Comments on Ph.D. thesis, "Aspects of RNA editing in *Trypanosoma brucei*" by Mohamod Hassan Hashimi
Laurie K. Read, Ph.D.

This thesis addresses several aspects of mitochondrial gene regulation in the kinetoplastid parasite, *Trypanosoma brucei*. It represents a large amount of high quality work, and is distinguished by the breadth of its scope. The research in this document ranges from molecular aspects of RNA processing, to physiology, to evolution. Mr. Hashimi does a nice job of tying all of these aspects together.

Chapter 2

In Chapter 2, the authors report a study of a mitochondrial protein termed, TbRGG1. TbRGG1 was previously implicated in RNA editing by its affinity for poly(U), and thus gRNAs, as well as by its association with high molecular weight complexes. Here, TbRGG1 is depleted in procyclic form (PF) *T. brucei* by tetracycline (tet)-regulated RNA interference and the effect on mitochondrial RNAs examined. Associated proteins are also identified in an effort towards understanding the protein's function. The strengths in include identification of some function for a novel trypanosome protein, as well as identification of a newly described multiprotein complex (put-MRB) that likely plays multiple roles in mitochondrial RNA metabolism. One weakness is the inability to discern from the data whether TbRGG1 affects RNA editing or stability. TbRGG1 is shown to be essential for PF growth. Analysis of mitochondrial RNAs reveals substantial decreases in all edited RNAs examined, suggesting a defect in editing or stability. There is little or no change in the corresponding preedited RNAs, although the authors mention they are increased. This brings up a point for discussion: what is expected regarding the preedited RNAs if the defect is in editing or stability? Does one necessarily expect an increase in preedited RNAs in the face of an editing defect? Might this differ for different RNAs and/or for the different targeted protein factors? What is observed for preedited RNA abundance upon downregulation of other editing related proteins, and if it differs between different factors, why might that be? Also following on these data, what future experiments could be performed to determine the precise role of TbRGG1 in mitochondrial RNA metabolism? Myc/His/TAP (MHT)-tagged TbRGG1 is expressed for purification of associated proteins. Cells expressing MHT-TbRGG1 presumably overexpress the protein. Was the level of overexpression ever checked by anti-TbRGG1 western blot? What do you predict would happen to mitochondrial RNAs upon TbRGG1 overexpression and do you think this would be informative? Why or why not? Mass spectrometry analysis of proteins associated with MHT-TbRGG1 revealed a large number of proteins including two that are related to each other and were previously suggested to be involved in RNA editing (GAP1 and 2; Chapter 3). By reciprocal tagging, 14 proteins were identified in common with TbRGG1 and GAP1/2. These did not include any proteins from the core editing machinery. TbRGG1 is associated with GAP1/2 by an RNA linker. It will be of interest to hear how one would more completely sort out the composition and interactions between components of the put-MRB. Finally, is there a (highly speculative) model for the point(s) in the lifetime of a mitochondrial RNA at which it associates with TbRGG1?

Chapter 3

Chapter 3 follows directly from identification of TbRGG1 associated proteins in Chapter 2. Here, the authors examine four subunits of the MRB complex that is associated with

