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Ph.D. Thesis: The impact if iron-sulfur assembly on the mitochondrial tRNA import in *Trypanosoma brucei*
Zdeněk Paris

Evaluation report

I have critically read the thesis **"The impact if iron-sulfur assembly on the mitochondrial tRNA import in *Trypanosoma brucei* "**, which was submitted for defense to obtain a Ph.D. degree by Mgr. Zdeněk Paris. I believe that the thesis is of high standard, bringing a number of original information about biology of trypanosome.

Overview

The experimental part of the work is preceded by an introductory chapter, which provides the reader with essential up-to-date information about molecular mechanisms of tRNA import, possible role of FeS cluster assembly in tRNA thio-modifications and their impact on tRNA cellular localization. The text of this chapter is well written and clearly certifies that the candidate gained a deep knowledge in the filed of tRNA modifications and transport and he is skilled to synthesize information to a compact logical text.

There are just a few typos which should be corrected:

Page 12 cystein = cystein**e**
Page 15, line13, the = **T**he only common
Page18, line 22 oter=ot**h**er

Questions:

Page 10
Second paragraph, "A similar observation was made in *T. brucei*…(Schneider et al., 1994). Is this correct reference?

Explain the last sentence: "However, this mutant tRNA was properly imported into the mitochondrion suggesting that the modified cytidine residue is **indispesable for import**…" Did you mean indispensable or dispensable?

Page 13 cluster
"Finally a role of CIA pathway itself in tRNA thiolation cannot be excluded since Nbp35 and Nar1 are known to contain Fe-S clusters." Are there other CIA components with FeS clusters?

Page 14
"This finding supports the notion that Nfs-Isd11 complex works as a functional equivalent of the bacterial TusABCDE sulfur relay system". Nfs-Isd11 is required for thiolation, but also for FeS cluster assembly, while TusABCDE system is required specifically for thiolation. Is it really "an equivalent"? If so, and Isd11 is involved in sulfur transfer, what is a role of mitochondrial Nfu proteins or Tum1? Are there other possible roles of Isd11?

Page 18
"..absence of most TOM subunit.."
Which components are present? Reference "Schneider et al., 2008" is missing in the reference list.

Results

Thiolation and the Rieske protein (an essential component of the *Leishmania* RIC complex) play negligable roles in *Trypanosoma brucei* iRNA import.

In this paper, the authors tested whether thiolation of tRNA, which was described in *Leishmania* as negative determinant for mitochondrial import, also operates in *T. brucei*. They used cell lines with knock down mitochondrial cysteine desulfurase TbIscS-2 to test whether thiolation affects tRNA import.

Questions:
1. As thiolation takes place in cytosol, is it clear whether TbIscS-2 is present in trypanosomes exclusively in mitochnondria or is it present also in other cell compartments?
2. What is the ratio between thiolated and non-thiolated $tRNA^{Glu}$ and $tRNA^{Gln}$ in the mitochondria in TbIscS-2 knock downs?

Is it possible that cytosolic TbIscS-1 is involved in tRNA thiolation?
This obvious question was answered in following elegant study:

Thiolation controls cytoplasmic tRNA stability and acts as a negative determinant for tRNA editing in mitochondria

In this paper authors clearly showed importance of TbIscS-2 for thiolation in both cytosolic and mitochondrial compartments, while TbIscS-1 seems to be not involved in this process. Most importantly, they demonstrated that thiolation is a negative determinant for $tRNA^{Trp}$ editing.

Questions:
3. Why $tRNA^{Glu}$ and $tRNA^{Gln}$ are thiolated in cytosol and not in mitochondria?
4. Is it known whether *T. brucei* possesses thiosulfate sulfurtransferase such as Tum1 to transport sulphur outside of mitochondria?
5. What is your opinion on proposed dethiolation of $tRNA^{Glu}$ in the mitochondria published by Bruske et al?

The Fe/S cluster assembly protein Isd11 is essential for tRNA thiolation in *Trypanosoma brucei*

This paper further extend the idea that part of FeS cluster assembly machinery is also involved in tRNA thiolation.

6. Frataxin is known to interact with IscS and IscU. What is your explanation that in trypanosomes, frataxin does not form a complex with above proteins.

Futile import of tRNAs and proteins into the mitochondrion of *Trypanosoma brucei evansi*.

