REVIEW STATEMENT – COURTNEY STAIRS

This thesis entitled **Characterization of the protein import into Giardia intestinalis mitosomes** authored by Eva Pyrihova aims at exploring different aspects of the mitosomal proteome of the human pathogen *Giardia intestinalis.* It represents an impressive body of work encompassing various bioinformatic, molecular biological and biochemical techniques. It was a pleasure to read. It always surprises me that such a small organelle can have such a complicated biology – and that we have only scratched the surface of its true function(s). Theses such as this provide the foundation for future work exploring the 'wild frontier' of mitochondrion biology.

Below I have summarized each of the chapters and provided recommendations and questions for the candidate. I have put much of the focus on the unpublished chapters. Most of the questions are more philosophical in nature and are aimed at stimulating discussion on the topic.

CHAPTER I. INTRODUCTORY CHAPTER

Summary:
In the introduction of this thesis, Eva Pyrihova tackles the vast amount of literature detailing mitochondrion evolution and what is currently known about protein import machinery of mitochondria. She successfully introduces the composition of various membrane complexes and addresses their evolution, distribution, and function in diverse eukaryotes. In addition, she touches on various evolutionary scenarios to explain some of the *weirder* observations of mitochondrial protein import in divergent eukaryotes.

Recommendations and questions:
A figure or table illustrating the various components of the protein import machinery and their origins could greatly improve the readability of the introduction. While the evolution of mitochondria is not the main focus of this thesis, I think that the introductory section 1.1 could go into more detail on the endosymbiotic theory. Some critical citations are missing such as Sagan (1967). See Roger et al. (2017, Current Biology) for some inspiration.

> *[Q]: Please clarify this statement "This endosymbiont of α-proteobacterial origin was gradually transformed to current day mitochondrion by gene loss and gene transfer to host nucleus"*

Towards the end of the first section, malawimonads are mentioned to have ancestral bacterial features however no references are provided. An extra sentence describing such bacterial features found in jakobids and/or malawimonads could be added.

On page 11, the following discusses the evolutionary processes of mitochondrion reduction. *"The driving force for the reductive mitochondrial evolution has predominantly been the anaerobic environment, in some cases accompanied by the parasitic lifestyle."*

> *[Q] Why is it anaerobiosis that drives mitochondrion reduction? How does the loss of respiratory function impact the protein import machinery?*

The section continues "*The comparative analysis of different mitochondrial forms enable us to distinguish the minimal essential set of components of mitochondrial import from the dispensable set of peripheral subunits."*

> *[Q] Discussion question: When examining anaerobic metabolism of diverse protists, many of us infer the pathways originate from **lateral gene transfer** from prokaryotic or eukaryotic donors. However, when examining protein import machinery we rarely consider lateral gene transfer. Why do you think this is? Are import machinery components resistant to transfer?*

Pyrihova adds that by studying the simpler protein import machineries, we can learn more about the import machinery of the protomitochondrion.

> *[Q] What is the danger here? What if we applied that same logic to other pathways in Giardia?*

Throughout the introduction, there are some MRO-containing organisms that are not discussed (e.g., *Mikrocytos*; *Stygiella;* Breviates: *Pygsuia, Breviata, Lenisia; and Blastocystis sp.)*. Indeed, each year, the list of MROs gets larger so it is impossible to include of them. Given that the protein import machinery has been predicted for some of these organisms and even explored experimentally in *Blastocystis* (Tsaousis et al. 2011) I think they should be mentioned.

Section 1.3 goes on to describe the protein import machinery of some protists. In the section on Entamoeba: *"It was proposed, that Entamoeba lost typical OM receptors such as Tom70 or Tom20 due to the loss of presequences and gained the lineage-specific receptor Tom60 instead"*

> *[Q]: This is a classic chicken-and-the-egg scenario. What was lost first? How do you think that such a transition could occur without creating so-called 'evolutionary dead ends'?*

While discussing Trypanosomes, Eva suggests that *"noteworthy, that even though the mitochondrion is aerobic and contains genome, it has undergone dramatic independent evolution likely due to the parasitic lifestyle."*

> *[Q]: What is it about a parasitic lifestyle that would alter the mitochondria? How could you test this hypothesis? Is there available data that could confirm or dispute this statement?*

The discussion section provides an excellent overview of what is currently known about the evolution of the protein import machinery. Since many of the protein import components are eukaryotic innovations, it might be that we will never truly know how this system evolved. If possible, this section would benefit for more discussion of the evolution of protein import in the context of eukaryogenesis. A brief discussion or reference to the protomitochondrion theory could also be added.

