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Referee's review of the doctoral dissertation of Hana Pavlisková entitled "Identification and characterization of flagellar tip proteins in *Trypanosoma brucei*"

Evaluation of thesis:

The doctoral thesis of Hana Pavlisková seems to me to have a wider scope than the title suggests, dealing also with tool development to facilitate the subsequent investigation of the flagellar tip proteome to reveal the role of this diminutive but important part of the eukaryotic cell. The thesis comprises the following:

- a comprehensive introduction written by the author covering the flagella generally in eukaryotes and specifically in trypanosomes, focusing on *T. brucei*
- three peer-reviewed articles in quality, impact-factor journals where Hana serves as first author and prominent second and third co-authorships; these present the development of sophisticated and valuable tools for investigation of flagella in the well-suited model *T. brucei*
- a preliminary manuscript in which Hana is first author presenting what can only be described as a *tour de force* investigation of up to 75 proteins that have been identified in a genome-wide, subcellular map of *T. brucei* proteins that localize to the flagellar tip.

The content of the thesis is in my opinion adequate for the requirements of Hana being awarded a doctorate degree. However, I do have some reservations. I have directly assessed four PhD theses, including this one, and would rank this one as among the weakest. The reason I mention this is not because I think my personal ranking is intrinsically important, but to convey to Hana what I consider to be relevant constructive criticism–I hope–for her future career. Before continuing, I should state I have been fortunate to be asked to review interesting and stimulating theses, this one included. Thus, I do not consider this a bad thesis. However, there is a major flaw that does detract significantly from the thesis unfortunately.

But first the strengths of the thesis. The introduction is more or less well-written and organized, demonstrating that Hana has acquired the requite background during her PhD studies investigating the flagellum. I especially appreciated that she covered several aspects of this organelle, *e.g.* key proteins, the biological functions, and ultrastructure, and just restricted herself to just the tip. There are some ambiguous and/or awkward sentences (see one example in appendix below), but I also take this as a positive sign that Hana wrote the introduction herself. I also would like to point out that I have known Hana since she did her pre-graduate studies in Budweis and I must state her English has substantially improved. She certainly is capable of writing scientific text, allowing her to successfully gain independent funding through the Charles University Grant Agency.

Another excellent quality demonstrated in the thesis is Hana's impressive ability to produce high-quality data via a wide-spectrum of technically advanced methods. Furthermore, Hana produced a large volume of data, showing she is efficient as well as proficient. I duly note that Hana made very significant contributions to all the presented articles and the manuscript, with her declared contributions comprising the vast

majority (chapters 5.2 and 5.4) or significant chunk (chapters 5.1 and 5.3) of the content. Especially impressive is that Hana spearheaded the very complex and vast undertaking of characterizing the flagellar tip proteome, which I feel will be a highly valuable and well cited article addressing significant gaps in knowledge in the cytoskeleton and molecular parasitology fields after this work is polished.

And this brings me to the major and impossible-to-ignore weakness of Hana's thesis: the very poor quality of chapter 5.4, the manuscript that attempts to report key findings about the flagellar tip proteome. Given that this chapter (1) comprises the data that is the title of the thesis and (2) is Hana's central project, this is a significant detraction from the quality of the thesis. It is also a shame, because it does not give Hana's herculean effort much justice.

The major problems are:

- The chapter is presented as a quasi-manuscript with an Introduction, Results and Methods sections plus dedicated citations. Missing is the important Discussion section that could have been employed to tie all the disjointed parts of the Results together
- As mentioned in the previous point, the results are presented in the main text in a rather disjointed and haphazard way. Problems include:
    - Confusing nomenclature, where accession numbers and ortholog names are used interchangeably. Furthermore, the data from the RPE-1 cells use human gene names, making it impossible to map these to the investigated *T. brucei* proteins. Some abbreviations are undefined and/or used inconsistently throughout the text.
    - No link is made between the various results that are shown. Temporal and special patterns of the tagged proteins are presented separately from subsequently reported RNAi phenotypes, with no connection really made between localization and functional studies. Also, for some reason, kymographs addressing the processivity of tagged motor proteins are included with RNAi phenotypes but again with no link in how this augments interpretation of phenotypic analyses.
    - The figures are not only inconsistent in the format (e.g. fonts of figures 11 and 10), but are poorly annotated and in some cases poorly rendered (e.g. line scans in figure 3). The legend of the key figure 4, summarizing flagellar lengths upon RNAi-silencing of a label of 35 proteins is poorly annotated. What do the colored accession numbers mean? Why are some in bold? Adjacent columns show p-values, but of what? What populations are compared? New flagella versus old? Demarked cell line versus parental cell line? I also feel figure should be a panel, with the different elements labelled A, B, etc.

