University at Buffalo
The State University of New York
Department of Microbiology and Immunology

Laurie K. Read, Ph.D.
Professor
Phone: (716) 829-3307
Email: lread@buffalo.edu

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Review of Ph.D. Thesis of Jiří Týč
"Kinetoplastids biology, from the group phylogeny and evolution into the secrets of the mitochondrion of one representation: *Trypanosoma brucei* – the model organism in which new roles of evolutionarily conserved genes can be explored"
Reviewer: Dr. Laurie K. Read

This is an outstanding thesis, which represents valuable contributions to two very disparate areas of the biology of kinetoplastid protozoa. The first is a global perspective on the phylogeny of monoxenous trypanosomatids that represent a huge increase in our knowledge of the diversity, geographic distribution, and host specificity of this group of organisms. The second major area addressed is the molecular biology of mitochondrial DNA replication in the human parasite, *Trypanosoma brucei*. Minor chapters also touch on proteins required for mitochondrial translation and mtHsp70 solubility. The candidate has also contributed to two review articles that span these diverse areas, and which constitute chapters in the thesis. As such, this thesis is exceptional in the breadth of knowledge that it covers.

Introduction (Chapters 1-4)
The Introduction provides sufficient information to understand all chapters of the thesis. It incorporates a large number of relevant references.

Question: Regarding section 1.6.2: Are mtHsp70, mtHsp40, and or Mge1 typically found in stable complexes? If so, is this their primary organization or are any of these proteins present outside these complexes? Do cytosolic and bacterial homologues exhibit a similar organization?

Paper 1: Growing diversity of trypanosomatid parasites of flies (Diptera:Brachycera): Frequent cosmopolitanism and moderate host specificity

To address the phylogenetic distribution, host specificity, and geographic distribution of parasitic trypanosomatids, 40 new isolates monoxenous (insect only) parasites were collected from Dipteran hosts from nine countries on four continents. Isolates were typed using small subunit rRNA genes. Strengths of the study include the very wide geographic distribution used and the restriction of samples to non-predatory dipterans, which confined the flagellates under study to those found in brachyceran flies. Several significant findings were made. First, 24 new Typing Units that may represent new species were identified. Additionally, the authors found strong host specificity at the genus level and determined that brachyceran parasites from geographically distant locations are more highly related to each other than they are to trypanosomatids from heteropterans from the same location. Another important contribution is the attempt to cultivate these 40 new trypanosomatid isolates. Not surprisingly, many could not be cultured; however, eleven isolates were successfully introduced into axenic culture. This effort now sets the stage for future in depth studies, including whole genome sequencing.

Question: Understandably, the media used for cultivation was "designed for the broadest range of parasites". Using the knowledge you have gained here, would it be possible to design more specific media that might permit the growth of specific isolates? Can you speculate on some media components that you would modify in an attempt to better support some of the isolates that have so far eluded culture?

Paper 2: Mitochondrial heat shock protein machinery Hsp70/Hsp40 is indepensible for proper mitochondrial DNA maintenance and replication

Here, the candidate uses *T. brucei* to dissect the functions of mitochondrial proteins that are highly conserved among eukaryotes, but whose functions are not well understood. *T. brucei* is especially well suited to such studies because of its robust RNAi machinery. The subject of the present study is the role of mitochondrial heat shock proteins (mtHsp70, mtHsp40, and their co-factor Mge1) in mitochondrial DNA maintenance and replication. Again, *T. brucei* is an excellent system due to its single mitochondrion that replicates just once during the cell cycle. Both cytoplasmic and bacterial homologues of mtHsp70 and mtHsp40 have been implicated in DNA replication. Here, the candidate uses RNAi-mediated knockdown of *T. brucei* mtHsps and their co-factor followed by detailed analysis of mt DNA to show that these proteins are indispensible for mtDNA maintenance and replication, with a prominent effect on the maxicircle component of the mtDNA network. Strengths of the study include the use of more than one clonal line for analysis of mtHsp70 and mtHsp40, the combination of multiple ultrastructural and biochemical means to analyze the phenotypes of knockdown cells, and the rigorous attempts to exclude secondary phenotypes. The chapter would be strengthened by inclusion of a model figure illustrating the proposed biochemical functions of the mtHsp70/40 machinery in mtDNA maintanence and replication. Overall, the studies here constitute a significant contribution to our understanding of mtDNA replication in kinetoplastids. Due to the conserved nature of the mtHsp70/40 machinery they likely have relevance in higher eukaryotes as well.

Question: What happens to the abundance and localization of the other two proteins when one is knocked down? If not known, what would you speculate?

Question: Can you elaborate on your model for the roles of mtHsp70/40 machinery in kDNA replication based on your data? What do the accumulation of covalently closed replication precursors, nicked/gap replication products and oligomeric minicircle free catenes tell you about the potential biochemical functions of mtHsp70/40? How does the decrease in maxicircle DNA upon knockdown of these proteins factor into your model? If you were suddenly given a million dollars ($24.5 million coruna!) to continue work on this project, what experiments would you do next?

