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: **Konstantin Kaltenböck**

Thesis title: *CRISPR/Cas9 gene editing in Drosophila melanogaster*
Supervisor: Mgr. David Doležel, Ph.D.

Referee: **RNDr. Pavlína Věchtová, Ph.D.**
Referee`s affiliation: **University of South Bohemia in Ceske Budejovice, Faculty of Science AND Biology Centre, Czech Academy of Sciences, Institute of Parasitology**

	Point scale[1]	Points
(1) FORMAL REQUIREMENTS		
Extent of the thesis (for bachelor theses min. 18 pages, for masters 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	1.5
Quality of the theoretical part (review) (number and relevancy of the references, recency of the references)	0-3	1
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	0
Quality of the annotation	0-3	2.5
Language and stylistics, complying with the valid terminology	0-3	1.5
Accuracy and completeness of figures/tables legends (clarity without reading the rest of the text, explanation of the symbols and labelling, indication of the units)	0-3	0.5
Formal requirements – points in total **21**		9
(2) PRACTICAL REQUIREMENTS		
Clarity and fulfilment of the aims	0-3	1.5
Ability to understand the results, their interpretation, and clarity of the results, discussion, and conclusions	0-3	1
Discussion quality – interpretation of the results and their discussion with the literature (absence of discussion with the literature is not acceptable)	0-3	1.5
Logic in the course of the experimental work /review	0-3	2

* *Choose one

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

Completeness of the description of the used techniques	0-3	0.5
Experimental difficulty of the thesis, independence in experimental work	0-3	1.5
Quality of experimental data presentation	0-3	0
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	0.5
Practical requirements - points in total **18**		10.5
POINTS IN TOTAL (MAX/AWARDED)	**39**	19.5

Comments of the reviewer on the student and the thesis:

Presented bachelor thesis by Konstantin Kaltenböck describes the process of CRISPR/Cas9 genome editing procedure of a timeless gene in the model insect species Drosophila melanogaster. The CRISPR/Cas9 tool is currently the most popular and easy-to-use genome editing tool and its implementation in the well-established genetic model such as Drosophila provide good base to learn and practice the procedure for proper implementaton of this editing tool. This work was done under the supervision of dr. David Doležel. The thesis ranges 30 pages and thus complies with the formal requirements for bachelor thesis at the Faculty of Science.

The aims of the thesis are formulated in four clearly defined thematic points. The introduction is six pages long and briefly describes the principle of CRISPR/Cas9 genome editing tool and the role and mechanism of Circadian clocks.
The Materials and Methods chapter is written on six pages and contains the description of all methods used during this bachelor work.
The Results chapter presents all the collected data on eight pages.
The Discussion chapter extends over two pages followed by short conclusion and a list of 31 references.

There are several formal flaws I would like to highlight.

Introduction
1) The information about CRISPR/Cas System would deserve a more detailed information. I am missing a lot of important details such as the description of a Tracer RNA which is, in my opinion, a crucial part of CRISPR/Cas9 system, it is only mentioned later in the introduction, however, without any description of its role or function in this system. I am also missing a note about the possibility to design a guide rRNA to both DNA strands or the identity of bacteria where the CRISPR/Cas9 was identified or which PAM sequences are currently known and used, etc. All these information are, in my opinion, important and a less knowledgable reader has to spend a lot of time by searching for these information elsewhere, in order to be able to understand what is described in the rest of this thesis.

Materials and Methods
1) In the chapter 5.1 gRNA design there is a mention that NetNES 1.1 and NetPhos 3.1

were used to predict nuclear localization signal and phosphorylation sites of the TIM protein. However, I did not find out until the discussion chapter what was the motivation to perform the prediction of these regions. Ideally a subchapter describing experimental design including motivation underlying chosen experimental procedures would help to prevent the reader's confusion about the presented methodology and results.

2) **Chapter 5.2 "Vector design" would be more understood if a figure with the map of the vector would be present. It took me quite a while to find all the information necessary to understand the procedure in this chapter For example, why was the BbsI restriction endonuclease used? Why was the gRNA designed with 4bp overhangs or which antibiotic resistance was used for transformants selection in subchapter 5.2.1?** On the contrary, there are unnecessary descriptions of manufacturers' protocols from DNA gel extraction and plasmid DNA isolation kits present where the method section would much more conveniently manage only with a reference to the manufacturers instructions or protocol.

3) None of the used materials is properly named and referenced. Each of the used chemical should be named using its entire official name followed by the name of the manufacturer in the brackets. **For example, there is not a single word describing a bacterial strain of *E. coli* used to propagate the plasmid containing the designed gRNA.** Similarly, I could not find a name of the manufacturer of SOC or LB medium, the manufacturer of PPP master mix is listed incorrectly and in completely different paragraph of the method subchapter. These flaws can be repeatedly found throughout entire methods section. The names of used instruments, such as PCR thermal cyclers or gel documentation systems are completely absent throughout entire work.

