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Referee report on the Master thesis of Lucie Kafkova entitled:

**Functional characterization of two paralogs that are novel RNA binding proteins influencing mitochondrial transcripts of *Trypanosoma brucei***

The Master thesis of Lucie Kafkova describes the functional characterization of two proteins belonging to the MRB1 complex in the mitochondria of Trypanosoma Brucei.
The thesis starts with a short introduction describing the life cycle of Tryponosoma Brucei and the kinetoplast composition. The special structure of the mitochondrial RNA and the editing mechanisms are described in some details. Lucie reviews the current knowledge about the protein factors needed to carry out and control the complicated editing process. She describes the RNA editing core complex (RECC) and the RNA editing accessory factors forming the mitochondrial RNA binding complex 1 (MRB1). She briefly describes the known/hypothesized function of some of the proteins and summarizes the network of protein interactions previously revealed by the host laboratory from a yeast-two-hybrid screening.
After the introduction, a manuscript is attached that has been submitted for publication in the journal "RNA". In this manuscript Lucie is shared first author. Before the manuscript, she clearly states the goals of the project. In the following I will walk through the main investigations and findings of the manuscript, highlighting Lucie's contributions to it, as indicated at the beginning of the thesis.
The aim of the work is to characterize the function of two paralog proteins, which are part of the MRB1, kMAP1 and kMAP2. First, Lucie compares the coding sequence of the two proteins and identifies the N-terminal region as the only region differing between kMAP1 and kMAP2. Second, Lucie finds that both kMAP1 and kMAP2 bind to single-stranded RNA. In this investigation she uses UV cross-linking and gel electrophoresis.
The next steps in the characterization of the function of the kMAP1 and kMAP2 are: 1. Measurement of the affinity of kMAP1 for pre- and edited mRNA, as well as gRNA, via a double filter binding assay. The result of this experiment shows that kMAP1 binds mRNA preferentially over gRNA; 2. Proving the inclusion of

kMAP1 in a high molecular weight complex together with other subunits of MRB1 (via separation of complexes on glycerol gradients and subsequent visualization of the proteins by immunodecoration). The inclusion of kMAP1 in a larger complex is demonstrated to be RNA-enhanced; 3. Demonstration that kMAP1 and kMAP2 are part of distinct complexes of similar composition (via TAP-tag purification of kMAP1 and kMAP2 containing complexes and subsequent analysis by gel electrophoresis or mass spectrometry). Lucie has not contributed majorly to this part of the work, as stated at the beginning of the thesis.
Next Lucie generates cell lines with the capability of RNAi inducible single and double knockdown of kMAP1 and kMAP2. She quantifies the abundance of the transcripts in both single and double knockdown cells by qPCR, and monitors cell growth over two weeks. She finds that the double knock-down cells grow slower than wild-type, while the single knock-down behave normally, indicating that kMAP1 can compensate for the loss of kMAP2 and vice-versa. On the basis of these results, Lucie investigates the integrity of the high molecular weight RNP complexes involved in RNA editing in the knock-down cells, using separation of the complexes on glycerol gradients and immunoblotting for protein visualization. In agreement with the cell growth curves, she finds that in the double knock-down cells the assembly of the high molecular weight sub-complexes of the MRB1 is impaired, while in the single knock-down cells the effect is much less prominent.
In the next experiment Lucie investigates whether the knockdown of the kMAP proteins impairs the stability of gRNAs. To this end she uses Guanylyltransferase labeling of gRNAs and Northern analysis. She finds that the kMAP proteins are not relevant for gRNAs stability. In her last experiment Lucie investigates the effects of the knockdown on maxicircles and RNA editing using both qPCR and poisoned primer extension (PPE) assays. In summary, she finds a different effect of the double knockdown for moderately-edited or pan-edited RNAs and also different effects in kMAP1 and kMAP2 single knock-downs. This points to a non-overlapping role of kMAP1 and kMAP2 in vivo.
A discussion of the results follows in the manuscript, although it is not clear how much Lucie contributed to it. A second discussion is found outside of the submitted manuscript in the thesis. Here Lucie reviews her results, describes a possible role of the kMAP proteins in the final steps of editing of pan-edited RNAs and in the initial step of editing of moderately-edited RNAs, discusses possibilities for the differential role of kMAP1 and kMAP2 in sustaining protein-protein interactions. She also suggests some future experiments that should help to better characterize the role of kMAP proteins during the editing process.
The work done by Lucie in this master thesis is impressive. This adjective "impressive" refers not only to the quantity but also to the quality of the work. It rarely happens that a master student produces data in publication quality. Evidently Lucie was able to do so and in the short time of the master thesis (I suppose 6 to 12 months) achieved a first authorship on a very nice piece of work. I have not seen Lucie working in the laboratory and I am not able to judge her independency. However, the quality of the work reveals a high degree of scientific maturity.