Question: The careful attempts to ensure that the observed defects in mtDNA replication are primary effects of mtHsp70/40 knockdown is a major strength of the current study. RNAi is a very commonly used technique in trypanosome biology. In general, can you elaborate on the approaches a researcher can take to determine if what they are measuring is a primary or secondary effect of knockdown of their target protein? Are there any other experiments you could do to further test that the effects you see on kDNA replication are primary effects of mtHsp70/40 knockdown?

Manuscript 1: RSM22, mtYsxC, and PNKD-like proteins are required for mitochondrial translation in *Trypanosoma brucei.*

Little is known about mt translation in kinetoplastid flagellates, and here the candidate presents data pertaining to three mt proteins with links to the ribosome and that are conserved between humans and kinetoplastids. These studies are in progress, but when completed will significantly add to our understanding of the understudied mt ribosomes in *T. brucei*. He shows that the proteins are essential for growth under conditions where the cells need a fully functional mitochondrion to survive, and that mt translation is compromised when these three proteins are knocked down. Sedimentation profiles of rRNAs provide strong evidence that RSM22 is critical for stability of the mt ribosomal small subunit. As expected, the levels of mt RNAs do not significantly change upon depletion of these proteins. However, there is an unusual finding with respect to the positive control in Figure 4.

Question: Why does mtRNAP depletion cause a decrease in EDITED Cyb and RPS12 RNAs but no change in pre-edited levels (Fig. 4)?

Manuscript 2: Aggregation of the Hsp70 chaperone in the mitochondrion of *Trypanosoma brucei*

In this chapter, the candidate shows that mtHsp70 solubility is temperature dependent and is also influenced by the presence of the known chaperone Hep1, as well as Mge1 and mtHsp40. He also makes

the interesting finding that procyclic form *T. brucei* can recover their normal growth rates even at 34°C.

Question: What percentage of total mtHsp70 is found in complex with Hep1? Does Hep1 bind to mtHsp70 concurrently with Mge1 or are these interactions mutually exclusive?

In sum, this thesis document and the work presented therein would fulfill the requirements for the Ph.D. degree at the University at Buffalo School of Medicine and other similar institutions for which I have reviewed, and would rank in the top 20% of such Ph.D. theses.

Sincerely,

Laurie K. Read, Ph.D.
Professor

May 11, 2015

Review Report for a Ph.D. Thesis submitted to the Committee of PhD studies in Molecular and Cell Biology and Genetics of the Faculty of Sciences of the University of South Bohemia.

Title: Kinetoplastids biology, from the group phylogeny and evolution into the secrets of the mitochondrion of one representative: *Trypanosoma brucei* – the model organisms in which new roles of the evolutionary conserved genes can be explored.

Author: Jiří Týč.

Dear Prof. Miroslav Obornik, Head of the Committee

I kindly accept the invitation to review the Ph.D. thesis submitted above by Jiří Týč and in this document I provide 1) general comments, 2) specific comments and 3) evaluation statement.

I. General Comments:

The topics developed in the thesis of Ph.D of Jiří Týč addresses important questions regarding the diversity and evolution of parasitism in trypanosomatids and the function of mitochondrial proteins, highly conserved among eukaryotes, which are involved in the maintenance and replication of the organellar DNA and its translation machinery using as a model the parasitic protists *Trypanosoma brucei*.

The introductory part of the thesis has an extensive and complete background summary of the literature and gives the reader a good perspective for the easy understanding of the objectives, results and discussions. However, probably it should underline a bit more strongly the importance and justification of the objectives pursued and emphasize the strength and appeal of this work to the reader (see specific comments). The objectives are well delineated and the summary of results and discussions are in agreement with the provided body of data except in very few punctual cases where apparent discrepancies between results would suggest a bit more cautious argumentation (see specific comments). In relation to the manuscripts in preparation, although the data is in preliminary stage the inclusion of statistical values in some of the experiments might be necessary to define their significance and the proper legend description for some figures should be included (see specific comments).

Considering the wide scope of the thesis, it achieves to add important contributions to the growing body of knowledge in several fields of biology ranging from areas like protistology and parasitology to evolution and cell biology. Indeed, the importance of the findings exposed on the present thesis have been already recognized by the scientific community as proven by one research paper and a review published in peer reviewed journals for the studies regarding the diversity of monoxenous trypanosomatids, and a book chapter and a research article dedicated to the findings regarding the importance of the mitochondrial heat shock protein machinery Hsp70/ Hsp40 (mtHsp70/ mtHsp40) in the maintenance and replication of the kinetoplastid DNA (kDNA). Regarding the functional studies of highly conserved proteins in eukaryotes, two manuscripts are in preparation that being at preliminary stage already show promising results.