      RNAi-phenotypes such flagellar loops and flagellar extensions are mentioned in text but not pointed out in the imaged cells. The position of central pair of microtubules are malformed relative to what (I suppose PFR?)? What am I looking at in Figure 16A?
    - Some data are mentioned but not shown for some reason, such as motility tracking. This seems like valuable data that should be included given that these proteins are presumed to affect the motile flagellum of *T. brucei*. We have to take Hana's word for it that some RNAi-phenotypes are faster and slower swimming in certain knockdowns. Also, the methods are sparse or even missing, especially pertaining to how the RPE-1 work, in which the author cites a thesis that is difficult to access.

- There is no flow in the text. Conservation of flagellar tip proteins is first discussed, which reads more like a list with no attempt to connect these findings with the wet lab data. Then localization patterns and RNAi/tagging phenotypes are reported, the latter organized based on their effect on flagellum length. This I appreciated, but found confusing when Hana delved into important differences in other RNAi phenotypes. Then the text continues to localization of a handful of proteins (without any clear rationale why these were chosen) in human RPE-1 cells with induced primary cilia. This order seems more arbitrary than a true attempt to rationally organize these complex data.
- The thesis abruptly ends without any attempt to tie everything together.
- A table summarizing the tagged proteins, RNAi phenotypes, evolutionary conservation, etc. would have been greatly appreciated to facilitate both reading and clear writing.

As a result, this central part of the thesis is very difficult to read. I found myself forced to infer information from the data and was lost in what the ultimate meaning of all this data was. I also had to re-read this chapter several times to at least get a grasp of the bigger picture, which again I had to infer and thus lack Hana's vision of this project.

It seems that Hana appreciated that to convey importance this story, both broader trends and deeper investigation into key details of certain proteins were both needed to understand the overall picture. However, this chapter fails in combining these two vital parts, thus precluding overall understanding of the flagellar tip proteome, as well as how key molecules contribute to the biogenesis and function of this vital and fascinating organelle.

Given the relative quality of the introduction, I assume the poor state of chapter 5.4 is due to rushing to complete the dissertation before the deadline. The synopsis of chapter 5.4 nicely gave an understandable overview of this project, which I also utilized to get a grip of the chaotic chapter that followed.

However, despite the quality and organization of the introduction, from which I learned a lot of new things about the flagellum, I still felt that it was more a list of facts and not a true synthesis of the information, connecting the dots to get true insight into the flagellum and the vital importance of the tip in its biogenesis and function. This tendency was even more apparent in chapter 5.4.

This is a good sign to me as Hana has reached the important yet difficult step in a scientist's career to synthesize data to gain true and deeper understanding. I admit I find this task difficult as well, but appreciate how importance this skill is. **Thus, I make this request for the PhD defense, which of course is pending the requests of the Hana's other reviewer. I would ask if Hana better connects the dots of the vast and impressive data presented in chapter 5.4. Please try to better link evolutionary conservation, spatial and temporal localization patterns and RNAi phenotypes and other data (*e.g/* TIRF microscopy, motility tracking) to give us insight into a few proteins that Hana finds key. Do these key proteins show any general trends other than being enriched in the flagellar tip?** Perhaps she can relate this to the overall trends she observed. I actually think these data need to be presented this way: giving an overall picture of major trends in localization and tagging/RNAi phenotypes and then delving into deeper investigation the protein categories Hana has already defined based on flagellar length phenotypes, covering protein architecture, evolutionary conservation and other data together and not spread throughout the text as it is now. This may make it easier for Hana (and subsequently the reader) to connect the dots. **It is the writer's and not the reader's responsibility to make their case clear by connecting the dots.**

Despite my reservations, I reiterate my opinion that **Hana should be awarded the doctoral degree** for her hard and thorough work, which has advanced our knowledge of both trypanosomes and also the evolution

of the flagellum. I believe that Hana has it in her to make the next step and synthesizing data into information and convey it in a clear and understandable way, and only give this constructive criticism to encourage her in this direction.

