Přírodovědecká fakulta
Faculty of Science

Jihočeská univerzita v Českých Budějovicích
University of South Bohemia in České Budějovice

OPPONENT'S REVIEW ON BACHELOR/DIPLOMA* THESIS

Name of the student:

Thesis title: "Assembly of the unique *Trypanosoma brucei* F_1-ATP synthase"
Supervisor: RNDr. Alena Panicucci Zíková, PhD

Referee: Ignacio Durante, PhD
Referee`s affiliation: Laboratory of Molecular Biology of Protists, Institute of Parasitology, BC-CAS

	Point scale[1]	Points
(1) FORMAL REQUIREMENTS		
Extent of the thesis (for bachelor theses min. 18 pages, for master's theses min. 25 pages)**, balanced length of the thesis parts** (recommended length of the theoretical part is max. 1/3 of the total length)**, logical structure of the thesis**	0-3	2
Quality of the theoretical part (review) (number and relevancy of the references, recency of the references)	0-3	2
Accuracy in citing of the references (presence of uncited sources, uniform style of the references, use of correct journal titles and abbreviations)	0-3	2
Graphic layout of the text and of the figures/tables	0-3	1.5
Quality of the annotation	0-3	2
Language and stylistics, complying with the valid terminology	0-3	2
Accuracy and completeness of figures/tables legends (clarity without reading the rest of the text, explanation of the symbols and labeling, indication of the units)	0-3	2
Formal requirements – points in total		13.5
(2) PRACTICAL REQUIREMENTS		
Clarity and fulfillment of the aims	0-3	1.5
Ability to understand the results, their interpretation, and clarity of the results, discussion, and conclusions	0-3	2.5
Discussion quality – interpretation of the results and their discussion with the literature (absence of discussion with the literature is not acceptable)	0-3	2
Logic in the course of the experimental work	0-3	3
Completeness of the description of the used techniques	0-3	2.5

* Choose one

1 Mark as: 0-unsatisfactory, 1-satisfactory, 2-average, 3-excellent.

Experimental difficulty of the thesis, independence in experimental work	0-3	2
Quality of experimental data presentation	0-3	2.5
The use of up-to-date techniques	0-3	2
Contribution of the thesis to the knowledge in the field and possibility to publish the results (after eventual supplementary experiments)	0-3	1.5
Practical requirements – points in total		19.5
POINTS IN TOTAL (MAX/AWARDED)	**48/33**	**33**[2]

Comments of the reviewer on the student and the thesis:

Vladyslav Lazebnyk showed to have performed experiments aiming to dissect the assembly of the F_1-ATP synthase of *Trypanosoma brucei*. As explained in the thesis, this complex exhibits some unique features as compared with other model organisms as *S. cerevisiae*. The rationale of studying its assembly in *T. brucei* is therefore justified as attractive for the identification of possible drug targets.

The experimental strategy to analyze the assembly of F_1-ATP synthase was to knockdown by means of RNAi ATP11 and ATP12 assembly factors, involved in the assembly of F_1 alpha and beta subunits in yeast. The RNAi cell lines were already available in the laboratory, although no information about the previous identification of these factors in *T. brucei* is provided.

ATP11 and ATP12 mRNA knockdown was evaluated by quantitative PCR, showing that the knockdown of the former was less efficient. ATP11 and ATP12 protein levels after mRNA knockdown were not assessed due to unavailability of antibodies directed against these factors.

Evaluation of the growth phenotype showed that in both cases, growth was affected upon knockdown, as compared with uninduced cells. The effect was to a lesser extent for ATP11, and this maybe due to the reduced efficiency of the knockdown. More drastic effects might be expected under culture with low glucose medium conditions, although this was not done.

According to the work hypothesis, the steady state levels of some F_1-ATP synthase complex subunits were assessed by WB analysis on purified *T. brucei* mitochondrial extracts of induced and uninduced ATP11 and ATP12 RNAi cell lines. For ATP11 knockdown, the levels of both alpha N-term and C-term were affected early, and as well as Tb2 and OSCP later; p18 levels remained unaffected during the induction. The results for subunit beta were difficult to interpret. For ATP12 knockdown, similar results were obtained.

Complementarily to this analysis, blue native gels were prepared to evaluate the stability of the complexes in their native conformation. In this case, increment of beta and p18 subcomplexes were observed for ATP12 RNAi induced cell fractions, while lesser effect was observed for ATP11, probably due to inefficient RNAi knockdown. WB blot analysis of blue native gels improved the beta subunit read-out for both ATP11 and ATP12 knockdowns.

Lastly, given the observed reduced efficiency of ATP11 RNAi knockdown, an alternative RNAi construct was generated targeting a different region of ATP11 mRNA. It is stated that this new construct transfected in procyclic forms exhibited better knockdown efficiency evaluated by qPCR and a more drastic growth phenotype, but the results are not shown.

