Review Report for Mr. Shaojun Long's thesis

Reviewer: Huangen Ding

1. In this thesis, have you found an original and creative intellectual imput of the student?

***In this thesis, Mr. Shaojun Long described a series of well-designed approaches to explore the physiological function of a highly conserved protein frataxin in protist *Trypanosoma brucei*. He successfully knocked-down frataxin in *Trypanosoma brucei* using the inducible RNAi approach and demonstrated that frataxin is essential for the iron-sulfur cluster assembly in both cytosol and mitochondrion and for prevention of excess production of reactive oxygen species in *Trypanosoma brucei*. He further expressed the frataxin homologue from protist *Trichomonas vaginalis*, diatom *Thalassiosira pseudonana*, plant *Arabidopsis thaliana*, and humans in the frataxin-depleted *Trypanosoma brucei*, and demonstrated that all these frataxin homologues are able to complement the function of native frataxin in *Trypanosoma brucei*. Mr. Long also investigated the function of the iron-sulfur cluster protein Isa1 and Isa2 in the iron-sulfur cluster assembly by using the same RNAi knock-down approaches in *Trypanosoma brucei*. The described studies clearly demonstrated the author's originality and creativity in pursuing the knowledge of physiological functions of frataxin in eukaryotic organisms.

2. In your opinion, has the student been autonomous in performing the experiments as well as in interpretating and presenting them?

*** While I have not direct knowledge on Mr. Long's specific roles in individual experiments and interpreting the results, it appears to me that Mr. Long made major contributions to the studies described in the three top-tier publications as first-author.

3. Are results of the project original, and what is their main outcome?

***The results of the research project are original in many aspects. The major findings are: **A)**, frataxin is essential for the iron-sulfur cluster assembly in mitochondrion and cytosol and for prevention of excess production of reactive oxygen species in *Trypanosoma brucei*. **B)**, the frataxin homologues from diverse organisms including humans are able to complement the

function of native frataxin in *Trypanosoma brucei*, indicating the functional conservation of frataxin.

4. **Would this thesis be adequate for PhD degree in your home institution/country – please answer Yes or No.**

***Yes.

5. **How would you evaluate the work of this student considering other PhD candidates whom you have known: among best 10%, among best 25%, average, among worst 25%.**

***I would rank Mr. Long's thesis among the best 10% among the PhD. candidates whom I have known in different research institutes and universities in US.

6. **Please do not grade the thesis, but state clearly whether or not it meets criteria for PhD degree.**

***The thesis meets the criteria for PhD degree.

Additional comments:

Although it has been shown that depletion of frataxin in human cells is associated with Friedreich's ataxia, the physiological function(s) of frataxin in mammalian cells is far from clear. The single-cell eukaryotic organism *Trypanosoma brucei* could be an ideal model system for elucidating the function of frataxin. The studies described in this thesis represented significant advance in understanding of the function of frataxin in the iron metabolism and iron-sulfur cluster biogenesis in eukaryotic organisms. Mr. Long not only developed new approaches to down-regulate specific genes in *Trypanosoma brucei* and but also explored the function of diverse frataxin homologues in the frataxin-depleted *Trypanosoma brucei.* The conclusions are based on solid experimental results followed by careful discussions. Overall, the study provided new foundation for further understanding the physiological role of frataxin in context of human neurodegenerative disease Friedreich's ataxia.

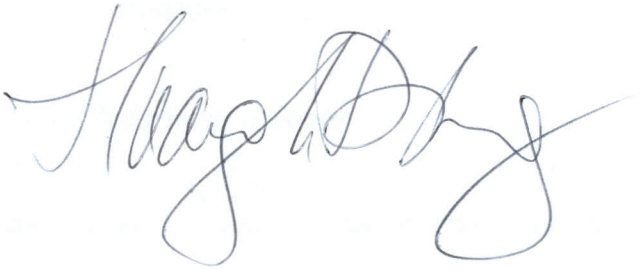
Specific questions for Mr. Shaojun Long:

1) Accoridng to your own studies published in **Molecular Microbiology**, what could be the mechainsm that frataxin alleviates the production of reactive oxygen species in *Trypanosoma brucei*? In this context, what are the potential links between the iron-sulfur cluster assembly and production of reactive oxygen species in *Trypanosoma brucei*?

