Evaluation of Ph.D. thesis of Mgr. Hana Pavlisková

The submitted thesis by Ph.D. candidate Hana Pavlisková focuses on components of the flagellar tip in a parasite *Trypanosoma brucei.* The thesis represents a comprehensive and solid work built on three published papers (one first-author and 2 co-author papers) and one first-author "in preparation" manuscript. The published work primarily deals with improving relevant methodologies (namely an approach to overexpress proteins in the Trypanosoma, a strategy to distinguishing the daughter cells after Trypanosoma division, and the adaption of expansion microscopy technique to analyze fine details of cilia ultrastructure). All of these represent important steps on the way toward the main goal of this thesis, which has been the systematic identification and characterization of proteins comprising the ciliary tip, capitalizing on access to the tryptag.org project data.

The student has opted for a more concise thesis format of "commented summary of papers", which in my opinion has its pros and cons. Overall, the thesis is carefully written, with ample attention paid to individual details. As a "non-Trypanosoma person" I would appreciate more synthesis of information in the provided text. For instance, the candidate states in the "Introduction" part (related to the Flagellar tip in Trypanosoma (page 24)), that the tip is associated with several important functions (e.g., IFT turnover, construction of the flagellum, social motility, etc.). This is followed by a detailed part describing individual proteins comprising the tip. Clearly stating what is the current model (if any) of how the individual components may act together to underlay the listed functions of the tip, what aspects (if any) of the flagellum tip function can be explained by the activity of the currently known components, and what function currently lacks any mechanistic inside would be very helpful here. The "Results and Discussion" part very well outlines the key findings of each paper comprising the thesis. However, while the results are well summarized, I somewhat miss here a genuine discussion of the obtained results and their interpretation (perhaps even some speculations would be welcomed), that would go beyond the frame of the text of each of the included papers. In the version I have obtained, the resolution of the attachments (original article files and the manuscript) is very low. Importantly however, none of these comments (or the following ones) should be taken as a sign of major criticism, but rather as a suggestion or a different point of view.

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In the first paper, the authors used a combination of antibody staining and fluorescence tagging to distinguish “old flagellum daughter” and “new flagellum daughter” cells. This strategy allowed to analyze differences between the daughter cells, leading to the discovery of remodeling of a “new flagellum daughter” during the G1 cell cycle phase. Here I would appreciate some more context in the corresponding summary text, e.g., a discussion on the importance of such differential remodeling for Trypanosoma biology. Importantly, the used pipeline has opened up a possibility to examine differential effects of candidate protein depletion for a new and old flagellum. The paper was published in *Molecular Microbiology*. Hana Pavlisková contributed as the second author to this work.

In the second paper, the authors used an elegant strategy to achieve inducible overexpression of proteins of interest from endogenous loci in Trypanosoma. They modified an existing PCR-only tagging (pPOT) system by introducing a T7 Polymerase Promoter together with Tetracycline-responsive elements. This way, they were able to drive expression of the selected gene from such promoter integrated at 5´end of the corresponding ORF. Subsequent analysis convincingly showed that this approach allows overexpression of fairly large proteins, thanks to perhaps surprisingly high processivity of the T7 polymerase (with runs well beyond 10,000 base pairs). It is not clear to me though whether or not the integration of the T7 promoter and Tet-response elements may in any way interfere with the transcription driven from the “endogenous/native” promoter of the selected ORF. The authors further mention that their strategy always (10 out of 10 examined ORFs) led to the targeting of one of two alleles. I wonder what is the estimated targeting efficiency of the used approach; if any significant differences in the targeting of individual ORFs were observed, and what adjustment would be necessary to allow efficient bi-allelic targeting? This paper was published in *Molecular& Biochemical Parasitology*, with Hana Pavlisková as the first author.

The third study represents “proof of concept” of adopting an expansion microscopy (ExM) procedure for the study of flagella and cytoskeleton in Trypanosoma and Leishmania. Following a very careful and rigorous evaluation, the authors concluded the obtained ExM-based data are in good agreement with earlier observations based on electron microscopy and noted that cation is in place when measuring distances in ExM samples (as expansion factors may differ for individual subcellular structures). In addition, they were able to examine some of the rare cell stages (e.g., cells undergoing division). Pertinent to the final part of the thesis, they were nicely able to resolve the localization of flagellum tip protein KIN-E to the distal end of each doublet as well as the central pair. This paper was published in *Open Biology*, with Hana Pavlisková as the third author.

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In the final manuscript, the authors present a comprehensive atlas of components of the flagellum tip in Trypanosoma. Based on initial data from the tryptag.org project, the authors constructed a panel of Trypanosoma lines with endogenously tagged candidate proteins. Based on the obtained localization data (including ExM data and analysis of spatio-temporal changes) the authors clustered the candidate tip proteins into several groups. For over 50 candidates they generated inducible RNAi lines and obtained functional data. I very much appreciate the provided summary (Fig. 7) of prototypical localizations and phenotypes found. The corresponding manuscript (with Hana Pavlisková as the first author) seems to be still "in preparation" though, with the Discussion part missing and the Material and Methods part very brief, (which is a pity). Some of the manuscript figures (e.g., Fig. 4) could perhaps be better explained. In sum, I think this systematic and rigorous work without doubt represents a key milestone in the understanding of flagellum tip composition and function in Trypanosoma.

Further questions/points for discussion during the thesis defense:

1. What does the Ph.D. candidate consider the most biologically relevant finding/question about the Trypanosoma flagellum or cytoskeleton revelated by the performed ExM, that was perhaps not adequately addressed before in EM- studies?
2. What might be the factors in the composition of individual structures that contribute to the observed difference in expansion factor (4.6 for the whole cell body, 6 for flagellar microtubes on cross-section, etc..) in the reported ExM analyses?
3. Fig. 7 demonstrates that components specific for either the old or the new flagellum exist. The RNAi led to phenotypes in either both flagella or only the old flagellum. Is there a reason that no new flagellum-specific defect has been found?
4. RNAi of 16 candidates produced morphological phenotypes and/or changes in flagellum length. What experiment would address the specificity of RNAi targeting and exclude possible off-target effects?
5. Some of the tip complex candidates showed an intriguing IFT-like movement. Transport by IFT would be an elegant mechanism to concentrate the tip proteins at their target destination. What about tip proteins not detected undergoing such "IFT-like movement", is another mechanism expected to mediate their tip localization?
6. Could the Ph.D. candidate comment on the results in Table 1 showing that the majority of vertebrate tip protein orthologs were not detected in the cilia tip in the RPE-1 cell line? Were any RNAi effects examined/found in vertebrate cells?

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In summary, I fully and unconditionally recommend accepting this thesis for the defense and in case of a successful defense, awarding the author, Hana Pavlisková, the title Ph.D.

In Brno 22.12.2023

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