Questions for discussion and an appendix documenting some example errors in text follow.

Hassan Hashimi

Questions for discussion (address **bold questions** at defense if time is limited):

1) **Throughout the introduction, Hana writes that the PFR is a kinetoplastid-specific structure. Is the PFR truly restricted to kinetoplastids? Can any evolutionary inferences be made about the distribution of the PFR in eukaryotes?**
2) **The carrot-like plugs that decorate the plus-ends of the A-microtubules and central pair (CP) in trypanosomes is a very fascinating structure. Is there anything known about the composition and/or function of these structures? Hana speculated that the fact these also are found appending the CP may confer unique properties to the tip of the trypanosomes flagellum. What kind of roles may this be? Is this trait restricted to kinetoplastids or trypanosomatids?**
3) Why can the flagella connector (FC) can be exposed extracellularly in PCF? Is the FC exposed in intracellular trypanosomatids like *Leishmania* or *Trypanosoma cruzi* or encapsulated like in BSF?
4) Why is the FC needed if the cell division can proceed normally *in vitro* even when the OF and NF are not connected, as seen upon depletion of investigated (both here and in previous work) FC components?
5) Just curious: why was SBF-SEM employed instead of conventional SEM to observe the anterior end of the trypanosomes in Figure 5 of the Mol. Microbiol. Article?
6) **Will the endogenous overexpression approach developed in the Mol. Biochem. Parasitol. Article work for all nuclear genome-encoded proteins?**
7) I really liked the attention paid to the evolutionary conservation of the flagellar tip proteins. But what can be inferred from a wide distribution of certain proteins? What about those restricted to certain lineages?
8) **Is it a problem that the functional conservation of motile flagellar tip proteins was addressed in human cell lines where primary cilia where induced, given the motile type has a central pair and the primary cilia lack these microtubules? Are there any other differences in the tip of primary cilia and the trypanosome flagellar tip (*e.g.* how A and B microtubules plus ends and aligned to each other)? Does this situation influence how these data are interpreted?**

9) **Since the flagellar tip is also a site for environmental-sensing and subsequent signal-transduction, was social motility and chemotaxis assayed in RNAi cell lines (induced, non-induced)? How can these traits be assayed? Which category of RNAi-cell lines should be given priority for such assays.**

10) **The reported (but not supported by empirical data) increase in motility velocity in certain RNAi-silenced cells that also resulted in epimastingote-like cell architectures or longer axonemes and cell bodies. Any hypothesis as to why these cells swim faster? Does trypanosomatid body morphology (*e.g* trypanomastigote, epimastingote, promastingote) affect the motility and velocity, *i.e.* are certain body forms faster/more agile than others *in vitro*?**

Appendix–Exemple mistakes in thesis:

1) Ambiguous/awkward sentences in introduction and Chapter 5.4. I cite an example from page 19 of the Introduction:

> These became proliferative epimastigotes which divide asymmetrically giving rise to epimastigotes with a long flagellum destined to die and short (13 µm long) flagellum that migrate to the salivary glands.

   This text sounds like the flagellum migrates to the salivary glands, not the epimastingote stage.

2) "…homology recombination machinery… (page 17)". Rather "homologous", and adjective form of homology.

3) "Contrary to motile cilia, such as respiratory cilia in airways ensuring movement of mucus in lungs, or sperms (page 11)." Rather "sperm". Uncountable nouns are written in singular form, *e.g.* hair.

4) FC1 is stated to be a kinesin (page 23) but gas rather a kinase domain

5) In chapter 5.4 the following statement seems to point to the wrong Figure:

> Interestingly, cell lines expressing YFP tagged variants of these proteins had conspicuously long axonemes reaching the highest lengths among all tagged cell lines with median lengths of OF axonemes in 2F2K2N cells above 23 µm (Fig. 12A)

   Shouldn't a part of Figure 4 be cited instead?