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Habilitation thesis "Unique and conserved features of the *Trypanosoma brucei* mitochondrion" by Mgr. Mir Mohamod Hassan Hashimi, Ph.D.
Evaluation report Prof. Jan Tachezy

It is a pleasure writing an evaluation report for habilitation thesis by Hassan Hashimi. His collection of research papers that were published during last 10 years, represents the major contribution to the understanding of biology of *Trypanosoma brucei*, particularly to gain insight into specific functions of their unusual mitochondria. The core 13 articles are focused on RNA editing, a fascinating process that was discovered in 1986. Hassan is the first author of highly cited article, in which the mitochondrial RNA binding complex 1 (MRB1) was described (Hasshimi et al., 2008). The composition of MRB1 and function of certain MRP1 subunits is systematically analyzed in detail in following 7 original articles, and the results are summarized in 4 reviews and a single book chapter in "Structures and organelles in pathogenic protists". This part of Hassan's comprehensive research activities is certainly sufficient for writing the habilitation thesis, however, the thesis continues with 4 papers on dyskinetoplastic forms of *T. brucei*, another fascinating field of trypanosome biology. It these papers, I particularly appreciated molecular analysis of various forms of kinetoplast DNA during the kinetoplast-to-akinetoplast transition process and consequences concerning definition of *T. brucei* species versus subspecies. Next, there are two papers dealing with mitochondrial translation. Hassan is the first author of very interesting paper showing insensitivity of *T. brucei* mitochondrial translation to tetracyclin due to extreme reduction of ribosomal SSU. The inhibitory effect of tetracyclin on mitochondrial translation in other eukaryotes compromises widely employed experimental system based on tetracycline-controlled gene expression. Finally, Hassan included a single research paper on trypanosome physiology focused on function of Letm1 protein and a review. This part represents new direction of Hassan's research in which he would like to focused on more general questions of mitochondrion functions using trypanosomes as model organisms. Altogether, he is the first author of 8 papers and senior author of 4 papers.

Specific comments and questions
The topics and results of research papers are briefly discussed in short introduction that includes general introduction of mitochondrion as an organelle, evolutionary position and biology of *T. brucei*, specific function of *T. brucei* mitochondrion and future directions using *T. brucei* mitochondrion to address major questions of mitochondria as a whole.

The mitochondrion is introduced as a descendant of an α-proteobacterial endosymbiont, which is prevailing opinion. However, recently there was interesting discussion on origin of

mitochondrion as a pathogen: "Pathogen to powerhouse" (Ball et all., Science 2016), reply "Infection and the first eukaryotes", Gould (Science, 2016). What is your opinion?

Trypanosomes are often introduced as ancient early branching organisms. Would you provide examples, which mitochondrial characters could be considered to be really ancient and which resulted due to early divergence (if possible)?

T. brucei the pathogen (page 10). There is over 1000 genes in *T. brucei* genome coding for VSG, which is often used to demonstrate a vast repertoire for antigenic variation. However, does this number really reflect the repertoire?

tRNA import (page 16). It is really exciting that tRNA is still imported into Ak mitochondria. Is this result really well supported? Is it clear that detected tRNAs are inside of the mitochondria? Did you observe any dynamic of tRNA import?

The mitochondrion of the bloodstream form (page 17): *"While not involved in energy generation, ...mitochondrion..."* Is this view really correct? What is the role pyruvate? Why mitochondrial pyruvate carrier is expressed in bloodstream forms (Figure 7. should be updated, mitochondrial pyruvate carrier has been characterized)?

The mitochondrion of BSF are devoid of cristae, which corresponds with absence of respiratory complexes cIII, cIV. Although, there is correlation between presence of respiratory complexes and cristae formation, there is not much information about molecules that are directly involved in formation of cristae. How is it with MICOS complex in kinetoplastids?

Finally, page 19: Functional analysis of Letm1 is introduced as a nice example of the protein of general interest, function of which was studied in *T. brucei* that was used as an experimental model. Nevertheless, Letm1 is rather narrow single direction. As Hassan promised at page 6 to discuss future directions, I would like to hear a bit more about major questions that could be addressed using *T. brucei* model.