TbRGG1, each of which are essential in PF. Strengths include the large amount of work that represents the first insights into the functions of these four proteins. The potentially unavoidable weakness is that it is difficult to form a coherent model from the disparate phenotypes in the knockdown cell lines. The authors show that tet-regulated depletion of GAP1, GAP2, and RNA helicase 1500 each leads to almost complete absence of gRNAs. Interestingly, GAP1 and GAP2 proteins are mutually dependent on each other for stability, but the helicase is not. What does this suggest about the organization and coordinated function of these proteins? A fourth subunit, Nudix hydrolase, does not affect gRNA abundance. With respect to mitochondrial mRNAs in cells depleted for each protein, depletion of GAP1, GAP2, and RNA helicase 1500 each leads to substantial decreases those edited RNAs whose editing is specified by maxicircle-encoded gRNAs, with increases in the corresponding preedited RNAs. Again, an interesting question arises regarding the relationship between the edited and preedited mRNAs. For example edited CO3 RNA is decreased similarly in all three lines, but preedited is up 20-fold in GAP1/2 lines but only 2.5-fold in the RNA helicase line. What could be the explanation for this and, what might it say about the mode of action of GAP1/2 vs. that of RNA helicase 1500? In contrast, depletion of the Nudix hydrolase leads to a general destabilization of almost all mitochondrial RNAs. Thus, depletion of either TbRGG1 (Ch. 2) or Nudix hydrolase leads to apparent RNA instability. Although the set of target RNAs differs somewhat, is there any indication that these effects are manifestations of the same pathway? How could the similarities or differences in the mechanisms by which these two proteins stabilize mRNAs be addressed? Next, the effect of these knockdowns on the total gRNA population is examined. GAP1/2 and RNA helicase 1500 depletion all lead to a decrease in the abundance of gRNA, while Nudix hydrolase depletion has no effect. It also appears in Figs 4A and 4B that the gRNAs in the induced cells may be a bit shorter than those in uninduced cells? Was this reproducible or is it simply an artifact of the decreased overall signal? GAP1/2 sediment in glycerol gradients at much lower S values upon disruption of the RNA helicase but not the Nudix hydrolase. What is the MRB1 model for interactions between these proteins, taking into account these glycerol gradient results? What RNAs would you expect to immunoprecipitate with each of these proteins, and do you think those types of investigations would help in developing the model? Additional elegant *in vivo* evidence for association of GAP1/2 with gRNAs is provided by depletion of gRNAs through mtRNAP depletion. This is also the first demonstration that the same polymerase that transcribes maxicircles also transcribes minicircles. Interestingly, indirect immunofluorescence reveals a punctate distribution of GAP1 in the mitochondrion. It could be very informative to determine whether additional MRB components have similar distribution. Finally, GAP1 and GAP2 are essential in BF and their depletion in this stage leads to a loss of gRNAs, as in PF, indicating conserved function in the two life cycle stages. The authors conclude that the GAPs and RNA helicase appear to be involved in some aspect of gRNA processing and/or stability. How would you go about investigating and distinguishing between these two possibilities?

Chapter 4

Chapter 4 turns to investigation of *Trypanosoma* spp. that are lacking part or all of their mitochondrial DNA (*T. equiperdum* and *T. evansi*). These species are termed dyskinetoplastic or akinetoplastic, respectively. Work in this chapter leads to the conclusion that those trypanosome lines previously designated as distinct species should be classified as *Trypanosoma brucei* subspecies, and that these cells are petite mutants of *T. brucei*. Inclusion of evolutionary aspects of the work is a strength of this thesis. The maxicircle and minicircle complements of a dozen

different strains were examined, and minicircle homogenization was demonstrated for several strains. Editing of maxicircle genes was examined in a subset of these stains and was confined to those that utilize maxicircle encoded gRNAs. Compensation for the lack of A6 RNA editing was shown to be due to mutation or deletion of one allele a nuclear-encoded ATPase subunit. Interestingly, 90% of mRNAs encoding this protein were transcripts of the mutant allele. How is it thought that this is achieved or regulated? Next, it was shown that even in strains that lack both minicircles and maxcircles, multiple nuclear encoded proteins involved in editing and minicircle replication were still efficiently imported into the mitochondrion. Was it investigated in any case whether these genes were mutated in a manner that predicts they will be non-functional? Would you expect this to be the case? Why or why not? Finally, a striking result is that electron microscopy shows that kinetoplast structure is maintained in dyskinetoplastic strains? Is there any indication of how this is achieved or how the absolute number of minicircles in controlled?