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The quality of the written thesis is not as high as the quality of the lab work. The parts written by Lucie display a somehow "hard" language and the flow of thoughts is not very clear at all points. The discussion is conceptually sound but the presentation of the thoughts is contorted and requires a second reading in many instances. The manuscript is not very well integrated in the thesis. For example, I would recommend embedding the figures and figures captions in the text. I realize that the journal "RNA" requires the figures at the end; however, this makes the thesis quite unpleasant to read. Furthermore, the thesis does not contain any detailed description of the experimental procedures. The short description in "journal style" is usually not enough for future members of the laboratory to be able to repeat the experiments. Usually a master thesis should contain an appendix with experimental details and protocols. This would be very useful not only for Lucie but also for the hosting laboratory as future reference.
I certainly recommend accepting this thesis to grant Lucie the master degree. However, I recommend amending the thesis according to the comments of the previous paragraph (i.e. addition of detailed experimental protocols and embedding of figures and figures captions in the text).
Finally, I recommend evaluating Lucie's master thesis with 1, in view of the very high quality of the experimental work.

Yours sincerely,

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Summary of thesis

The Masters thesis of Bc. Lucie Kafkova entitled, '**Functional characterisation of two paralogs that are novel RNA binding proteins influencing mitochondrial transcripts of *Trypanosoma brucei***' describes a detailed analysis of two uncharacterised subunits of the recently described 'mitochondrial RNA binding complex 1' (MRB1), termed 'kinetoplastid mRNA associated proteins 1 and 2' (kMAP1/2). These paralogs are proposed to play roles, albeit them subtly different in terms of target transcripts, in mitochondrial mRNA editing.

General points

Without being overly familiar with Masters theses submitted at the University of South Bohemia, the format of a written introduction with project aims followed by an article (currently under consideration at the journal '*RNA*' that party contains Lucie's experimental results) finishing with a rather truncated discussion section (see also specific point 5 below) seems somewhat unconventional. I would have preferred it if the methods and results sections had been presented in dedicated sections of the thesis proper, with the submitted article attached as an appendix. In its present form it is difficult to know to what extent Lucie contributed to the writing of the submitted manuscript, that in its self forms the majority of her thesis.

However and notwithstanding this point it is commendable that her experimental work has been sufficient to merit consideration for publication in a worthy journal. Moreover, that the ambitious program of stated project aims appears to have been satisfied. Without being an aficionado of the RNA editing field, I did at times find the array of abbreviations and unique terms a bit bewildering but to Lucie's credit I found that she made a satisfactory effort to negate a problem that I suspect is inherent in the field. On the whole the written style of the thesis was good but it did lack a certain flow, being a little disjointed. Nonetheless it was certainly understandable. There were a number of typographical errors that I noted (listed below) but it is entirely possible I did not detect them all.

Specific points

1) Relating to the unique *T. brucei* chromosomal duplication event that has given rise to kMAP1 and kMAP2, can the author expand the reasons why they suspect the coding sequences of the kMAP paralogs have diverged so much relative to other neighbouring and related paralogous genes within the duplicated section (*i.e.* 77.1% homology in kMAPs versus 99.1% in MRB8180/4150 that are also found in the MRB1 complex)?

2) In connection to figure 2 and it's accompanying text, what is the reason for '*copious amounts of free GST-tag*' in the kMAP2 protein isolations used in the RNA binding assay (lane 8)?

3) Regarding figure 4 and the incorporation of kMAP1 into macromolecular complexes and the dependence of this effect on intact RNA. In the absence of RNAse cocktail, fractions containing kMAP immuno-reactivity do not always overlap with those containing immuno-rectivity to RECC markers. What does this tell you about kMAP1 incorporation into macromolecular complexes, particularly considering the immuno-reactivity from those denser fractions that lack RECC markers? Could this have implications for interpreting the RNAi knockdown phenotypes, for example the dKD kMAP1/2 growth retardation phenotype and its relation to perturbed mitochondrial gene derived mRNA editing?

4) In the thesis (although not manuscript) discussion, it is suggested the kMAP1/2 paralogs contain a conserved region that fulfills the same role in both proteins and a diverged N-terminal region that is responsible for additional interactions that could explain the differing effects on pan-edited mitochondrial transcripts in the RNAi mediated sKD of either kMAP1 or kMAP2 alone. How might one experimentally test this suggestion given what is known about kMAP1/2 interacting partners from the presented TAP-tag mass spectrometry experiments?

5) In the thesis discussion, a model is put forward suggesting that kMAP1/2 may play a role in the final stage of editing and that this would be related to the overlapping or non overlapping nature of individual editing blocks in pan-edited and moderately edited transcripts respectively. I would have found this easier to understand if the text had been accompanied by an explanatory figure. Can the author briefly explain this model now?

Conclusions

Taking everything into consideration, I can see now reason why this thesis of Lucie Kafkova cannot be recommended for conferment of the degree of Master of Science and I fully commend it to the examination committee.

Typographical/ grammatical errors

1) Page 1; NADH hedydrogenase (NADH dehydrogenase).
2) Page 2; Moderately-edited mRNA: transcripts that need editing only in a small part of thein (their) . . .
3) Page 7; Guide RNA (gRNA) hybridyzes (hybridises) . . .
4) Page 33; Because the kMAPs have been found in several . . . – *the sentence can not start with 'because'.*
5) Page 49, figure legend 2; (BSA indicated with "b") – *"b" is not in the figure.*
6) page 52, figure legend 9; . . . in which in which level above. . . – '*in which*' is repeated unnecessarily.

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