Minor comments
Please, correct in your list of abbreviations, and in the text: Dk - dykinetoplstic; S - Svedburg)

In conclusion, I have no doubt that Dr. Hassan Hashimi is excellent mature scientist who is fully qualified for habilitation with potential for further academic career.

Prof. Jan Tachezy, Ph.D.

Prague, 26 July 2016

A review of the Habilitation Thesis of Mir Mohamod Hassan Hashimi, PhD

Title of the Habilitation Thesis:

Unique and conserved features of the Trypanosoma brucei mitochondrion.

The Habilitation Thesis is divided into four parts. Part one focuses on trypanosome RNA editing, part two on dyskinetoplastic *Trypanosoma brucei*, part three on trypanosome mitochondrial translation, and part four on trypanosome mitochondrial physiology. These parts are based on original publications in international peer-reviewed journals: 14 primary research articles, 5 reviews, one book chapter and one popular article. HH is the first (co)author of 8 papers, and corresponding author of three papers. Currently, his publications have been cited >500 times, and his recent Hirsch-index is 11 (July 2016).

The Thesis is introduced with a short but informative text about trypanosomes with a focus on the mitochondrion, its function as the cellular powerhouse, its kinetoplast DNA – replication – transcription/editing – translation. This is set into the context of *Trypanosoma brucei*, its position in the phylogenetic tree and role as a pathogen.

The first part is the most extensive one of the four parts, and describes the discovery of the mitochondrial binding complex 1 (MRB1) and its subsequent topological and functional analyses. The MRB1 complex was identified as a multi-protein complex that associated with TbRGG1, an oligo(U)-binding essential protein. HH and colleagues discovered MRB1 independently of, and in parallel with two other laboratories. It turned out to be a major discovery. MRB1 is an evolutionarily old complex, and it plays key roles in initiation of editing and its early stages, such as the positioning of gRNAs and facilitating 3' to 5' editing progression. HH played a central role in the discovery and characterization of this complex.

***Q1:** Is there a reason why it is U that is the inserted/deleted letter? Could you, please, speculate on the evolutionary origin of editing?*

The second part deals with dyskinetoplastic *Trypanosoma brucei*. It consists of four papers, and the best cited paper (>100 citations), published in PNAS, investigated several *T. brucei evansi* and *T. brucei equiperdum* strains. These strains were originally believed to be separate species, *T. evansi* and *T. equiperdum*. The PNAS paper demonstrated that these two are in fact only subspecies that were relatively recently derived from *T. brucei*, and that

these two subspecies represent bacterial forms that lost part (dyskinetoplastic) or all (akinetoplastic) of their mitochondrial DNA. As a result, these forms are locked in the bloodstream form of trypanosomes, and do not need the tsetse fly as a vector. They affect camels, horses, and water buffaloes. They can be transmitted sexually, and thus escape the tsetse inhabited region. The paper also presented data that indicated that the trigger for Dk is the homogenization of minicircles and subsequent deletion in maxicircles. This is a beautiful work that combines modern molecular biology methods and applies them to a long standing biological question, and provides a simple and elegant answer.

Q2: *The Dk and Ak forms appear to represent evolution captured during a step. Do you envision that these two subspecies will eventually become divergent enough to be classified as species?*

The third part describes a unique domain of *Trypanosoma brucei* elongation factor Tu (EF-Tu) that is essential for its function. This domain consists of 30 amino acids (aa), is positioned close to the C-terminus. EF-Tu is a protein that brings aa-tRNA to the A-site on the ribosome. This 30 aa domain was also detected in EF-1a, and this EF-1a domain was able to functionally substitute the corresponding EF-Tu domain. The authors proposed that the function of this domain is to facilitate the fit of EF-Tu to the mitochondrial ribosome that has a unique intersubunit space. This and the following part illustrate the complex scientific interests of HH, and his ability to study diverse processes in these organisms.

Q3: *Is similar intersubunit space observed also in the cellular ribosome? (The expectation is yes, as EF-1a contains this domain as well.)*

The fourth part identifies the Letm1 protein as critical for the maintenance of potassium homeostasis by playing a role in K+ efflux out of the mitochondrion. Depletion of Letm1 results in osmotic swelling of the organelle. Letm1 absence is one of the known causes of the Wolf-Hirschhorn syndrome (WHS).