In general, the thesis is very well written and structured, with adequate citation, although

[2] Enter the number of points awarded.

often incomplete. The theoretical introduction is well suited to place the questions, aims and relevance of the study. The methodology is explained with greater detail and is of significantly higher extent than the results section. This was probably due to the COVID pandemic, that compromised the repetition of procedures and further characterization of the recombinant strains, as it is mentioned several times in the thesis. The results are well presented and commented. The conclusions are adequate considering the results obtained and the lack of repetitions and complementary experimental data.

Overall, this is a very good thesis, that shows proper knowledge of the matter by the student and adequate experimental work for a bachelor defense.

Suggestions and questions, and other observations are stated in the corresponding section.

Suggestions and questions, to which the student has to answer during the defense. Mistakes, which the students should avoid in the future:

For clarity, the observations are placed in order of appearance in the text, divided by sections. Questions to be answered by the student are in bold.

First Part – Introduction

- 1 (minor): "parasitic" is not a suitable term for an enzyme.
- 2: In the index, there are incorrectly numbered sections.
- 3 (minor): the size is not probably as relevant as other distinctive characteristics of *T. brucei*.
- 4: More elaboration could be provided for the "metamorphosis" (the proper term would be differentiation) during *T. brucei* life cycle. **Why is it complex?**
- 5: The morphological features of BFs seem to be linked to the fact that *T. brucei* can traverse BBB.
- 6: The tripartite attachment complex it is not defined properly as well as the location of the kinetoplast. In this section, citation is missing regarding the kinetoplast and kinetoplast encoded genes.
- 7: "The different environments provided by the insect vector or the mammalian host means that the parasite must utilize various carbon sources to produce energy in the form of ATP"... could be better formulated. The next sentence is not properly cited.
- 8: „Since BF parasites encounter excess levels of glucose in the blood, it is sufficient and preferable to just utilize glycolysis to generate 2 net ATP per 1 molecule of glucose" ... citation is incomplete.
- 9: „Since these proteins encode in the nucleus of the cell, BF mitochondria have to keep a membrane potential for the protein import. In such scenario, the FOF1-ATP synthase hydrolyzes ATP and rotates in reverse orientation, in order to pump protons into the mitochondrial inner membrane space. This activity of the enzyme is essential in the BF parasite." ... citation missing.
- 10: „Nevertheless, the main pathway for ATP production for PF *T. brucei* is through oxidative phosphorylation with proline as the main energy source. In this case, energy metabolism operates in its classical way, transforming the mechanical work of ATP-synthase into chemical energy in a form of ATP molecules" ... citation missing.
- 11: „converts potential energy into *physical* energy." … chemical or kinetic energy are proper terms. Kinetic in the example.
- 12: "Dimerization of ATP-synthase in yeast occurs at the angle of 86^{o} so two adjacent monomers resemble a V-shaped nexus. This specific angle determines the unique curved shape of the mitochondrial inner membrane and allows ATP synthase dimers to fold into the

rows, which in turn ensures the most effective ATP production during energy metabolism“ ... **Is it always like this in other taxa?**

- 13: In Figure 8, it is not indicated which represent A and B panels.
- 14: When the aims are presented, there is no mention to *T. brucei* ATP11 and 12 and neither in the rest of the thesis. **How were they identified? What are the corresponding GIs of these orthologues in *T. brucei*? Given the central role of this proteins in the thesis, this information should be provided. Is there something that could be elaborated regarding for example, presence of conserved, functionally relevant domains in *T. brucei* orthologues with respect to other taxa, as welll as differences, etc?**