Chapter 5

In Chapter 5, the authors turn to the physiological consequences of editing. Specifically, they analyze several knockdown cell lines deficient in editing to provide indirect evidence that the protein product of edited ATPase subunit 6 mRNA is incorporated into complex V. The connection of the molecular aspects of editing in Chs. 2 and 3 with the physiological consequences adds significantly to the well-rounded nature of this Ph.D. thesis, and the approach used to address difficult but important question is creative. Here, Mr. Hashimi uses in gel ATPase assays and western blotting are used to identify a 2700 kDa complex V oligomer and a 900 kDa F1 dimer. Knockdown of the nuclear encoded A4 subunit lead to loss of both complexes (thus, defining them as ATPase complexes) and slow growth of PF. It is unclear, though, why the growth phenotype is so modest in the absence of the ATPase. To indirectly assess the role of ATPase subunit 6 in ATPase assembly and function, the core editosome KREPA6 subunit is depleted (which abolishes editing of all RNAs, and thus the ATPase 6 protein). The MRP2 knockdown, which affects editing of some RNAs not including ATPase 6 RNA, is included as a very nice negative control. In the absence of ATPase subunit 6 protein, the abundance of complex V is greatly reduced and F1 dimers accumulate, as shown by in gel activity assays and western blot. To provide additional evidence that it is ATPase 6 in particular that is disrupted, 2D gels are performed to show that the ATPase 9 ring in absent in the KREPA6 knockdowns. This is quite conclusive and very exciting, providing important, albeit indirect, evidence for the function of protein products from pan-edited RNAs. Similar results are demonstrated for naturally occurring akinetoplastic cells, providing additional confirmation for the conclusions and nicely tying in studies in Chapter 4.

In summary, this body of work meets and exceeds the requirements for the Ph.D. degree.

Ph.D. Thesis: Aspect of RNA editing in *Trypanosoma brucei*
Mir Mohamod Hassan Hashimi

Evaluation report

I have critically read the thesis **"Aspect of RNA editing in *Trypanosoma brucei* "**, which was submitted for defense to obtain a Ph.D. degree by Mir Mohamod Hassan Hashimi. I believe that the thesis is of extraordinary quality, bringing a number of original new information about biology of trypanosome.

The experimental part of the work is preceded by an introductory chapter, from which it is apparent that candidate not only gain a deep knowledge in the filed of molecular and cell biology of the parasite but also that he is skilled to synthesize information to a compact logical text. The introduction is very well written (I learnt a lot) and it should be published as an independent contemporary review. The experimental results are embedded in four papers from which two were published in high rank journals (RNA, PNAS), the third was accepted and will be published soon in RNA, and the fourth is in stage of manuscript which will be certainly published soon as well. All accepted papers are of impressive quality, the last ms may need some minor trimming before submission.

To demonstrate that I did my job, some statistics: the thesis consists of 139 pages, the introduction itself is written on 46 pages with 13 Figures, covering 157 references. Most importantly, the paperback issue I have weighs 654 grams.

Comments and questions

Chapter 1.

Abbreviations
Some people love abbreviations, the people with bad memory like me hate them. I would avoid especially non-standard abbreviation such as "ak", "dk" , "KRAP, KREL, KREN, KREP, KRET, KREX": It is not easy to remember meaning of all these abbreviations. HAT …the usual rule is to create abbreviation if it is used in the text more then 5x.
A,C,G,T – is it necessary to explain?

Page 1.
The paper by Cavalier-Smith supporting your statement that Kinetoplastida is "…one of the earliest diverging eukaryotes with typical mitochondria" is more then 10 years old. Is there any new evidence supporting early branching history of Kinetoplastida? What is your opinion on Kinetoplastida evolution? What do you mean by "typical mitochondria"?

We all are using how certain unique feature of the parasite may uncover potential drug target. (page 5). Do you know any nice example of antiparasitic drug designed on such a rational approach?

Page 6 Atypical energy metabolism: "…two of the cytochrome-containing respiratory complexes .. are absent (in BS). Does it mean that the other complexes are present?

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Fig 3, Page 9 SSE, UMSBP abbreviations are not explained

Page 9. Upon replication of all minicircles, the remaining gaps are repaired and the network is split into two (refs). It sounds very simple, so what is the mechanism how the network is split?

What is an OB fold? Page 13

Fig 8 the abbreviation should be explained (OB fold, EEP, Pum…)

Page 17, "…in several of the several.."?

Page 20 "While the short tail destabilizes pre-edited molecules, it has the opposite effect in edited RNAs" Why, what is a mechanism?

Page 21
What means "diskinetoplastic" cells? A cell without kinetoplast?