2) If frataxin is an iron donor for the iron-sulfur cluster assembly in *Trypanosoma brucei* as you proposed, where does the iron in frataxin come from? Hypothetically, what would be approaches to approve or disapprove the notion that frataxin is the iron donor for the iron-sulfur cluster assembly in *Trypanosoma brucei*?

3) In your studies published in PNAS, the unprocessed forms of huamn frataxin are also able to rescue the iron-sulfur cluster assembly in the frataxin-deficient *Trypanosoma brucei* (Figure 7). This would indicate that maturation of frataxin may be unnecessary for the function of human frataxin in *Trypanosoma brucei*. In your opinion, could the unprocessed human frataxin still interact with other iron-sulfur cluster assembly proteins or bind iron just like the mature form? How would you test these ideas? Also, what could be the potential advantage of processing frataxin to mature form in human cells?

4) In a broad sense, what are the major questions remaining in the field of biogenesis of iron-sulfur clusters now?



Report on the Ph.D. thesis of Shaojun Long, "Iron-Sulfur Cluster Assembly in *Trypanosoma brucei*"

Dr Janneke Balk
Department of Plant Sciences
University of Cambridge

Summary
Mr Shaojun Long has, during his PhD, published three peer-reviewed first-author papers, one of which is in a very high-impact journal, Proc. Natl. Acad. Sci USA. This achievement demonstrates that Mr Long is able to perform experiments with high technical proficiency, and that the results obtained are of high quality forming coherent scientific arguments.
The three publications by S. Long et al. are related in topic and methodology and concern the knock-down of frataxin expression in *Tryponasoma brucei* (Tb), complemented with frataxin from other species. However, Mr Long's studies are not limited to Tb frataxin, but also include work on Tb Nfs1 (Wohlgamuth-Benedum et al 2009) and Tb Isa1 and Isa2 (draft manuscript).
The discussion of his thesis furthermore goes beyond the published papers, i.e. demonstrating that Mr Long has developed independent critical thinking, by forming testable hypotheses on the cellular distribution of Fe-S cluster assembly components in the bloodstream and procyclic stages.

1. Intellectual input in the thesis
The thesis consists of an Introduction (review of relevant literature, 40 pages) written by the candidate; four peer-reviewed, published papers jointly with co-author(s) and supervisor; one draft manuscript jointly with co-author(s) and supervisor, one Conclusion section (3 pages). Given the difficulty of assessing the personal intellectual input of the student in the co-authored papers, I will focus on the Introduction and Conclusions of the thesis.

The Introduction reviews the literature on Fe-S clusters and their assembly in vivo (Section 1), as well as the biology of *Trypanosoma brucei* with relevance to Fe-S-dependent metabolism (Section 2). The structure of Section 1 follows closely recent reviews by Prof R. Lill. The student demonstrates his own input in that the Introduction is tailored to the experimental work. Moreover, references to the student's own work are inserted in the appropriate context, clearly indicating his contribution to the field. The Introduction contains only minor errors, for instance in spelling, or occasionally a citation.

In the Conclusions and Perspectives, the student demonstrates, in the last paragraph, that he has taken ownership of the research topic by posing the question of "where in these cells [mitochondrial or cytosolic compartment] Fe-S clusters are assembled, and how they are made" based on "obtained evidence". Unfortunately, however, the preliminary data for this line of research are not included in the thesis (Question 2).

Overall, Mr Long should receive originality credits for developing *Trypanosoma brucei* as a amenable and insightful model organism for studying Fe-S cluster assembly, including an in-depth study of frataxin and the Isa proteins.