<u>Second Part - Materials and Methods</u>

- 15: Tet *operon* is not correct.
- 16: Citation for the Smox cell line is missing.
- 17: Table1. It is not possible to prepare the medium as it is formulated. It is sterile medium?
- 18: “Cell lines should generally not be maintained for more than 6-8 weeks as this can lead to the selection of mutants that have lost genetic controls introduced into the cell line or have altered metabolism due to extended time in the culture. To prevent the degradation of genetic modifications over time, cell lines could be cryopreserved in liquid N2“ ... **Is this documented somewhere? Where?**
- 19: Table 2. Again, the units, volumes and preparation of the solution are not correctly specified.
- 20: 2.3. The calculation of the cumulative growth is not clearly defined.
- 21: 2.4.1. **Is there an alternative method to follow the protein levels after knockdown that does not rely in the obtention (and characterization) of antibodies? How this method could be implemented in the current problem?**
- 22: 2.4.1 The principle of quantitative PCR is not clearly explained and citation is missing.
- 23: 2.4.2 **What is the principle of nanodrop quantitation?** The criteria for determining purity and concentration are never explained throughout the thesis.
- 24: Table 4. To be consistent with other Tables, DNAse should have units.
- 25: 2.4.4. „Depending on the concentration, each RNA sample was diluted appropriately to generate a working solution of 0.4 µg/µl. A total of 2 µg of each RNA sample was then aliquoted into duplicate 200 µl thin-wall PCR tubes“ ... for clarity, it should be added to the MMix after. I had to calculate that the remaining volume of 5 ul
- 26: Table 8. **The parameters of the qPCR are not explained.**
- 27: 2.4.5. *randomly* is not correct. **How the values „plugged“ in the formula vary and what is the meaning of this?** Eq. 1 is not clearly explained and the efficiency not clearly defined.
- 28: 2.5 *antibodies*.
- 29: 2.5 *to amplify the signal* is incomplete regarding secondary antibodies.
- 30: Given the relevance that the antibodies have in this study, information regarding their source, targeted antigen and other characteristics should be provided.
- 31: 2.6.2 *12,00* is not correct
- 32: **What does it mean protein „complexity“?**
- 33: Calculations for protein concentrations are not explained, nor its theoretical background, or a representative example of the calibration curve obtained. **Details of these procedures should be provided.**
- 34: „RNAi is a powerful molecular biology tool to rapidly discern the function of a protein.“ ... RNAi is a tool to knockdown the expression of a protein, it is a poerful reverse genetics tool, but discerning the function of a protein is a much more complicated process, specially for

hypothetical proteins.

- 35: „*Believing* these molecules to be viral in origin, the *T. brucei* RNAi defense mechanism employs dicer-like proteins to cleave the dsRNA into small interfering RNAs (siRNAs)“ ... Animism and finalism should be avoided as a general basis, the sentence should be reformulated in a more accurate scientific manner.
- 36: In general, the section describing RNAi knockdown is not cited.
- 37: Figure 14, the fragments of the ATP11 inserted in the 2 MCS of the vector do not seem to be identical, **Is there any explanation for that?**
- 38: „Other unique features of the pAZ055 RNAi plasmid include positive selectable markers for both bacteria and *T. brucei*. The plasmid encodes a secreted β-lactamase that degrades the antibiotic ampicillin, allowing only bacteria that retain the plasmid to grow.“ ... These are rather general features of shuttle vectors used in *T. brucei* and other organisms.
- 39: **Why the EP promoter is present in the vector?**
- 40: „Furthermore, to increase the transcript stability of the selectable marker, the *T. brucei* aldolase 5’ untranslated region and the actin 3’ untranslated region flank the coding sequence“ ... This is rather to the transcript to be expressed.
- 41: (Table16) Percentage of agar is missing.
- 42: „The length of the dsRNA should be between 400-600 base pairs (bp) for the optimal RNAi depletion of a target gene in *T. brucei*. It has also been demonstrated that targeting various regions of the coding sequence can lead to different levels of RNAi depletion for the same gene product.“ ... citations are missing.
- 43: „With these restrictions in mind, we utilized the powerful Primer3 software to first suggest the best primer pairs to amplify a region of ATP11 to target by RNAi.“ ... **What other VERY important precautions have to be taken into account when designing RNAi primers? Is there any additional tool that could have been used for primer design in that sense?**
- 44: Table 20. **The parameters of the PCR are not explained.**
- 45: 2.7.5 **How 3:1 insert:vector molar ratio for ligation was achieved? Eq. 2 is not explained.**
- 46: „Such an abrupt change of temperature created a pressure difference between the inside and outside of the cells, which in turn caused the formation of pores in the cell membrane through which supercoiled plasmid DNA could enter.“ ... This is not cited and I would say it is not the reason why the heat-shock favours DNA entering the cells.
- 47: 2.7.6 **Is there an alternative, more specific method to screen for positive clones after cloning?**
- 48: 2.7.8 **Is there any particular reason why the BTX procedure for transfection was used and not the AMAXA machine that is available in the laboratory?**

<u>Third part - Results</u>

- 49: „Expectedly, the growth phenotype was not overly dramatic, as the cells were grown in a glucose rich medium and thus were not completely dependent on ATP synthase for ATP production. Therefore, even this intermediate growth effect suggests that the function of these two assembly factors will be essential when the parasites are grown in a more physiologically relevant medium“ ... This is overstated and speculative, considering also that the growth in SDM-80 was not addressed. **Why the results are considered to prove essentiality of the factors? What other reasons could explain that the phenotypes are not so drastic?**
- 50: Figure 20. Rotules for the y axes are missing. It would be good to specify the percentages of knockdown in each case, since they are not obvious from the graphs.
- 51: „The milder growth phenotype observed for ATP11 RNAi is likely due to a less efficient

RNAi cell line. Therefore, the function of ATP11 is probably just as important as ATP12." ... This has to be taken carefully, since nothing is known about the protein levels.