Chapter 2:
In this paper the authors demonstrated involvement of TbRGG1 in mit RNA metabolism, most likely in RNA editing. Moreover, they found that TbRGG1 is a member of novel protein complex and they studied the complex by mass spectrometry.

To this part I have two questions: Page 971 **"The positively charged arginines are thought to electrostatically interact with the negatively charged amino acids, thus facilitating RNA binding." Could you explain in more detail character of this RNA –protein interaction?**

Page 973, "These two proteins were identified in the sample with high peptide coverage (data not shown)." I would like to know more about these "data not shown". What do you consider as high protein coverage? What were the criteria to distinguish between contamination and the protein of the complex? When you compare protein composition of the complex identified by Panigrahi et al., and Hashimi et al., did Panigrahi's complex contain proteins which were identified but excluded based on your criteria? Did you consider level of gene expression, which might be similar for the members of the same complex?

Chapter 3:
This part extended previous work in direction to investigate a role of several subunits of the putative MRB1 complex, they showed that they are essential for the growth of procyclic trypanosomes and required for minicircle-encoded guide RNA-dependent RNA editing. When I compare growth curve in previous paper page 971 and those in Fig 1, **I wonder how many times the cells were subcultivated in the first case (page 971), or did you induced RNAi at time 0 and then the cells were cultivated in the same tube for 14 day reaching density 10^{13} cell/ml as showed?**

Chapter 4.
Adaptation of *Trypanosoma brucei* to gradual loss of kinetoplast DNA…

This is very interesting paper which attracted attention of many parasitologists. The concept that dyskinetoplastic trypanosomes are ancestors of *T. brucei* has been suggested in various studies previously. The novelty of this paper is that *T. equiperdum* and *T. evansi* are rather recent derivatives and that the transition from eukinetoplastic trypanosomes to dys- or akinetoplastic is a recent and perhaps continual process. Thus *T. equiperdum* and *T. evansi* are in fact just a *T. brucei* mutants and not separate species. **How your SL RNA repeats based analysis fits with previous taxonomic or phylogenetic analysis of trypanosoma strains based on AFLP (RFLP), microsatellite, SSU etc. clustering? In some of these studies, *T. equiperdum* and *T. evansi* formed a separate clade.**
Second, the novel concept (at least for me) is that dyskinetoplastic trypanosome possesses kinetoplast with normal morphology. Does it mean that all previous reports showing that dyskinetoplastic trypanosomes have no kinetoplast based on various labelling, but just some dispersed mit DNA, were incorrect (for example "Demonstration of kinetoplast DNA in dyskinetoplastic strains of *Trypanosoma equiperdum*" Science 1976)? Does its means that if kinetoplast is not labelled by Giemsa, these trypanosomes are in fact akinetoplastic?
In "discussion" second page: "…between the organelle and the nucleus in these primitive flagellates." What the author means by "primitive" flagellates?

Chapter 5.
The protein product of pan-edited ATP synthase subunit 6 is…

This is the most fresh manuscript dealing with characterization of ATP6 subunit of ATP synthase, a subunit which seems to be critical for transition of kinetoplastic to dyskinetoplastic organisms.

How did you estimate molecular weight of the uper band in Fig 1 (2700 kDa) ? Fig1B: it is rather difficult to envisage a band of 800 kDa in the first line.

What authors mean that .."LC-MS/MS…is not amenable to identification of the hydrophobic peptides comprizing Fo (data not shown)"?
What is the message of the ultrastructure study (page 9).

In conclusion, the quality of the experimental performance and critical way in which the results and background knowledge have been presented in the thesis meet the criteria for obtaining a Ph.D. award. More importantly, this work by Hassan represents an excellent piece of science.

February 2009

Review of PhD thesis "Aspects of RNA Editing in *Trypanosoma brucei*"
by Mir Mohamod Hassan Hashimi

Reviewer: Achim Schnaufer Feb. 16 / 2009

Recommendation: PhD degree should be awarded

Summary

The candidate's thesis project was aimed at exploring two important aspects of trypanosome biology: (1) the biogenesis of mitochondrially encoded RNA molecules and (2) the consequences for the parasite of perturbation of this biogenesis by experimental means or in naturally occurring trypanosome mutants.