YB

2. Autonomy in performing, interpreting and presenting experiments
Publishing three first-author papers in such a short time-span requires a considerable amount of autonomy in performing the experiments, including performing the appropiate controls, therefore I have no doubt that Mr Long has the level of independence required for a career in research.
The autonomy in interpretation is difficult to assess from co-authored, reviewed papers, and I would therefore like to address this in the questions (Questions 2 and 3).
The figures showing the results of Mr Long's studies are well presented, giving the right amount of detail and consistence in quality.

3. Originality and main outcome
The originality of the project lies, in my opinion, in the choice of model organism for the study of the role of the frataxin and Isa proteins in Fe-S cluster assembly in eukaryotes. Trypanosomes do not synthesize haem, allowing dissection of these two Fe-utilizing pathways and their interaction with Fe homeostasis. Moreover, Trypanosomes belong to an early branching lineage in the eukaryotes, allowing studies on both evolutionary constraints as well as the basic mechanism on the mechanisms of in-vivo Fe-S cluster assembly.

The main findings of the thesis are i) that frataxin is an essential protein in *T. brucei*; ii) that frataxin is required for the activity of (non-essential) mitochondrial and cytosolic Fe-S enzymes; iii) that frataxin is localized in the mitochondria in the procyclic stage of *T. brucei*; iv) that its structure and function are highly conserved during evolution, since frataxin of a wide variety of distantly related eukaryotes can replace Tb frataxin, provided that it is imported into the mitochondria as shown for human frataxin; v) that functional complementation by human frataxin does not depend on N-terminal processing; vi) that the loss of frataxin does not result in mitochondrial iron accumulation, unlike in yeast and mammals, but it does result in increased ROS; vii) that *T. brucei* has two Isa proteins, Isa1 and Isa2, both localized to the mitochondria and essentia; viii) that the Isa proteins are required for the activity of mitochondrial, but not cytosolic enzymes; that the Isa proteins may play a role in Fe homeostasis.

These findings have, to my knowledge, not been reported by others. The data are a significant contribution to the debate on the role of frataxin and Isa proteins.

4. This thesis is adequate for a PhD degree in the UK, pending minor revisions (answer is yes).

5. The experimental productivity of the student is among the best 10%; The quality of this written work (Introduction/Discussion) among the best 25%.

6. This thesis meets the criteria for a PhD degree, in that it demonstrates an original, not previously published, body of work, contributing new data to important questions in Fe-S cluster assembly in general as well as the evolutionary conservation of frataxin in particular.

JB

Questions (J. Balk)

1. What are the outstanding controversies surrounding the function and structure of frataxin? What new insights regarding these controversies are provided by your results?

2. Please provide and discuss the experimental evidence to the following statement in your Conclusions: "by Western blotting we have obtained evidence that in the bloodstream stage both proteins are (mainly) localized in the cytosol, not the mitochondrion."

3. Interpretation of data:

S. Long et al (2008) Mol Biochem Parasitology 100-4.

Figure 2A: Why are the precursor forms of *T. pseudomona* or *A. thaliana* frataxin observed?

Figure 2B: What are the error bars (variance) in this growth experiment? Given that no error bars are shown, is the following conclusion justified: "the growth rate is [..] rescued by the expression of At-frataxin, although not to the same level".

Figure 4: What positive control would have benefited this experiment?

4. How can the de novo Fe-S cluster assembly be studied in Trypanomes? What methods need to be developed and is this technically possible?

5. Why, in your opinion, does frataxin knock-down in *T. brucei* not lead to mitochondrial Fe accumulation, in contrast to yeast and mammals? Can the lack of Fe accumulation in mitochondria from Arabidopsis *atm3* mutants also be explained by this mechanism?

6. What is, in your opinion, the single most important finding of your PhD work and why? What are the potential impacts on "improving the quality of life"?