Q4: *In an article from 2014 (Dis Model Mech. 2014 May;7(5):535-45. doi: 10.1242/dmm.014464.), the authors used a panel of samples from WHS patients. They demonstrated that Letm1 expression was reduced in mitochondria and this was associated with altered intracellular Ca(2+) levels. Could you speculate on whether Letm1 from Trypanosoma and humans can have diverse effects with respect to the identity of the affected ions?*

In the Introduction, HH mentions that he is at a crossroads in his scientific career. I would be very much interested if he could outline what types of questions attract his attention and sketch his future research directions.

Overall, this Thesis represents a huge body of work, and it was a pleasure to read. I appreciated the spectrum of research interests of HH and the close connection of the detailed molecular studies to the biology of the organism. HH is also an active and highly valued member of the RNA community, participating on the organization of the RNA Club meeting. HH is an inspiring person and University of South Bohemia can be definitely envied to have such a scientist. I fully recommend his Thesis to be accepted.

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Oponent's review of Habilitation thesis of

Hassan Hashimi

Unique and conserved features of the Trypanosoma brucei mitochondrion

in the **Field of Molecular and Cell Biology and Genetics**, Faculty of Science, University of South Bohemia in České Budějovice

In his Habilitation, Dr Hashemi addresses several aspects of the unique mitochondrial biology of Trypanosome. These pathogenic parasites belong to the kinetoplastids, a lineage which has diverged very early from other eukaryotes. Mitochondrial studies in evolutionary diverse groups are important to better understand the organellar function and gain more insight in conserved and evolved pathways. The characteristic mitochondrial features illustrating the evolutionary diversity of Trypanosome are the complex maturation steps of mitochondrial mRNA, the metabolic switches to adapt from vector to host[1] and the simplified organellar morphotype.

A major part of his dissertation is devoted to the very complex RNA editing system in the Trypanosoma species *T. brucei*. The work performed to understand the interaction architecture and activity of RNA editing players and characterize the mRNA binding complex1 or gRNA binding complex is technically extremely sound and relevant.

As further revealed in this dissertation, two more recently evolved subspecies, *T. b. equiperdum* and *T.b.evansi* display large deletion and total loss of DNA, respectively. The use of this genetic background is a very elegant way to assess mitochondrial functions, such as the ATPsynthase, in dependence of RNA editing, without having to manipulate the mitochondrial genome; or to investigate the futile import of tRNA into mitochondrial although devoid of mitochondrial DNA, and consequently lacking translation; or analyzing the global proteomic changes in function of mitochondrial translation. These research contributions are as innovative and original; revealing conserved essential functions and questioning the ancestral aspect of the crosstalk between mitochondria and nucleus[2].

While the research field of mitochondrial RNA maturation in *T.brucei* is broad due to its complexity, an experimental limitation in the *in vivo* use of this model system is the high motility of this organism. Dr. Hashimi has introduced a novel technique that was not routine in the lab allowing sophisticated microscopic applications such as FRAP or FRET in the immobilized but living protists. The method is well elaborated and validated by convincing experiments[3].