Question:
7. The authors showed that *T. evansi* cells lack thiolation of $tRNA^{Try}$ in the mitochondrion. Are TbIscS and Mtu1 present in mitochondrion of these organisms?

Comment
In my view the Fig1 should be improved particularly Northern blot for mitRNAP is not very clear.

Unpublished data
At the end of the thesis the candidate attached several interesting preliminary results suggesting that methylation serves as negative determinant for tRNA import, and he also studied whether other components of FeS cluster assembly such as IscU and Atm1 are involved in thiolation.

Question:
What is your interpretation that Atm1 knock- down causes decrease in cytosolic thiolation. Is it an indirect effect which is based on impaired FeScluster assembly of FeS proteins involved in thiolation or impaired export of persulfide sulfur from mitochondria via Atm1?

In conclusion, the thesis of Zdeněk Paris is of very high standard, the quality of the experimental performance resulted in three publications in recognized journals with high impact and additional results are promising to be published in the future. Thus, this thesis not only meet but certainly exceed the criteria for obtaining a Ph.D. award.

September 1st, 2010

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Marburg, September 6, 2010

Report of PD Dr. Antonio J. Pierik (external examiner)

On the PhD thesis titled: "The impact of iron-sulfur assembly on the mitochondrial tRNA import in *Trypanosoma brucei*" by Zdeněk Paris, University of South Bohemia, Faculty of Science, České Budějovice, 2010

Supervisor Prof. Dr. Julius Lukeš, cosupervisor Prof. Dr. Juan D. Alfonzo

tRNA import into mitochondria is an extremely exciting field which has gained an enormous momentum due to the increased availability of organellar and nuclear genomes. Contrary to several well-studied higher eukaryotes (like humans) it is now appreciated that in many eukaryotes an appreciable proportion of tRNAs is not encoded by the organellar genome, but that several if not most tRNAs have to be imported into the mitochondrion. *Trypanosoma sp.* present an excellent model system, since none of the tRNAs are encoded on the organellar genome. As tRNAs are highly complex molecules with a range of chemical modifications positive and/or negative determinants for the import appear to be difficult to unravel. The thesis of Zdeněk Paris has provided an important contribution to the understanding of such determinants. By extremely well-designed experiments described in a set of published articles Mr. Paris has adressed the impact of depletion of cysteine desulfurase, selenocysteine desulfurase, Isd11-like proteins and the thiouridylase homolog Mtu1 on tRNA thiolation as determinant. Since iron-sulfur cluster assembly of proteins (including some involved in tRNA modification) also requires Nfs and Isd11 the impact of depletion on this class of proteins depletion was also assessed. That tRNA import not always serves a purpose was shown in the chapter on futile import in *Trypanosoma brucei evansi.* In a hitherto unpublished but highly exciting chapter the m^1G37 modification of $tRNA^{Ile}_{UAU}$ by Trm5 is described as negative determinant. Mr. Paris has contributed to the field by clarification of determinants of tRNA import in eukaryotes.

Based on the extensive body of published scientific work and the plethora of experimental methods used, Mr. Paris most certainly has acquired the experimental skills required for a PhD in the natural Sciences. In the introduction of the thesis he has also shown that he is able to summarize the remarkably complex field of tRNA and communicate the general relevance in a scientifically sound and accurate manner. Comprehensive references to published work have been given and, if applicable, contributions of other researchers have been discussed and carefully phrased in case there was ambiguity about results of others.

My recommendation as external examiner is therefore to grant Mr. Paris the degree of Doctor, which is subject to the quality of the presentation, replies to the questions and discussion with the examiners during the PhD defence.

PD Dr. Antonio Pierik

PhD Thesis

The impact of iron-sulfur assembly on the mitochondrial tRNA import in *Trypanosoma brucei.*

Zdeněk Paris

Report from Dr. Mark van der Giezen, University of Exeter (September 2010)

Summary:
This thesis is of a high international standard and I thoroughly enjoyed reading it. When comparing to theses from the UK, Mr. Paris' thesis is of a similar standard if not better.