The first project studied composition and function of a recently identified ribonucleoprotein (RNP) complex termed mitochondrial RNA binding complex 1 (MRB1). The second project investigated structural changes of the ATP synthase complex in response to perturbation of mitochondrial gene expression and explored in considerable detail the extent of kDNA loss in various dyskinetoplastic (dk) trypanosome strains and the consequences of that loss for mitochondrial biogenesis and function.

The candidate's thesis provides very important insight into trypanosome biology, evidenced by three publications in high-quality peer-reviewed journals, with a fourth manuscript being under review. Although the precise role of the candidate in each work is not given, the significance of his contributions is evidenced by the fact that he is the first author of three of the studies and second author of the remaining one. The thesis is very well written and clearly structured, methods and interpretation of results are generally sound, and data and concepts are well presented. In summary, the thesis provides ample evidence that the candidate has acquired the ability to carry out independent research and demonstrates the professional maturity adequate for a PhD degree.

Detailed comments

The thesis starts out with a very good and nicely illustrated *Introduction* (Chapter 1) that first reviews life cycle and general aspects of trypanosome biology and the diseases caused by these parasites. It then provides an up-to-date review of energy metabolism and mitochondrial biology of these organisms, does a good job at setting the stage for the studies to follow, and demonstrates that the candidate has acquired an in-depth knowledge of the field. The *Introduction* also discusses to some extent the results of this thesis in the context of recent findings by other labs but this could have been done in considerably more detail, either here or in the *Conclusions* at the end of the thesis (see below).

Chapters 2 and 3 describe, in the form of two published papers, the identification of a novel RNP complex, termed MRB1 by these authors, an analysis of its protein components, and initial functional analysis of five such proteins. As described in the first paper, initial identification of this complex was the result of tandem affinity purification (TAP) of proteins associated with TbRGG1, a mitochondrial RNA

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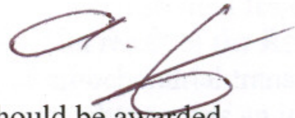
Reviewer: Achim Schnaufer  Feb. 16 / 2009

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binding protein identified earlier by another group. Expression of five components, including the original bait TbRGG1, was knocked down using an RNAi strategy, with the result that all proteins turned out to be essential for cell growth of the procyclic stage of the parasite. Another important result of the RNAi studies was that all knockdowns affected abundance of mitochondrial transcripts, but the species or types of transcripts affected and whether the change was an increase or decrease very much varied between the knockdowns. Very interestingly, three proteins appear to be critical for gRNA biogenesis. These differential effects, together with experiments investigating structural changes of the MRB1 complex in response to individual knockdowns or RNase treatment, suggest that the MRB1 complex is an RNP complex in which individual proteins or smaller complexes are associated via interaction with RNA.

These are important results, in particular since current knowledge about mitochondrial RNA biogenesis in trypanosomes beyond RNA editing is still very limited. The work presented here represents a significant expansion of our knowledge of the important players in this process and, together with recent studies from other groups, opens the door to future studies that promise to be very informative.

The corresponding section in *Conclusions and perspectives* offers a brief summary of these results in the context of related studies published by other labs and makes some suggestions for future directions. I think this would have been a good opportunity for a more in-depth comparison between the various studies that summarizes where the data are in agreement and how conflicting data might be explained and resolved. One example is the effect of the Nudix hydrolase RNAi in this study compared to Weng et al. (2008). Another major point is the heterogeneity of complexes isolated in different labs and Figure 9 in the *Introduction* is a nice attempt at visualizing these differences. The suggestion to investigate direct protein-protein interactions among the various identified proteins via yeast two-hybrid studies is a good one. Points that would have deserved some critical discussion are the reliance on mass spectrometry to determine association of a protein with a given complex and the specificity of the RNA interactions. Are these complexes the result of a dynamic interaction with specific substrates, or the result of random interaction with RNA upon cell lysis? How might one distinguish between these possibilities?