II. Specific comments:

1. Regarding the introductory part of the thesis, there is a good background history on the phylogeny and importance of the studies of trypanosomes in section 1.1.2 and/or 1.2 that could be used to introduce the relevance of understanding the diversity among monoxenous trypanosomatids, therefore emphasizing the importance of the present work. Similarly, sections 1.3-1.6.2 have an extensive bibliographical revision, however, this information could be presented in a way that emphasizes the specific topics which are to be introduced and studied, i.e, section 1.5.2 could be used to introduce the PNKD-like, RSM22 and mtYsxC putative ribosomal proteins.
2. Regarding the research article "Mitochondrial Heat Shock Protein Machinery Hsp70/Hsp40 is Indispensable for Proper Mitochondrial Maintenance and Replication", although in the discussions it is proposed that the monoclonal antibody against mtHsp70 used in this work might not recognize a possibly modified isoform that localizes to the kDNA, how to explain the lack of specific colocalization of the tagged version of mtHsp40 to the kDNA?
3. Regarding the manuscript I titled "RSM22, mtYsxC and PNKD-like proteins are required for mitochondrial translation in *Trypanosoma brucei*", the difference in growth phenotype shown in figure 1, A-F for the knock-down cell lines is difficult to judge having different values on the Y-axis. Is there really a more pronounced effect of the knock-down effect in the absence of glucose? Any statistical value to support this observation? The graphs shown in the figure are a representative of different experiments? Why the results of qPCR for PNKD-like are shown up to day 3 and not to day 6 like RSM22 and mtYsxC? It would be nice to include the p value in these qPCR experiments given the variations of the mRNA expression observed for RSM22 and mtYsxC.
4. On the same manuscript I, figure 3 shows the reduction of mitochondrially encoded subunits of respiratory complexes III and IV by 2-D electrophoresis analysis in the knock-down cell lines for PNKD-like, RSM22 and mtYsxC. It would be helpful to include measurements of their respective specific activities to support these observations.
5. In figure 4 and 5 there seems to be a discrepancy regarding the threshold value upon which a results is considered significant. In figure 4, approximately 30% decrease in the value for the Pan-edited RPS12 mRNA in the PNKD-like knock-down seems to be insignificant while in figure 5, a 30 or 35 % decrease in value for the 9S and 12S rRNA, respectively, seem relevant, why? For example, if treated with the same level of significance, the results of figure 4 might indicate that the PNKD-like protein could have a specific effect on the abundance of pan-edited mRNA. Figure 5 shows that RSM22 affects both the levels of 9S and 12S. Why it is suggested to interact only with the SSU and not with the LSU as mtYsxC?
6. Regarding the manuscript II titled "Aggregation of the Hsp70 chaperone in the mitochondrion of *Trypanosoma brucei*", in the aggregation assay of "Material and Methods" section the part where is written "(10 µl of buffer per mitochondrion purified from 1 x 10^7 cells)..." is not clear. In my opinion, it would be easier to express the relation of detergent: mg protein or the total protein amount included in 10 µl of the assay.

7. An increase in temperature translates into higher entropy in the system, in this case inside the cell, and as a consequence higher denaturation rate of proteins in general. This raises the question whether the observed aggregation of mtHsp70 is with itself or with other denatured proteins. Oligomers resulting from self-aggregation could be distinguished by density gradients, gel chromatography or and/or western blots from native gels.
8. On the same manuscript II, figure 1 does not include in the legend how much sample was loaded for the analysis nor is it written in the "Material and Methods" section. Also on figure 1, why the western for the cells treated at 27 °C at 6 days is missing?
9. Likewise, figure 2 of the same manuscript does not have a description for the difference between solid and dashed lines. It's a bit confusing. In figure 3, the aggregation of mtHps70 in the mtHsp40 knock-down is more pronounced at lower temperature which seems contradictory, is this result reproducible and, if so, how does it fit with the rest of other results?

II. Evaluation:

The present Ph.D thesis submitted by Jiří Týč have bring about clear evidence of the unexpected diversity of monoxenous trypanosomatids by defining at least 24 new typing units (TU) which could potentially be new species. Furthermore, his work has shown for the first time the higher host specificity of monoxenous trypanosomatids, at least at the genus level, compared to those of hemipterans. In the second part of his work, he has successfully proven the importance of the mitochondrial chaperones Hsp70/ Hsp40 for the correct maintenance and replication of the kDNA and has produced exciting data that further contributes to widen our knowledge on the biology of the unique mitochondria of trypanosomatids which could be translated in the future into potential new chemotherapeutic targets against this group of important parasites.

Based on these observations, I sincerely recommend the permission for the defense of the Thesis for Ph.D. to be granted to Jiří Týč. The suggestions given in this review are not mandatory, but can be accommodated in a possible revised version in case one is made.

I feel deeply honored and appreciate the invitation to act as external reviewer for this valuable piece of work.

Sincerely,

Jorge Morales, Ph.D.

Emmy Noether-Group "Microbial Symbiosis and Organelle Evolution"
Heinrich-Heine-Universität Düsseldorf
Universitätsstr. 1; Gebäude 26.12.01; Raum 70
40225 Düsseldorf, Germany.
Tel: +49 0211 81-13582/13425