JB

Corrections

Page 3, H-cluster: this is considered to be a [4Fe-4S] cluster coupled to a di-iron centre, rather than [4Fe-4S] + [2Fe-2S]. Please update with more recent references.

Page 5, top. "Initial insights about the pathway for the Fe-S assembly in vivo were gained through the analysis of proteins required for the activation of nitrogenase". Is this really true? Please consider the findings by Strain et al (1998) and Schilke et al (1998) in Baker's yeast.

Page 6, end of 1.2.2.1. The citation Richard et al 2006 should be formatted as Richard and van der Giezen, 2006 (with z not s), since it is the same.

Page 6, last line: "cells make full use of" should be "cells have an array of".

Page 7, end of 3rd paragraph: I cannot find the reference Schilke, 2009, neither in the references or on PubMed. It think it should be Adinolfi et al 2009.

Page 9, IscU proteins. Please consider recent studies by Dean and coworkers on Azotobactor IscU.

Page 10, end of 1.2.2.4. When citing Leon et al 2003, Yabe et al 2004 should also be cited.

Page 12, end of 1.2.2.6. Bych et al 2008, not 2009.

Page 13, line 12. "Recently, in *A. thaliana* mitochondrion, Atm1 has been identified etc". This section has many mistakes! A plant orthologue of the yeast ATM1 gene was first identified by Kushnir et al 2001, not by Bernard et al 2009. The protein, called STA1, was localized to mitochondria using GFP fusion. It has also been identified in mitochondria in several proteomics studies, see the discussion of Bernard et al 2009 for references. Philip Rea et al (Chen et al 2007) renamed STA1 as ATM3. Please also note that in plants, proteins are denoted as all caps, no italics. Furthermore, there is no evidence that ATM3 is required for Fe-S cluster and Moco assembly machineries in the cytosol, but for the activity of Fe-S and Moco enzymes.

Page 14, Cfd1. Which cytosolic and nuclear Fe-S proteins did Roy et al 2003 analyse? Is this "virtually all"?

Page 14, Nar1. What is the similarity (approximate percentage) of Nar1 and hydrogenases? Is this "some" or more than that? What is the evidence that Nar1 does not function as a hydrogenase? Also, Urzica et al 2008 needs to be referenced here.

Page 16, line 7. 'petit', not 'peptite'

Page 41, Table 1. *A. thaliana* does NOT have a homologue of Cfd1, see Bych et al 2008 JBC.

Page 41-2, Table 2. What is the source of these data? This study and/or others? Please give unit of Size (column 3).

JB

Page 26, bottom: "We dare to predict that surprising differences exist between the Fe-S assembly pathways between these two stages." Please motivate this statement, taking into consideration experimental evidence, as well as the remarkable evolutionary conservation of Fe-S cluster assembly pathways. In what way are the predicted differences 'surprising'?

JB

Ph.D. Thesis: Iron-sulfur cluster assembly in *Trypanosoma brucei*
Shaojun Long

Evaluation report

I have critically read the thesis "Iron-sulfur cluster assembly in *Trypanosoma brucei* ", which was submitted for defense to obtain a Ph.D. degree by Mr. Shaojun Long. The thesis consists of three parts: Introduction, which is a well written overview about FeS cluster assembly covering also own results on *T. brucei*; three full papers, a short communication and a manuscript (I am also sure that he has a lot exciting results which are not published yet) and finally a short chapter devoted to conclusions and perspectives.

Part 1 Introduction

From the introductory chapter, it is apparent that the candidate gain a deep knowledge in the filed of FeS cluster biogenesis and he is able to synthesize large mass of information to a compact logical text.

Comments and questions:
Page 5
"Initial insights about the pathway for FeS cluster assembly…were gain through the analysis …of nitrogenase in *Azotobacter*…(Christensen et al., 2001). Subsequent …analyses…(Agar et al., 2000)."
In this context, it would be more appropriate to use the original paper by D. Dean instead of a review by Christensen et al.

