication by Mr Basu shows that these technical problems are apparently now solved. It remains therefore a pity that Mr. Basu fails to mention whether he revisited his Erv1-depleted strains to answer this important question. Despite these weaknesses, this part of the thesis is an important contribution to both the physiology of *T. brucei* and mitochondrial protein import. The publication is of high quality, and represents an important advance in the field.

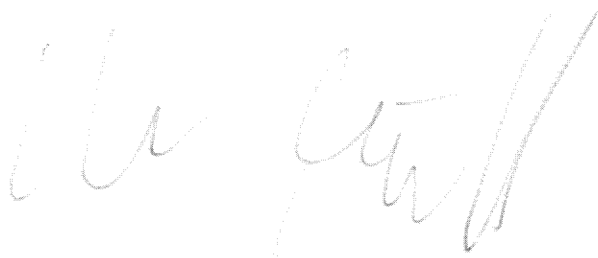
The second major part of his thesis, (accepted for publication in Molecular Microbiology) dissects the components of the cytosolic CIA system for the maturation of cytosolic Fe/S proteins in *T. brucei*. This work is again a combination of *in vitro* analysis of recombinant proteins (in this case Tah18 and Dre2) and a physiological study of *T. brucei* depleted for potential CIA components by RNA interference. Mr. Basu indentified 6 components of the CIA machinery by homology search and showed that these are essential for viability and for the activity of cytosolic Fe/S proteins of *T. brucei*. These two criteria are sufficient to identify these *T. brucei* homologs as *bona fide* CIA factors. For a further proof, Mr Basu shows that *T. brucei* Tah18 and Dre2 accommodate the same co-factor as their yeast counterparts. This comprehensive analysis is flawless and convincing and represents an important advance in the field.

Using the same RNAi approach of candidate genes of ISC components, Mr Basu identified several other CIA and ISC components of *T. brucei* and *Giardia intestinalis*. These are described in the remainder sections of the thesis and are convincing, albeit occasionally preliminary. Besides this convincing experimental work, Mr Basu contributed to two reviews, one as the first author.

The thesis by Mr Basu is well written and fulfils the formal standards expected for a Ph.D. thesis. The Overview is well organized, informative, comprehensive, focused and well illustrated. I personally learned a lot about *Trypanosoma* from this introduction. The final Conclusions section is a bit thin in that it is more a summary of the work done in the context of this thesis rather than an elaborate discussion that puts the new findings of this thesis into the general context of the state of the art of the field. This is a clear weakness of the written part of the thesis.

Taken together, the thesis by Mr Basu is excellent with respect to both the quality of the experimental work and the presentation of the data.

Sincerely yours,



Dr. Ulrich Mühlenhoff