> *[Q] How would protein import evolve in a syntrophic (e.g., Hydrogen hypothesis – Martin & Muller 1998) vs phagocystis (e.g., protomitochondrion – Gray 2015) model for mitochondria/eukaryote evolution?*

> *[Q]: What about dual localization strategies? We know that much of the yeast mitochondrial proteome is dual localized? Could this happen in metamonads too? What would you envision the mechanism to be?*

CHAPTER II – PUBLICATION #1 ON CYTOCHROME B IN GIARDIA

Summary:
Here, Pyrih and colleagues perform a thorough analysis of the heme-containing cytochrome b5 proteins encoded in the *Giardia*. Despite lacking the canonical cytochrome utilizing pathways and the means to synthesize heme de novo, *Giardia* encodes 4 orthologues of cyt b5. The authors perform primary sequence analysis as well as spectrophotometric and cell biological characterization of these proteins particular focus on the incorporation of heme into the mature proteins. They (i) demonstrated that the Giardia cytb5 proteins are capable of incorporating exogenously supplied heme, (ii) identified the residues necessary for heme binding, (iii) determined the subcellular localization of each cytb5 protein and (iv) identified a potential redox partner for the cytb5 protein.

I think this chapter represents a thorough analysis of these proteins and only have a few questions for this work:

> *[Q] How could you identify the other redox partners of cytb5? Are there bioinformatics or experimental techniques you could do?*

> *[Q] What is the reduction potential for these reactions?*

> *[Q] Similarly, how do you think heme would be imported in vitro? What sort of experiments could you do to check for import?*

CHAPTER III – PUBLICATION #2 ENZYME TAGGING BirA.

Summary:
Here, Pyrihova et al., describe an innovative method for detection of mitosomal proteins in Giardia. Critically, they identified a Tim44 homologue, previously missed by bioinformatic surveys. Indeed, this revolutionary technique is critical for the advancement of our understanding of the mitosome of Giardia and provides the foundational research for exploring mitochondrion-related organelle proteomes in non-model organisms.

[Q] What do you think is happening with the overexpression of the MOMP35 homologue?

[Q] Thought experiment: If the BirA reaction is dependent on mitosomal ATP, could you use this as a reporter to identify the ATP importer?

[Q] Do you think you could apply this method with modern "de novo proteomics" techniques? That is, deducing the sequence of peptides without the guide of a genome.

CHAPTER IV. PUBLICATION #3 Cytosolic FeS clusters

Summary:
Herein, the authors employ bioinformatics and cell biological techniques to explore the cytosolic FeS cluster system (CIA) of Giardia. This publication outlined the phylogenetic distribution of the core CIA system components across diverse eukaryotic lineages. Using these phylogenetic profiles, the authors observed clear patterns of "co-loss" or retention in different metamonads. Indeed, most of the CIA components still present in Giardia showed predicted cytosolic localization. Unexpectedly, at least two components (Nbp35 and Cia2) demonstrated dual localization to the mitosome and cytosol.
One of the components that is apparently missing from the Giardia CIA pathway, is the NADPH oxidizing Tah18, although a divergent homologue (GiOR) can be detected by homology probing. The authors demonstrate that this divergent GiOR protein can complement Tah18 depleted *Trypanosoma brucei* and is localized exclusively in the cytoplasm (despite having known mitosomal localization in Giardia). The authors caution the reader to be wary of thinking that GiOR is an analogue of Tah18 given its organellar localization and thus separation from the CIA system.

[Q] Thought experiment: In some protists (Entamoeba, Monocercomonoides) we see that the ISC system has been functionally replaced by a cytosolic system or alternative mitochondrial pathway (Pygsuia, Mastigamoeba). From this paper, it appears that at least two components of the cytosolic system are perhaps involved in the mitosomal FeS cluster system. Do you think this could be evidence for another replacement of ISC, this time by a CIA pathway?