- 52: Figure 22. The y and x axes rotules are missing. **How is the quantitation performed? The results for beta subunit are simply stated as „hard to interpret", is there an explanation why in some cases, the quantification shows an increase in the later time points?**
- 53: **Why it is claimed that Tb2 is not changed when according to the quantification it seems to decrease?**
- 54: The amounts of protein loaded in the BN gels are not specified. It would be good to quantify the results for other subunits showed in the complementary WB analysis.
- 55: 3.5 There is an incomplete sentence
- 56: Figure 27. There are two lanes indicated as one. The MW weight of the digested product is not indicated.
- 57: „The digested pAZ055 migrates as a single band above 5000 bp, which is consistent with the size of the linearized plasmid." The size is not indicated.
- 58: „Any undigested plasmid contaminating our linearized plasmid prep would preferentially be taken up by bacteria in the downstream transformations, thus making our cloning efforts very difficult. After the gel extraction, the isolated linearized plasmid was determined to be 154 ng/µl, with an A260/A280 ratio of 1.92." ... purity criteria not explained.
- 59: „Based on the GC content of the primers, we chose an annealing temperature of 52 ^{o}C for the PCR reaction." ... **According to what criteria?**
- 60: Figure 28. **What is the difference between the lanes?**
- 61: „With this confirmation, the linearized plasmid was then transfected into the PF pSMOX *T. brucei* cell line. After the positive selection of transfected clones, the growth curves and qPCR analyses were completed by others in the lab (data not shown). As expected, the qPCR analysis quantified a much higher level of ATP11 depletion, similar to the levels that I calculated for the ATP12 RNAi cell line. Due to this more efficient ATP11 RNAi cell line, the observed growth defects in the tetracycline induced cells was also larger, on par with those detected in the ATP12 RNAi cells." ... It is a pity that this data is not shown at all.

<u>Fourth Part - Discussion</u>

- 62: Citation missing in the first paragraph.
- 63: „However, once the RNAi cell lines are adapted to SDM-80, a low glucose medium, the parasites become more dependent on the FoF1-ATP synthase. Furthermore,the ATP11 and ATP12 RNAi plasmids could be transfected into the blood-stream form stage of the parasite, where the ATPase activity is absolutely essential for maintaining the mitochondrial membrane potential. It is only under these conditions that it can been demonstrated that the assembly factors ATP11 and ATP12 provide a vital function in the parasite [23]." ... **What would be expected, based on reported data, to observe in such scenario?**
- 64: „To answer this, we performed a qPCR analysis that revealed the ATP12 transcript was indeed significantly more reduced compared to ATP11." ... this was not statiscally contrasted, therefore significance cannot be stated.
- 65: „While the qPCR indicated discrepancies between the RNAi cell lines, the quantification of the targeted transcripts is not always an accurate predictor of protein expression." ... I would say it is hardly ever a good predictor of protein expression. **How the strategy could be modified to overcome this limitation?**
- 66: „Indeed, the new ATP11 RNAi cell line I generated targeting a different region of the ATP11 coding sequence was later found by my colleagues to more efficiently deplete ATP11 and produce a more significant growth defect (data not shown)." ... Again, it is a pity this is

not shown. **What was the percentage of improvement of the knockdown efficiency? Is there any possible explanation why different regions of a transcript could show different knockdown efficiencies when targeted for RNAi?**

- 67: „Since we anticipate that these gene products will be essential“ ... **Why?**
- 68: I would say that the *T. brucei* F_1 catalitic subunit is divergent rather than unique.

Additional questions

It would be interesting to provide some insight into the evolutionary background of ATP synthase. Are there some other „peculiarities“ in other taxa with respect to other model organisms as for example, yeast?

Why only beta and p18 subunits are evaluated by BN-gels?

What methods other than BioID could be applied to identify new factors involved in ATP synthase assembly? How these strategies would be implemented?

It is clearly established that COVID imposed several restrictions to the concretion of the experimental part of the thesis. Apart of the mentioned growth evaluation in SDM-80 medium, what other couls be performed in the RNAi strains that would relate to ATP11 and ATP12 RNAi-produced alterations? How these assays would be implemented?

Final comments

I congratulate Vladyslav Lazebnyk for a very well written, well-structured, and amenable thesis that deserved careful reading and introduces very interesting preliminary results and questions regarding the assembly of ATP synthase in *T. brucei*.

Conclusion:

In conclusion, I r e c o m m e n d * the thesis for the defense and I suggest the grade very good.[3]

In Ceske Budejovice date 20th Jan 2022

……

signature

[3] You can suggest a grade, which can be modified during the defense based on the presentation. However, if the reviewer is not present at the defense, the grade will not be counted. Grades: excellent (1). Very good (2), Good (3), Unsatisfactory/failed (4).