Background:
This thesis deals with an important aspect of the biology of the causative agent of African Sleeping Sickness, *Trypanosoma brucei*. *T. brucei* has an alternating life cycle in two different hosts. One is the Tsetse fly, the other hosts are humans. During the transfer from one host to the other, this parasite dramatically reorganises its biochemistry and cell biology. In the fly, it behaves as a standard text-book eukaryote with classic mitochondria performing oxidative phosphorylation and which gain energy via an electron transport chain. However, in the human host, these pathways are all lost and *T. brucei* solely relies on glycolysis for ATP production. Its mitochondria are highly reduced and are not performing any of the classic biochemical pathways such as citric acid cycle, oxidative phosphorylation and even do not have a functional electron transport chain. And here lies the central tenet of this thesis; previous work by others has postulated that part of the electron transport chain is involved in the import of tRNAs in kinetoplast mitochondria. As bloodstream *T. brucei* has no electron transport chain in their mitochondria, how would they import tRNAs in order to be able to translate mitochondrial mRNAs? This is a crucial aspect of the trypanosomal cell biology as they have transferred all mitochondrial tRNA genes to the nucleus and are therefore dependent on the proper targeting and import of these tRNAs for survival. As mentioned in this thesis, here lies a potential Achilles' heel that might be exploited to tackle these human parasites. This thesis excellently addresses the questions surrounding tRNA import into *T. brucei* mitochondria and has provided a wealth of information of a high international standard which has already been, or will be, published in internationally recognised scientific journals of high standing and impact.

Chapter 1:
This chapter is a brief and clear overview of what to expect to and wet the appetite of the reader.

Chapter 2:
In this chapter, a thorough overview is given of trypanosomes, their mitochondria and tRNA import. It is a well written chapter and with some minor modifications, I would expect it to stand a good chance to be acceptable as a review on this topic.

Comment: page 6, line 11-14. It is mentioned that all remaining mitochondrial genes encode highly hydrophobic proteins but leaves it at that. As there are several hypotheses as to the reasons behind organellar genomes, I wonder if Mr. Paris has any thoughts on these. I would like him to focus on the hydrophobicity one from Gunnar von Heijne and the CoRR hypothesis from John Allen. This might be especially interesting considering the existence of *T. brucei evansi*.

Comment: page 9, figure 2. This figure would have benefitted from an expanded legend which explains all the various abbreviations used in the figure (such as Gm, S^4U, acp^3U, etc.).

Question: page 11, first paragraph. It is suggested that the cytosolic CIA system is required for thiolation of cytosolic tRNAs and that this system is not required for thiolation of mitochondrial tRNAs (work by Bruske et al). Apparently, Bruske et al claim that mitochondrial tRNAs are imported as thiolated versions which are subsequently dethiolated. If this is the case, how can the CIA system *not* be involved with mitochondrial tRNAs? Obviously, they must first be thiolated in the cytosol in order to be able to be dethiolated later?

Comment: page 14, first paragraph: As eukaryotes have lost the Tus complex, the uniquely eukaryotic Isd11 is given as potential replacement. I am not sure I would agree as it has been shown that Isd11 is an essential 'co-factor' of IscS. Its role in thiolation might solely be due to its tightly bound nature to IscS rather than causal. It is interesting to note that bacteria have a similar small helical protein that is bound to IscS and essential, YfhJ.

Chapter 3.1:
This chapter has been published in 2009 in RNA under the title 'Mitochondrial tRNA import in *Trypanosoma brucei* is independent of thiolation and the Rieske protein.

The justification for this work was the observation that tRNA thiolation was a negative determinant for import into *Leishmania* mitochondria and the notion that the Rieske protein was part of a supposedly essential RNA Import Complex (RIC).

This work clearly puts doubts on RIC being involved in RNA import and also clearly shows, using Nfs (=IscS) knock-outs that thiolation has no effect on mitochondrial tRNA import.

Question: This might be due to my ignorance but why does Nfs knock-out not result in death? If procyclics have normal full-blown mitochondria than one would expect lack of Nfs to have disastrous results? In figure 1B it can be seen that although Nfs protein has disappeared, there is still quite a lot Rieske protein left. This makes me wonder what the half-life of FeS proteins is? Could it be that despite having no Nfs, there are still enough FeS proteins around to do some essential chemistry? What if you would have repeated the Western blot after eight days? Finally, would Nfs or Rieske knock-out convert procyclic mitochondria into bloodstream ones?

Chapter 3.2:
This chapter was published in the Journal of Biological Chemistry in 2009 under the title 'Thiolation controls cytoplasmic tRNA stability and acts as a negative determinant for tRNA editing in mitochondria'.

