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A reviewer's opinion of a PhD thesis written by Lucie Ridlon.

Thesis title: Functional analysis of newly described 45S SS* complex in *T. brucei*

This study takes a journey to an unexplored jungle of mt translation in *Trypanosoma brucei*. This unicellular organism has been studied extensively during the last 30 years. Due to this research we have gained enormous amount of knowledge of its biology. From the surface VSG coat, cell division, cell cycle, metabolism, transporters, drug resistance to composition and function of its organelles. However, for some reason, the mt translation is still covered by a coat of mystery. What can be the reason? It has been proved that it is extremely difficult to detect mt translation and its products due to their biological peculiarities. Moreover, mt translation is highly integrated with the mt mRNA expression and maturation, which causes troubles in result interpretation. However when you look under the imaginary coat, there is a promise of something big.

Major points & questions

The presented study attempts to define (a) possible function(s) for a novel and unique ribosomal complex called 45S SSU*, which has been originally identified in *L. tarentolae*. It is composed of two lobes, the first lobe represents the SSU moiety and the second lobe is called SSU-like (Maslov et al., 2006 and 2007). Even if the EM figures look very nice, I am still wondering how the authors do know, which lobe is which one?

The author also mentions (page 18) that one of the lobe is IDENTICAL to the functional mitoribosomal SSU –to which SSU (from what organism?)

What is the composition of just the 30S SSU moiety and what are the major differences (proteins) between SSU and SSU-like complexes?

Page 18
The author introduces a new term non-SSU lobe – I prefer labeling „SSU-like", because this complex contains 9S rRNA, a hallmark of a SSU moiety, and in addition to rRNA, it also contains specific MRP proteins. Is there any reason, why this SSU-like is suddenly called non-SSU?

One hypothetical function of the 45S SSU* complex is to prevent a reassociation of a free SSU with a free LSU, how this is prevented in other organisms?

JBC paper, Figure 1A

- The peak labeling is intriguing to me. I was wondering if the authors have any additional evidence to the assignment of the peaks. E.g. silver/sypro ruby staining of the individual peaks followed by a mass spec, re-sedimentation as it was done for *Leishmania* ribosomes.
  - If the 25S peaks represents free SSU, would not author assume to detect this peak upon a RNAi knock-downs of PPR29, 200Kda and Rhod (Fig. 3A, B and C)?
  - If the 50S peak represents monosome, how it is possible that this peak is clearly detected upon L3 knock-down (Figure 3D), in which 12S rRNA is diminished?
  - The peak for 45S SSU* and 50S monosome are moving by a few fractions in Figure 3. Is that just within the error of the sedimentation patterns?

JBC paper, Figure 1C

- I have noticed in M&M section that authors used less BF cells than PF cells, this is surprising considering the down-regulation of mitochondria activity, metabolism and most likely translation in BF cells. I was wondering is there any reason why did you use twice less material . Furthermore, lyses of whole cells by DDM is also surprising, as DDM is usually used to solubilize the mt membrane and not the plasma membrane. Any reason behind this decision? How was the relative amount of 9S and 12S rRNA (Figure 1C, inset) compared to PF and what was used as a standard ?

JBC paper, Figure 1B

- As written in the text, the 0.5M KCl treatment should have dissociated mitoribosomes to the individual complexes, however 50S monosome is still labeled in this figure. Does this mean that *T. brucei* mitoribosome is more resistant to a high salt concentration ? Is the same resistance observed also in *Leishmania* mitoribosome ?

JBC paper, Figure 2 – qPCR data

- What does the mean represent ? How many independent RNAi inductions were prepared ?
- What is the author opinion about a decrease of 12S rRNA (done by qPCR) in the 45S SSU* RNAi knock downs (except for 200Kda) and S17 ? Moreover, in Figure 3 it seems that the 45S LSU peak remains at the same (or very similar) height in noninduced and RNAi induced cells (except L3, of course) Btw. the figure 3A is mislabeled ☺.

Why did authors choose to knock-down S17 as a representative knock-down for the SSU part, as this protein is also a component of the SSU-like moiety ? Would not be better to pick a protein that is SPECIFIC just for the 25S-30S SSU ?

