MASARYK UNIVERSITY
FACULTY OF SCIENCE
DEPARTMENT OF EXPERIMENTAL BIOLOGY

REVIEW OF THE PhD THESIS

Title: Gene targeting in Silkworm (*Bombyx mori*) by Engineered Endonucleases

Applicant: Suresh Sajwan, MSc.

Field: Molecular and Cellular Biology and Genetics

The topic of the thesis is original, current and relevant in the context of up-to-date research in the gene targeting technologies based on engineered nucleases. The author established methodology of directed mutagenesis by two classes of engineered endonucleases, ZFNs and TALENs, using the color marker genes of the silkworm as targets. Main conclusion of the thesis is that both ZFNs and TALENs are useful and promising tools for further gene targeting applications in the silkworm. Considering the fact that the field of the thesis has been almost unexplored, there is no doubt that to reach this conclusion, the author had to undergo laborious experimental work.

Formally, the text of thesis is composed of two main parts. In the first part, the world of the endonuclease engineering is introduced by review of the current knowledge in this field. This paragraph takes less than 6 pages (plus one figure) and to my surprise, it seems to be the only part of the thesis written solely by the applicant. Then, the second part dealing with material and methods, results and discussion follows. Importantly, these key parts of the thesis are presented simply as copies of two original papers published in Insect Biochemistry and Molecular Biology and supplementary data provided to the journal. The applicant is one of six (one of nine, respectively) co-authors of these papers. Although his contribution to at least one of them is undoubtedly significant (shared first authorship), it is impossible to judge which parts of the papers were written by the applicant himself and, therefore, it is not clear to me what should be the subject of my review. Since the papers were successfully published in well-respected international journal and therefore underwent rigorous reviewing by its editor, there is no reason to raise any criticism. Using published papers as parts of the PhD thesis is acceptable. However, the author should provide

the reader with additional information dealing with the thesis, such as aims of the thesis that do not necessary need to overlap with the aims of the multi-author papers, comments on the results obtained that were not included in the papers, discuss more general aspects of the studies and so on. This would definitely improved quality of the thesis and documented presenting capability of the applicant clearly.

Overall, the experimental work performed by Suresh Sajwan that resulted in two valuable research papers is fully in agreement with requirements for PhD. thesis in the field of Molecular and Cellular Biology. In contrast, the written part of the thesis is too short and below the standards in the field. To acknowledge experimental effort and pioneering work in the mostly unexplored field of the research and after successful defense of the PhD thesis, I recommend it to be accepted and doctoral degree be awarded to Suresh Sajwan.

Questions and points to discussion:

1. The color marker *BmBlos2* and *Bmwh3* genes were targeted by engineered endonucleases in this study. Based on "know-how" and experience obtained, what other Silkworm genes would be recommended by the applicant to target in a future and why?
2. Is there any way how to reach tissue-specific gene knock-out using the engineered nucleases?

March 7, 2013

Prof. RNDr. Jan Šmarda, CSc.

Review of the Ph.D. Thesis by MSc. Suresh Sajwan:
Gene targeting in Silkworm (*Bombyx mori*) by engineered nucleases

The Ph.D. Thesis by Suresh Sajwan focuses on the use of novel methodologies to perform genome engineering, namely the use of zinc finger nucleases .
Ph.D. Thesis consists of two papers published in impacted journals (one with Suresh Sajwan as the equal first author). Specific contribution of Suresh Sajwan in the two published papers is stated. Overall, the presented Ph.D. Thesis contains a very short introduction (7 pages including one Figure) and two papers in their original format as printed in the journal including all published supplementary information. Following the two papers there is one page (p.56) devoted to conslusions. Appendix contains maps and sequences of the two plasmid vectors prepared by Suresh Sajwan. Not considering already published texts, which are of course of high quality, the pages in the thesis written by Suresh Sajwan are well readable and comprehensively written.

Minor comments to Introduction text and Formal questions.

1) Introduction contains some new literature references (CRISPR paper, 2013) which is very good. On the other hand I would appreciate references and thorough discussion on the use of targeting vectors or single stranded oligonucleotides for genome manipulation in conjuction with ZFNs and TALENs.
2) On p. 4, RVD NN targets G or A not only G.
3) Reference Mandell and Barbas is out of order in the reference list.
4) ready to use ZFNs are not provided by Sangamo but by Sigma-Aldrich, that uses Sangamo algoritms.
5) The example on p.5 on ZFNs specificity. Argument that ZFN with three fingers „would recognize 23-24bp, long enough to ensure specificity in any genome" is not correct because only 18bp are specifically recognized (spacer can not be counted as a specific DNA sequence because any nucleotide sequence can be present in spacer region).
6) The claim that TALENs are more specific as they bind longer sequences is in most cases true but formally such statement is not correct-it depends on the design of particular TALEN or ZFN (Sigma provides 5 or even 6 finger ZFNs).
7) On p.7 it is argued that yeast assay can select engineered nucleases with low toxicity. I am not sure about that claim. Yeast assay can hardly say anything about toxicity in a model organism for which ZFNs/TALENs are developed. Toxicity in a given animal is due to the nonspecific (off-target) cleavage of genomic DNA by ZFNs/TALENs. Given the difference between genomic sequence of yeast and *Bombyx* it is unlikely that toxicity in yeast can be correlated with toxicity in *Bombyx* (or any other animal).
8) Contribution of Suresh Sajwan to publication Sajwan et al. is perhaps understated, as listed experiments cover only a very limited area. Who did TALEN construction by Golden Gate cloning? Who did injection of TALENs into *Bombyx* ? Who did mutational analysis and sequencing? Who did genotyping, crossing of *Bombyx* and scoring of the phenotypes? Perhaps some of these tasks were performed by the defender and not claimed in the thesis.

Scientific questions.

1. TALENs have very relaxed rules and well-behaving code. Still, not every sequence can be targeted by TALEN. What is the most important constraint?
2. What was the reason to design TALENs with so many RVDs (e.g. BLT-1, 28 and 24 RVDs).
3. Perhaps the most important contribution of the defender was the deveopment of a yeast assay. However, there is no correlation observed between data in yeast and Bombyx – BLT-1 TALEN was by far the worst in yeast but did work the best in Bombyx. Notably, there is no discussion in the manuscript Sajwan et al., 2013 of this discrepancy. Please discuss your ideas about why the tests in yeast do not correlate with data in *Bombyx*.

Conclusion: Ph.D. Thesis by Suresh Sajwan is recommended for defense at JCU.

RNDr. Zbynek Kozmik CSc.
Prague, March 22, 2013.