4) In the subchapter 5.2.1 "Plasmid transformation" an X-gal is used, however, I could not find anywhere in the preceding or the following text the motivation for its usage or the evaluation of the resulting LB plate using this chemical.

5) **In the subchapter 5.2.2 "Plasmid DNA isolation" the description implies that the vector propagation was done directly from single colonies without previous amplification of the selected bacterial colonies in liquid media.** I believe, that bacteria amplification prior plasmid DNA isolation is done in the majority of the existing plasmid DNA isolation protocols.

6) **I could not find any information about the identity of amplicon that was used in the colony PCR in subchapter 5.2.3 "Colony PCR". Usually, the PCR primers along with their sequence and the length of the expected amplicon should be supplied either in the method or in the results chapter.**

7) **In the subchapter 5.4. "Germ line screening" I could not find any reference to the mysterious "squishing buffer"** and again, **I could not find any description of the PCR product and the respective primers that were used to screen the transformed germ line cells.**

8) In the subchapter 5.5. "Crossing scheme" a Cyo/Sco balancer is used. I could not find anywhere in the thesis what it is and what it is good for. Additionally, I don't understand what the "PCR SDS-Page screening", mentioned in this chapter, means.

Results

1) **In subchapter 6.1 "gRNA", I could not find any information about the nature and position of PAM sequence in the second gRNA.** This information is present at the description of the first gRNA and I believe it is important to describe it also at the second gRNA as well at least for the sake of consistency.

2) The Figure 5. documentation was not done properly. The exposition was too long and it is nearly impossible to see the fragments of both products as well as of the size reference. Additionally, the individual fragments of the size reference are not marked with their appropriate sizes, which does not allow for the proper size identification of the presented amplicon. The size labelling of reference fragments is also absent in all other figures presenting the results of agarose gel electrophoresis.

3) **The figure 7. in chapter 6.3. presents the agarose gel electrophoresis results. The figure is supposed to demonstrate the selection of G_0 flies which carried the wild type and mutant alleles of the target amplicons thus forming heteroduplexes that can be detected by the presented results of the heteroduplex mobility screening. Unfortunately, the quality and the absence of any labels or indicators in the figure disallows the reader to identify any difference between each sample. It would be much more convenient to draw at least arrows that would point at the differences that the author used to select the candidate flies carrying the aforementioned heteroduplexes. A complementary description of the heteroduplex appearance, including its size, would also be greatly appreciated.**

4) The description of primers and amplicon in subchapter 6.4 is also absent. The results presented in Figures 10 and 11 are completely absent in the main text and are only limited to the legends of these figures.

Discussion

1) Very important information describing the results collected in this thesis are mentioned in the Discussion chapter for the very first time such as the use of transformed flies expressing gRNA and Cas9 nuclease that were already available in the laboratory. The description or at least a reference to a publication which describes these transformed fly strains should be present in the thesis.

2) The information in the first four paragraphs were completely omitted in the introduction and are only presented as a part of discussion which forces a reader to read entire thesis again in order to understand the collected results in the context of the topic which should be fully covered in the introduction.

3) I believe that a useful discussion point would be the comparison of the results with other works that also used the nuclear localization signal or phosphorylation sites to interfere with the function of the targeted genes.

General comments

1) There is not a single reference to any figure throughout the text of the thesis. I believe that it is a generally accepted consensus to refer to a figure, table, or graph in the preceding text in any scientific publication.

2) There are lots of stylistic and grammar mistakes, which sometimes disallow to understand the meaning of the sentence entirely, as for example the fourth sentence of the

introduction: "In the first step, the viral DNA is recognized a fragment, the protospacer incorporated."
There are several more sentences like that throughout the thesis.

Conclusion:

In conclusion, I would like to highlight that the thesis topic is very interesting, the amount and the quality of the experimental work that underlies this thesis is also good. However, the thesis itself seems to be written hastily and majority of my comments regards plentiful formal flaws, typos and obviously overlooked mistakes. I also appreciate the good level of English used to compose the thesis despite the typos and other language mistakes mentioned eralier. Despite the number of comments that I presented above I

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the thesis for the defence and the grade will be suggested after the defence presentation and also based on the answers to my comments above that are written in bolt and also based on the questions that are given below.

Questions:

1. What are the common procedures to prevent off-target and increasing on-target effect using CRISPR/Cas9?
2. Please, suggest an experiment that would verify that the function of the nuclear localization signal of the gene of interest was successfully eliminated by genome editing in your experiment in the Fruit fly.
3. Please, describe the Cyo/Sco balancer and its function in *D. melanogaster* mentioned on page 14 of your thesis.
4. What is the function of X-gal mentioned on page 12?
5. Could you, please, describe the detailed procedure of Fruit fly ovaries injection by injection mentioned on page 13?
6. Could you present any other research work that targeted either nuclear localization signal and/or a phosphorylation site to eliminate/modulate any gene function in any other organism?

In **České Budějovice** 14th of June, 2021

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