JBC paper, Figure 4

- I have a difficulty to accept the author's conclusions regarding this section. They are claiming that RNAi of L3 and S17 has more profound effect on mt translation than RNAi of PPR29, 200Kda and Rhod and thus down-regulation of 45S SSU* does not cause a disruption of mitoribosomes. Looking at gels from PPR29, S17 and L13 IND cells, they look to me quite similar. Yes, 200Kda and Rhod RNAi cells exhibit some translational activity at day 3 after RNAi, however looking at the gels displayed in page 51, almost no cyb and COI products were detected at day 4 after RNAi. Surprisingly, authors never discuss the efficiency of RNAi (it varies significantly between the RNAi cell lines as obvious from the qPCR experiments) and the stability of the proteins of interest. Without specific antibodies and without a quantification of remaining monosomes, it is difficult to

state such a strong conclusion as the effect can be easily attributed to this. Can author comment on this ?

The major conclusion of the paper is that COI and CyB LT mRNAs are selectively reduced by ablation of 45S SSU* while RPS12 mRNA is not affected. This is very interesting observation which opens possibilities about a selective translation initiation of certain mRNAs. I was wondering, since the author detects general destabilization of 45S SSU*, if the same effect would be observed upon a knock-down of other 45S SSU* subunits. Furthermore, does the author have a hypothesis/opinion about the possibility of RPS12 being the link between RNA editing and translation (Aphasizheva et al., 2013) and how this would apply to her results ?

Is there a reason why only the edited Cyb transcript (and not also pre-edited Cyb) was analyzed by RT-qPCR ?

Page 61 – The major conclusion of the sedimentation profiles is that RNAi of 45S SSU* components selectively influence only the targeted ribosomal complex without noticeable impact on other ribosomal complexes. Can the author explain this statement using her sedimentation profiles? Honestly, I was looking at the graphs for some time and I don't comprehend how this conclusion was made.

Page 82

- I strongly disagree with the author that by day 5, the growth phenotype is FULLY apparent for 2710 and 2650 RNAi. There is almost NO growth phenotype for 2710 and very mild phenotype for 2650. Considering that in the case of 2710 there is a significant decrease of 9S rRNA hybridization signal, while nothing is happening for 2650, these results seem to me inconclusive and more work is needed to support the conclusions on page 83 and 89.
- What was the glucose concentration in the SDM 79 medium ? Would be worthy to perform this study under low-glucose conditions ?

Page 85

- How does the author know that the three proteins of interest are specific just for SSU-like lobe ?

Page 87

- Could author explain a little bit more the following statement? „.....to find out if SSU* ablation is the trigger of the observed translation collapse or rather represents a secondary effect of the gene down-regulation due to depletion of mature mRNA". If the author means that the loss of translation is due to loss of LT mRNAs, what experiment she would suggest to distinguish between the direct effect on mt translation and stability/recruitment of LT mRNAs.

Page 90

- I agree with the author that the specific antibodies are crucial for monitoring the complex composition and integrity, however I was wondering how is she planning to investigate a protein localization (within the 45S SSU*) using the pAb ?

Minor points & technical flaws
These points won't be read during the defense as they are solely for the author to improve her style for a next publication.
The text contains a lot of mistakes and typical flaws include missing citations, spelling errors, not a common theme to one paragraph, abbreviations at the beginning of sentence or title etc.
Unfortunately I have to mention that the text was not easy to read as confusing statements and not-enough explained features were used.

Page 1 – 3rd paragraph about common characteristics of all trypanosomatids
- 41 chromosomes apply only to *T. cruzi* and not to other trypanosomatids
- the organelle description applies only to *T. brucei*

Page 9
- Chapter is called „Mitochondrial ribosome", however the first sentence starts with „Ribosomes of every cell....." - if this should be mitochondrial ribosomes (as title would suggest) then the first sentence is not correct, as not every living cell contains mt ribosomes
- Sharma et al 2003 is incorrect reference as it does not refer to bacterial ribosome, but to mammalian mitoribosome
- A, P and E sites are formed by LSU, not SSU
- If the major FUNCTIONAL features are L1 protuberance, the central protuberance and base stalk, it should be also explained, what are the functions of these features.