Furthermore, he approached with LETM1[4] another topic related to a difficult subject in physiology.
While the evolutionary remarkable position of T. Brucei justifies each of addressed research topics, it specifically highlights the importance of the work on LETM1 in this model system. *LETM1* is a conserved and essential eukaryotic gene, as stated by separate recent studies, here it is also defined as an ancestral gene. This is a relevant aspect from the perspective of the debated function of *LETM1*. Indeed, LETM1 was first characterized as a protein controlling the mitochondrial volume homeostasis. A large body of work convincingly demonstrated that LETM1 is an essential factor of the mitochondrial potassium-proton exchanger, (KHE). The mitochondrial KHE is a vital element of the Chemiosmotic Hypothesis of Energy Conservation postulated by Peter Mitchell and thus for the mitochondrial functionality. Decreased mitochondrial functionality in absence of LETM1 was also illustrated by other studies revealing decreased mitochondrial translation and suggesting a role of LETM1 in translation and membrane assembly of proteins encoded by the mitochondrial DNA. The role of LETM1 in KHE activity was then challenged by a screen identifying LETM1 as calcium-proton exchanger. Since then several contradictory papers were published. However, the importance to understand the role of LETM1 is reinforced by its involvement in the pathology of seizures in the complex Wolf-Hirschhorn Syndrome.
It is this context that Dr. Hashimi addressed the role of LETM1 in Trypanosoma, taking advantage of the exclusive mitochondrial properties of this organism. Using RNAi to effectively knockdown LETM1 to almost undetected expression in *T. brucei*, he showed a phenotype of swollen mitochondria, similarly as previously described in yeast, worms, flies, rodents and humans. He also noted decreased mitochondrial translation and growth. An important piece of evidence for a primary role of LETM1 in KHE rather than in translation was provided by silencing LETM1 in *T. b. evansi*, demonstrating growth impairment and swollen mitochondria in the background of a strain lacking mitochondrial DNA. In any case, all morphological and physiological phenotypes could be reverted by treatment with the ionophore nigericin, a synthetic KHE, or expression of human LETM1, supporting the ancient role of LETM1 in KHE as well as the ancient origin of mitochondrial volume homeostasis. This data is critical to understand the function of LETM1 in eukaryotes as it provides further evidence that LETM1 is involved in the KHE, well after having been implicated by others in calcium-proton exchange.

The dissertation includes a variety of research questions and proves the broad range of expertise of Dr. Hashimi. The techniques used are remarkably sound and numerous, ranging from classical to more sophisticated and self-established like: RNAi KD, Southern, northern, western blots, glycerin gradient fractionation of mitochondria, in vivo translation, tandem affinity purification, recombinant protein purification, silac proteomics, RNA cross linking or double filter binding assays, guanylyltransferase labeling, yeast 2H, measurements of respiration, membrane potential, ATPase activity, preparation of submitochodnrial particles and trypanosome immobilization for FRAP analysis.

The record of publication from 2005 to 2016 is impressive with altogether 22 publications: 14 peer reviewed research articles with 6 first (1 co-corresponding) and 3 last authorships, 6 peer reviewed reviews including 2 first (1 co-corresponding) and 1 last authorships, 1 book chapter and one popular article (first author). Of note, before engaging into his PhD, he already appeared as undergraduate student as co-author on 2 papers with different topics. Altogether, this demonstrates a high commitment to science.

Overall, I strongly support the approval of his Habilitation at the University of South Bohemia

Questions refer to the superscript numbering throughout the review and are as follows:

Question 1: Metabolism became a hot topic in cancer research and the metabolic reprograming observed during oncogenic processes in primary or metastatic cancers are currently widely studied. Can we learn some lessons from the bioenergetics regulation of *T.Brucei* switching between procyclic and slender bloodstream forms?

Question 2: What is known on retrograde signaling in the *T. brucei* sub species?

Question 3: Can the immobilization method be applied with comparable success to other motile unicellular or small organism for live microscopy?

Question 4: in early studies, Dr. Hashimi found Tb927.3.4920 (LETM1 RBD) along with other members of the multi protein complex associated with the RNA metabolism factor TrGG1 in procyclic *T.brucei* cells. If I am correct, this was no longer mentioned. Could Dr. Hashemi comment on this association with TrGG1?

Summary of the review:

In summary, this Thesis presents a thorough and expert dissertation on manifold and important aspects of the unique mitochondrial biology of *T. Brucei*. Dr. Hashimi explored in depth fundamental genetic questions of RNA maturation, a topic in which he was extremely well trained. He then moved to the exciting field of energy metabolism and cell biology. Further, he became interested in mitochondrial ion transport. With his work on LETM1 in *T.brucei* as a model system, he demonstrated the ancient origin of the regulation of mitochondrial volume and K^+ homeostasis. Moreover, he notably contributed unravelling the debate on the protein function of *LETM1*, a gene implicated in the pathology of seizures in the Wolf Hirschhorn Syndrome.

The large variety of techniques, engagement in collaboration and record of publication has consolidated his reputation. I am impressed by the dissertation and strongly recommend the approval of his Habilitation.

Sincerely,
Karin Nowikovsky

Vienna, 14.09.2016

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