Chapter 4 describes, in the form of a published paper, a detailed analysis of the kDNA content of a substantial number of trypanosome strains from the *T. evansi* / *T. equiperdum* group. The study confirms the dyskinetoplastic status of these strains since they have either, in case of *T. evansi*, completely lost the mitochondrially encoded genes or, in case of *T. equiperdum*, the ability to edit most of these genes as a consequence of gRNA loss. The study then presents partial sequences from the ATPase gamma subunits of four dk strains to suggest that in these parasites, similarly to a laboratory-induced dk strain of *T. brucei*, mutations in this protein may be associated with compensating for the loss of normally essential mitochondrial gene products. The study finally attempts to assess whether *T. equiperdum* / *T. evansi* represent a mono- or polyphyletic group by a comparative comparison SL RNA genes from several strains and concludes that they are indeed paraphyletic and that strains may have developed individually from *T. brucei*.

The detailed analysis of kDNA content from a large number of strains previously identified as *T. equiperdum* or *T. evansi* and confirmation of their dk status is very useful for the field and therefore important. The observation that four of these strains have a mutation in the C-terminal region of the nuclearly encoded ATPase gamma subunit while more distantly related trypanosomes do not is striking but, without functional confirmation or a knowledge of the overall sequence variation between the dk strains and *T. brucei*, the significance of this finding is uncertain. Did the researchers look at the gamma subunit of all the *T. equiperdum* and *T. evansi* strains at hand? Did mutations occur in other parts of the protein as well? What is the general sequence divergence between proteins from the *T. brucei* group vs. *T. equiperdum* and *T. evansi*? Finally, if the dk parasites are indeed paraphyletic and have emerged repeatedly, how can the fact be explained that the various strains appear to share the same remaining minicircle classes?

Chapter 5 describes, in the form of a submitted manuscript, the attempt to confirm that the pan-edited mitochondrial transcript designated as ATP6 indeed encodes that subunit of the ATP synthase complex. The overall strategy was to assess the impact of loss of ATP6 editing, mediated by knockdown of a critical editosome component, on the ATP synthase complex. A cell line with ablated MRP2 protein, which shows decreased abundance of a number of edited and never-edited mitochondrial mRNAs, but not of ATP6, served as a negative control. The study first shows that several forms of the *T. brucei* ATP synthase can be detected on Blue-native (BN) gels, ranging from apparent sizes of 2700 to 900 kDa. Induction of RNAi for 6 days against KREPA6, but not MRP2 converted most of the largest form into the smallest form. Further fractionation of the native complexes by denaturing polyacrylamide gel electrophoresis and analysis with antibodies against F_1 subunit beta and F_0 subunit ATP9 provided convincing evidence that the largest form represents at least a partial F_0F_1 complex whereas the smallest one lacks the ATP9 ring of F_0. ATPase assays carried out with mitochondrial fractions derived from the same cells in the presence of specific ATPase inhibitors supported these conclusions. BN gel analysis of dk cells detected only the smallest form of the complex.

This study addresses an interesting problem regarding the products of mitochondrial gene expression in these organisms. So far, only very few products of mitochondrial translation could be detected directly. Products of pan-edited transcripts, including ATP6, have remained elusive. The candidate has chosen an innovative and technically challenging approach to provide additional evidence that the ATP6 mRNA is indeed translated into that subunit of the F_0 moiety of the ATP synthase complex. The work does provide compelling evidence that knockdown of RNA editing results in conversion of an F_0F_1 complex into F_1 and this is indeed consistent with RNA editing being necessary for synthesis of a protein critical for F_0 assembly. One potential problem of the study is the choice of the MRP2 RNAi cell line as control. The rationale for this control only holds if a lack of an effect on ATP6 editing is the only difference between this and the KREPA6 RNAi cell line. However, this is clearly not the case, illustrated, for example, by the fact that the former cell line shows a less severe growth phenotype upon RNAi induction. This makes it quite problematic to directly correlate loss of edited A6 mRNA with conversion of F_0F_1 into F_1. Interestingly, MRP2 RNAi resulted in a more pronounced increase of F_1-associated ATPase activity than KREPA6 RNAi. Nonetheless, the study provides compelling additional evidence that kDNA encodes a critical subunit of the F_0 subcomplex.