CHAPTER V. MANUSCRIPT ON A NEW TIM TRANSLOCASE

Summary:
Here, Pyrihova et al., provide the first evidence for a TIM translocase in the mitosomes of Giardia intestinalis. Using a suite of sophisticated bioinformatics methods, they identified the sequence of a Tim17 homologue. They characterized the primary sequence elements that are typical of a bona fide Tim17. Both fluorescent microscopy and their well-established BirA-tagged mass spectrometry method were used to localize the protein to the mitosomes of Giardia. To investigate the sub-organellar localization, they used STED-microscopy and observed the Tim17 protein does in fact localize to the membrane of the mitosomes despite the absence of detectable transmembrane domains. The authors continue and provide an evolutionary scenario for the Tim 17/22/23 protein family in Excavata involving differential loss from a Tim 17/22/23-containing LECA.

In my opinion, this chapter represents state-of-the-art mitosomal protein characterization that the field has become accustomed to from the Dolezal research group. The intersection of bioinformatics and experimental characterization is unique in the field and continues to raise the bar for mitosomal research.

Recommendations and questions:
The supplementary data was not provided to the reviewers, could this be added to the final thesis?

The story begins with using HMM profiles to find Tim17 candidate proteins in the Giardia genome.

[Q] Hypothetical: Assuming you have a HMM profile that will detect the Giardia Tim17, what is the limitation of using just the predicted proteome of Giardia? What sorts of barriers are there to this bioinformatics approach?

After finding the protein, Pyrihova et al., identified the key residues for protein function yet failed to identify obvious transmembrane domains despite its predicted membrane location.

[Q]: How could you test the function of some of these residues? What experiments would you do?

[Q]: Do the CLO proteins have TM domains?

Attempts to solubilize the inner membrane of the mitosome were not successful. To explain this Pyrihova et al. present an interesting hypothesis *"The overall resistance of the mitosomal inner membrane to detergent treatment suggest its highly unusual lipid composition, when compared to the properties of canonical mitochondria [23]. In light of these results it is possible that the non-conformity of putative TMDs in GiTim17 are results of adaptation to the unusual composition of mitosomal inner membrane. "*

[Q] Have there been studies of the mitosomal inner membrane (or membrane composition) of Giardia? What do you expect the major lipid components are? Is this unique to Giardia? Diplomonads? Metamonads?

[Q]: Are there more techniques aimed at characterizing membrane proteins? (styrene maleic acid for example)

To improve this chapter, I recommend a careful inspection of the methods section for typographical errors, abbreviation explanations and overall clarity. I think that, in general, the manuscript would improve with more introduction to each of the techniques so that a non-expert reader could understand the experiments. For example, the section explaining Tim17 dimerization has a lot of information that is difficult to process in its current state, I think it could be elaborated on. There could be more information on the plasmids too – what were the auxotrophic markers for the yeast plasmids? A table summarizing the genotype of yeast and the auxotrophic markers of each plasmid could help. Concentration of 3AT was not provided (a brief statement of what 3AT does would also help). Such elaborations could be done in the main text of the paper or in the figure legend. I realize for publication these details might end up in the supplementary material, but they will likely be requested by the reviewer if omitted.

[Q]: Could you elaborate on the MS quantification methods?

MINOR COMMENTS FOR THE DOCUMENT

Both the introduction and the manuscript would benefit from proofreading and grammatical guidance – I have made some suggestions on a hard copy I will provide to the candidate.

(i) ISC pathway is not universal to all eukaryotes, we know at least two exception in the archamoebe and breviates. Consider rephrasing in the abstract and introduction.

(ii) Eubacteria vs. bacteria consistency. It is convention to only use one of these terms. If you would like to use eubacteria, you should not use bacteria as well. Similarly, if you use 'eubacteria' you also cannot use 'archaea' you must use 'archaebacteria'.

(iii) Western should not be capitalized – only Southern. Southern blotting was named after Edwin Southern while western and northern blotting were just named as such as a clever homage.