Page 5
"In typical eukaryotic cell, the *de novo* assembly of Fe-S clusters required….Nfs1 and Isd11…"
What is not typical eukryotic cell? Do you consider cells of *T. brucei* or *T. vaginalis* to be typical or atypical eukaryotic cells? Do they have Nfs1 and Isd11?
At this point, the acronym Nfs1 should be explained.

Page 6
"In both the mitochondrial ISC-assembly machinery (Nfs1) and all three bacterial systems (IscS, NifS and SufS)…."

In the whole work (and a number of papers), names given for ISC assembly machinery in *S. cerevisiae* are used as general names for mitochondrial orthologs. In my view, this is basically incorrect and misleading. There are three, clearly defined FeS cluster assembly machineries: ISC, NIF and SUF. A cysteine desulfurase present in mitochondria was inherited from proteobacterial ISC system. It is not an ortholog of neither NifS nor SufS. Why to use a different name Nfs1? The same for IscU versus Isu, IscA versus Isa. However, the most inadequate is using names Yah1, Yfh1, Arh1, and Ssq1 for [2Fe2S] ferredoxin, frataxin, ferredoxin:NADH oxidoreducatse and HSP70, respectively. Ferredoxin was known before discovery that it is involved in FeS cluster assembly. Why to call it now Yah1?

Using of this inappropriate names leads to statements such as at page 10: "The second step of FeS cluster biogenesis in mitochondria *(in general sense)* involves….Hsp70 chaperone Ssq1…"
Questions: Is Ssq1 generally present in eukaryotic mitochondria?
Why do you think that we should use different names for mitochondrial and bacterial orthologs of ISC machinery?

Page 6
"Even organisms lacking mitochondria…." Inapropriate statement, they have reduced or modified mitochondria.
"…have a homologue of Isd11…"
Are there organisms without Isd11?

Page 7, 9, 11

Statement at page 11: "…IscA is supposed to be a scaffold protein for *de novo* FeS cluster assembly…" etc.

The exact function of IscA is a matter of discussion. Nevertheless, in your opinion, what is a role of IscA, (i) is it an iron source, (ii) an alternative scaffold for *de novo* FeS cluster biogenesis, (iii) carrier of FeS cluster from IscU to target apoproteins?

Do you think that the role of IscA in bacteria and mitochondria is same or different? Is the role of IscA1 (Isa1) and IscA2 (Isa2) redundant or do they have specific roles?

I miss more recent references:
Vinella et al., 2009
Jiang at al., 2009 – residues involved in [4Fe4S] cluster,
Tan et al., 2009

Page 8
In addition to Yah1, there is not reason to call human ferredoxin adrenodoxin. It is [2Fe2S] ferredoxin.

Page 9
"Crystal structure for any of these proteins are not available."
I agree that crystal structure of Arh1 and Yah1 is not known. **However, did you check for crystal structure of other mitochondrial [2Fe2S] ferredoxin-ferredoxin reductase complexes?**

Page 11
What is a specific role of Jac1? (paper from A. Dancis group?)

Page 11
"…The exact function of Grx5 is unclear…" yes, but some more information is known.
Is it possible thet Grx5 provides reducing equivalents for FeS cluster assembly? Is it possible that Grx5 binds FeS cluster in mitochondria and mitosomes?

Page 12

"…As all three Isa proteins…"
Iba57 is not Isa protein.

Page 14
What is the main difference between Cfd1 and Nbp35? It would be good to mention this in the text

Page 21, 22
Text on page 22 concerning cellular location of fumares in *T. brucei* is not consistent with figure 4. Why?

Page 24
Ferritin…."However, trypanosomes do not possess its homologue in their genomes."
Is it specific feature of trypanosomes? How is it in other protists with ferritin?

Page 24
Table 1
The information in this table summarized knowledge in - 2005. It should be updated.