Here, the interesting problem that there only exists one tRNA for tryptophane while the nuclear and mitochondrial codons are not identical is addressed in an elegant manner.

Question: It is presented that unedited $tRNA^{Trp}$ pairs with UGG and that the edited $tRNA^{Trp}$ pairs with UGA. This is postulated to be a mechanism to differentiate expression of different genes when for example the trypanosomes up- or down-regulate their mitochondria when moving life-stages. Have you checked whether the codon usage for mitochondrial encoded genes follows this patters and that there is 'clear' distinction between certain genes that use UGG and others that use UGA?

Chapter 3.3:
This chapter has been published in 2010 in the Journal of Biological Chemistry as 'The Fe/S cluster assembly protein Isd11 is essential for tRNA thiolation in *Trypanosoma brucei*'.

Here, it is shown that Isd11, similar to Nfs, is required for tRNA thiolation in general and Mtu1 for mitochondrial tRNA thiolation in particular. The authors need to be congratulated with this paper in JBC.

Question: page 22400, right column, top paragraph. It is argued Nfs1 and Isd11 might be present in the cytosol at undetectable levels. That way, they would still be capable of thiolating tRNAs. It is also suggested that an unknown component moves via Atm1 from the mitochondria to the cytosol and which is involved in thiolation. There is no evidence for the former comment while that latter has support from Atm1 RNAi studies. What is then the reason for the sentence starting with 'This creates a scenario…'?

Chapter 3.4:
This chapter has apparently been submitted to Molecular and Biochemical Parasitology. It deals with an interesting *T. brucei* strain called *evansi* which has lost its complete mitochondrial genome and would therefore be eternally be locked in the bloodstream stage (but not require an insect vector either…).

This study provides further ammunition for the notion that the RIC is not required for mitochondrial tRNA import (not say that it doesn't exist at all).

Question: page 54, 2nd paragraph. You have shown *T. brucei evansi* imports some proteins and tRNAs into its mitochondria. As there is no longer a genome in these mitochondria, it is indeed remarkable tRNAs are imported. However, you then go on to say it imports 'dozens or even hundreds of different proteins condemned to non-functionality and thus produced and imported in vain'. This is incorrect, many, if not most, mitochondrial pathways do not contain mitochondrial encoded proteins and are completely nuclear encoded (ISC assembly, amino acid degradation, urea cycle, heme biosynthesis, beta-oxidation, etc). Import of proteins and RNAs involved in the mitochondrial genetic apparatus would indeed be in vain but this does not hold true for other pathways. Or do you perhaps have more information about the *T. brucei evansi* mitochondria?

Comment: page 57, top lines. A Western blot should have been presented showing the drop in Rieske protein.

Chapter 3.5
This chapter contains some material that hopefully one day will see the light as published papers, most notably the tRNA methylation story.

Comment: page 66, figure 5. Why figure 5? What happened to figure 1-4?

Comment: page 70, figure 10. This work needs to be repeated until there is no enolase signal present in the mitochondrial fraction as shown in the previous chapters.

Question: page 71-72. The presented data is complex and I am not sure I follow the logic of the presented argument. I would interpret the data that there is *no* methylated tRNA present in the mitochondria. This is based on my assumption that the blue probe cannot hybridize a tRNA if the methylation is present but only binds in the absence of the methylation. If I understand that correctly, then the data suggests that methylated cytosolic tRNAs cannot be imported into mitochondria. Removal of TRM5 (in the cytosol)

results in a huge increase of non-methylated cytosolic tRNA which suddenly can be imported into mitochondria, hence the increase of mitochondrial signal when RNAi is induced. Please talk me through your argument that methylated tRNA does exist in the mitochondrion.

The small paragraph on Atm1 down-regulation is promising.

Chapter 4:
The conclusions clearly present the achievements of this thesis. However, the importance of the mitochondrial membrane potential has only been mentioned as an aside and has not received a lot of detailed attention.

Overall conclusion and impression:
This is a high quality thesis with important original contributions and I am certain it will meet Czech requirements of a PhD thesis, it certainly would in the UK or the Netherlands. It was a pleasure reading it and being involved in the assessment of this work. I would like to wish Mr. Paris a successful continuation of his promising scientific career.

Dr. Mark van der Giezen