Page 10-12 Chapter called Trypanosomatid mitochondrial ribosome
- Mess – if there would be possible to re-write the PhD thesis, this would be a chapter that would benefit strongly from this opportunity
- Figure 7 and others – „adapted" does not mean copied

Page 14 – the mitoribosomes appear in six different ribonucleoprotein complexes –In the second paragraph I have counted only 5 (LSU dimer, monosome, monosome-dimer, 45S and 45S dimer), which one is missing ?

Page 16 3rd paragraph
- The paragraph explains a translation initiation in bacteria, however in the 4th sentence, author suddenly mentioned mitoribosome, is that correct ?
- Missing citation for Charriere et al., 2005, JBC
- IF1 and IF3 have not been detected in T. brucei genome based on a homology search, it is too strong conclusion, that those two factors are lacking

Page 17
- A cap on the 5'end helps to recruit mRNA to CYTOSOLIC ribosome – I am not sure why this is mentioned here
- 5'end of mRNA is always phosphorylated, mt mRNA do not contain caps
- Poorly described section
- 3rd paragraph – what does recycling mean? – I doubt that it means that newly created protein is released and incorporated into the membrane as described in this paragraph

Page 19
- Tb.927.5200, Tb927.4930 and Tb927.1793 are incorrect gene IDs

JBC paper

- This paper represents important study that opens an exciting avenues to study translation initiation, regulation and its tight connection to mRNA expression. In addition what is presented in this paper, the author performed the same experiments using different time points after RNAi induction. I was wondering if it would not be more beneficial to choose just two time points, but repeat the individual experiments at least twice to be sure about the reproducibility of the experimental procedures (especially the gradient sedimentations, northern blot analysis to detect LT and ST versions of mRNA and qPCR)

Page 43
- What does it mean that the function of 2650 and 2710 remain unchanged ?

Page 47 – 48
- graphs are not labeled, labeling within the graphs is wrong (everywhere is listed PPR29)

Page 51-52
- Different labeling, very confusing!

Page 57
- confusing labeling, the axes are not the same, difficult to compare the figures with each other

Page 68
- Wrong picture of the plasmid used. The author used pLEW79MHTAP vector, which does not contain the luciferase gene.

Page 71
- KRIPP10 – 86.5 Kda, however on a next page is stated that KRIPP10 is 96.6 ? Which information is correct ?

Page 72
- How the mitochondrial lysates were prepared ? Without a loading controls no conclusion should be made regarding the targeting of the proteins of interest to mitochondria.

Page 75
- I have never seen that somebody would induce bacterial cells for 24 hours and expect that the protein of interest would be soluble

Page 85
- RNA interference is not a tool to monitor effect of silencing genes, it is a tool to silence gene expression

In conclusion, I recommend this thesis to be defended by Lucie Ridlon on Sept 18th, 2014.

In Ceske Budejovice, 11.9.2014

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Review of PhD Thesis

Author: Lucie Ridlon

Title: Functional analysis of newly described 45S SSU* complex in *Trypanosoma brucei*

Supervisor: Prof. RNDr. Julis Lukeš, CSc.

Co-supervisor: Prof. Dmitri Maslov, PhD.

Topic of the presented Thesis is very interesting. Mitochondrion of trypanosomatids is full of surprises and 45S SSU* complex is without any doubts one of them. Results presented in the work are novel and valuable. Presented data suggest the diversity of regulation of expression of mitochondrial genes and participation of the 45S SSU* complex in this process. Although the function of this complex is far better understood, presented results are essential steps to its knowledge.

Experiments are performed very precisely and final figures in already published paper as well as in part describing unpublished results are on excellent level. Very clear line at which the results are presented, however, quite strongly contrasts with the way the rest of the theses is written.

First what comes to the eyes of referee is non-standard layout of the work. Unpublished results are not presented as a whole, but are divided (I would say even broken) into individual experiments; list of abbreviation is inserted even after References, description of number of Figs is not sufficient for their proper understanding, ... My overall impression is that the work was written in a hurry, and/or there was not paid enough attention to its writing.

I have found several inaccurate statements already in Introduction. I show only two examples and am not going to describe everything (I indicated some more of them directly in the printed Thesis).