Page 25
"Moreover, Nfs1 and Isu…"
Hydrogenosomes possess IscS, IscU.
Was it directly demonstrated that hydrogenosomal IscS and IscU are involved in FeS cluster assembly?
In *E. cuniculi*, is it clear whether mitosomes possess Ssq1 or general Hsp70?

Page 25
Repeated sentence about alfa proteobacterial endosymbiont.

Page 26
Line 13
..of f.e. Jac1 and Isd11 …???

Page 42
Table 2
There are three HSP70 in mitochondria of trypanosomes. Do you expect that they have different functions like HSP70s in certain fungi or they are redundant?
I miss information about Iba57 and Ind in *T. brucei*.
A number of squares in the table are blank. What this indicates?

References:
The candidate included unusually large set of references: 241, and from the text I believe that he knows majority of them. There are some formal errors, such as inconsistent usage of "š" in the name Lukeš, "ü" in names Muller, Muhlenhoff.., italic in latin names etc. As an author of two recent book chapters covering FeS proteins and FeS cluster assembly, particularly in protist, I am sad that the candidate ignored these reviews, and as an reviewer I have a good occassion to punish him for it, but I understand that books are, unfortunately, not the medium which is readily accsessible and these days nobody reads books.

Part 2 Publications

The candidate published his results in three full papers in high rank journals: JBC, PNAS, and Mol.Microbiol. and another study has been published as a short communication in MBP. Importantly, he is the first author on three of these four publications. The main contribution of these papers concerns function of frataxin in mitochondria of *T. brucei* and in general conservation of frataxin function in euakryotes. These findings are original, very interesting and certainly more then sufficient for Ph.D. degree. I will not comment these papers in detail, as they went throughout peer-review evaluation an I will focuse on the last manuscript concerning function of IscA1 and IscA2 in *T. brucei*.

The authors report on interesing results that depletion of IscA proteins caused decrease in mitochondrial [4Fe4S] proteins and that assembly of this clusters in not essential for blood stream trypanosomes. However, the manuscript will need some trimming before submission.

Introduction:

"A subset of these *(understand mitochondrial)* de novo formed clusters is exported into the cytosol and nucleus…."

Is it likely that FeS cluster is the compound which is exported across two mitochondrial membrane into cytosol?

I would consider to include references Vinella et al., 2009, and other more recent papers about function of IscA in [4Fe4S] cluster assembly.

Results

„ ….for 173 and 272 amino acids-long…proteins with ….29.5 kDa and 19.kDa, respectively."

There is something wrong.

pSORT, should be Psort

What do you thing about specificity of Isa1 and Isa2 binding based on Fig1 B?
What about presence of other mitochondrial proteins in this preparation?

It is difficult to compare growth curve in Fig2 and Fig7 B,C,D. **Why you did not use the same graphical style?**

Knock-down of Isa1 (Fig 2) caused some decrease in growth rate of trypanosomes, however, only double knock-down caused complete growth inhibition. **What is your interpretation? The role of each Isa paralogue should be discussed (redundant, non-redundant etc.)?.**

Discussion:

"…succinate dehydrogenase was strongly hit in all the knock-downs, which went unnoticed in other eukaryotes…"

See recent paper about function and cellular location of IscA1 in human cells by Song et al., 2009

Part 3 Conclusions and perspectives

I appreciate particularly the part concerning perspectives which indicates that the most exciting results are in fact not published including systematical comparison of FeS cluster

assembly between vector and blood stream trypanosomes, an idea which we suggested with Ondřej Šmíd in the initial paper about FeS cluster assembly in trypanosomes in 2006.

In conclusion, the quality and quantity of the experimental performance is on very high level, and the way in which the original results and background knowledge have been presented in the thesis meets the criteria for obtaining a Ph.D. award.

December 2009

Prof. RNDr. Jan Tachezy, Ph.D.