- As one of a common characteristic of all trypanosomatids is ..."single nucleus with 41 pairs of chromosomes" (page 1, following citation is not written properly either).

- Very similarly Fig 4 (page 6) seems be a typical representative of of the kp DNA to all trypanosomatids

So my first question is. Could you indicate how many chromosomes have the most studied trypanosomatid species and shortly characterized their kinetoplast DNA (common features and differences)?

There is comparison of bacterial and mitochondrial trypanosomal ribosomes. But there is no data about other mitochondrial ribosome. Why you didn't compare them with mitochondrial ribosomes from other organisms (e.g. yeasts and/or mammals) that should be more similar than bacterial? Could you shortly compare it now?

I see even as a larger defect the way how unpublished results are presented. Each method/experiment is presented as a separate unit. This part seems to be more like manual of different techniques that are illustrated with some outputs than results in PhD thesis. I see no connection between them. Discussion could partially corrected this deficiency, but it is more like result summary than real discussion about the Lucia's achievements. So I strongly recommend Luci to prepare her defense as a one story where her results would be as a mosaic of overall picture of 45S SSU* rather than current image of a number of unconnected data.

Here I have last questions. Lucie concludes on page 83 that product of genes and Tb11.02.2710 are essential for PS *T. brucei*. What is the base for that conclusion if grow curves shows only small (in case of Tb927.8.2650) or almost no phenotype (in case of Tb11.02.2710)? How do you explain the discrepancy, that drastic destruction of 45s SSU* complex after induction has so undetectable effect on cell growth? Especially if similar strong effect in the genes published was always accompanied with strong growth phenotype

Absolvent of PhD study should be able independently perform standard laboratory experiments as well as to process and evaluate obtained results. Lucia has demonstrated the ability to carry out very well also quite tricky experiments. I am sorry, that I couldn't find the same perfection in the way how she has written assessed Thesis. I strongly believe, that she convinces us during her presentation, that she is in her results well versed and that she is able correspondingly to discuss them. That is why I recommend her work to the defense. After successful defence she will be eligible to obtain rank of Doctor Philosophy - PhD.

12. September 2014

Assoc. Prof. Anton Horváth, PhD.

In the last few years much progress has been made in understanding the various mechanisms by which ribosomes engage in protein synthesis and to a great extend many of the steps of translation have been clarified. This progress has been made possible by the elucidation of ribosome structures from a diverse group of organisms, but most prominently bacteria. Despite all this, little is still known about the nature of the mitochondrial ribosomes and we are still ways away from establishing a mitochondrial in vitro translation system. Part of the complication has been the inability by many researchers to purify intact ribosomes, despite many efforts. The present thesis is significant in that for the first time trypanosomatid ribosomes have been isolated and as expected they offer several nuances that go great length in telling the story of the possible evolution of the mitochondrial translational systems.

A big puzzle in the trypanosomatid mito ribosomes has been how is it possible that the ribosome assembles into a functional particle when many of the conserved domains in rRNA are missing. One suggestion is that in such cases the function of such domains has been taken over by proteins, thus in a way the trypanosomatid mitoribosomes harbor the minimal rRNA sequence information required for translation. Overall this is a very well written thesis that definitely would meet the standards of a PhD-level thesis at my own institution.

I just have several questions (below) that can be address following the oral presentation of the thesis.

1. Explain the difference between "similarity" and "homology"

2. On page 9, you write that the "prokaryotic ribosome is 70s in size".
What is the size of an archaeal ribosome, the size of a bacterial ribosome and explain what is the meant by "prokaryotic"?

3. On the same page you provide two models for the origins of mitochondria, both involved a very established process of endosymbiosis. However, the difference between the two models is on the origin of the proto-eukaryal cell.
Please explain the pros and cons of the two models with emphasis on the origin of the eukaryotic nucleus

4. Lastly, on the ribosome structure on page 15, one of the most conserved ribosomal proteins is RPS12. In trypanosomatids this protein is encoded in the mitochondria, posing a potential conundrum: a protein that may serve an essential function in translation still has to be made by ribosome that potentially lacks it. Please speculate on how would the mitoribosome achieve this why obviating the need for S12.

JUAN ALFONZO