



Efficient genome editing by CRISPR-Mb3Cas12a in mice

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Original submission

First decision letter

MS ID#: JOCES/2019/240705

MS TITLE: Efficient multiplex genome editing using CRISPR-Mb3Cas12a in mice

AUTHORS: Zhuqing Wang, Yue Wang, Shawn Wang, Andrew J Gorzalski, Hayden McSwiggin, Tian Yu, Kimberly Castaneda-Garcia, Huili Zheng, and Wei Yan
ARTICLE TYPE: Short Report

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: <https://submit-jcs.biologists.org> and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Wang et al describes the use of Cas12a for genome editing in mice. The rationale is that Cas12a targets a different PAM motif than the more commonly used Cas9 and is

proposed to have reduced off-target effects. However, the use of Cas12a has been hindered so far by inefficient editing and the long PAM sequence (TTTV), which restricts the number of gene loci that could be edited. Here, the authors have tested two different PAM motif sequences and different length of the spacer. They find that both TTV and TTTV PAM motifs result in efficient genome editing, and that an increased spacer length of 23 nt is superior to the previously used 20 nt spacer. Further, they show successful editing in mice and germline transmission of edited loci in mice. Overall, this is a very good paper, technically well done and of interest to the community. There is not much insight into new biology, but that's not the point of this paper as it mainly presents an improved method.

Comments for the author

I have one major concern: the methods are not appropriately described. Often, the authors refer to "as previously described" or alternatively just say "PCR reactions were conducted...", "T7EI assay was followed..." without describing how these assays were done. Similarly, the legends do not give enough detail to understand what the Figures show. For a person who is not familiar with genome editing, these Figures will be very difficult to understand.

Reviewer 2

Advance summary and potential significance to field

In this manuscript the authors demonstrated that the *Moraxella bovoculi* (Mb) Cas12a enzyme can be utilized to edit the genome of murine zygotes to generate both knock-in and knock-outs. The authors demonstrated >70% editing efficiency of the murine genome using an alternative TTV PAM sequence (as opposed to the TTTV PAM typically utilized for this enzyme). The authors demonstrate that MbCas12a can also utilize a streptavidin conjugated approach to generate knock-ins.

Although previous reports by the Zhang group demonstrate that this Cas12a enzyme can efficiently edit the genome in HEK293 cells, the authors do advance the field by demonstrating that direct injection of the Mb3Cas12a mRNA into murine zygotes can indeed edit the genome utilizing a more flexible PAM sequence. Nevertheless, several questions need to be addressed in the revision.

Comments for the author

Major Points:

1. The authors indeed demonstrate editing in vitro in HEK293 cells in figure 1b, 1c, 2b and 3c via T7E1 endonuclease assay and show sanger sequencing tracks for their in vivo editing in figure 1d, 2c and 3c. However they do not quantify editing at the genetic level. Editing should be quantified via TIDE or other means of quantifying indels of at least 3 independent replicates as editing efficiency can be vary with transfection/injection efficiency in the above-mentioned figures.
2. The authors mention that the advantage of utilizing enzyme is decreased off-target editing however they show no off-target analyses to substantiate this claim. Off-target analyses should be performed for the in vivo studies at a minimum.
3. In order to truly establish that mice generated in Figure 1 and Figure 3 are knockouts and knock-ins respectively, analyses at the protein level is required i.e. western blot or immunohistochemistry.
4. What is the knock-in efficiency utilizing this enzyme without the biotinylation conjugation? This is an important control that is missing from the study.

Minor Points:

1. Why did the authors utilize two different sites in Figure 1a and Figure 1b? The authors should demonstrate editing in a mouse fibroblast cell line in vitro of the same locus as they edited in Figure 1b in-vivo.

2. Was there a non-target control utilized for these studies? Editing using the non-target control at the target locus should also be shown in the figures.
3. The authors seem to define editing efficiency as the number of animals successfully edited. This is slightly misleading in the CRISPR field as editing efficiency implies quantification of genomic editing. The authors should consider revising this throughout the manuscript to “% of animals successfully edited.”
4. Can the authors speculate why the TTV PAM sequenced targeting was mono-allelic?
5. How large were the large deletions identified in this study? The large deletions identified should also be validated via PCR.
6. The authors identify four residues that they claim are responsible for conferring Mb3Cas12a with the ability to process its own crRNAs and elegantly demonstrate that the enzyme can indeed process its own crRNAs. In order to fully substantiate the claim that these residues are responsible in this enzyme, the authors should mutate these residues in order to abolish that function.

First revision

Author response to reviewers' comments

Point-by-point reply to reviewers' comments:

[We appreciate both reviewers for their meticulous review of our manuscript. Following their comments, we revised our manuscript by performing more experiments and polishing the text.](#)

Reviewer 1 Advance Summary and Potential Significance to Field:

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Reviewer 1 Comments for the Author:

I have one major concern: the methods are not appropriately described. Often, the authors refer to “as previously described” or alternatively just say “PCR reactions were conducted...”, “T7EI assay was followed...” without describing how these assays were done. Similarly, the legends do not give enough detail to understand what the Figures show. For a person who is not familiar with genome editing, these Figures will be very difficult to understand.

[Reply: Agreed. We added more details in methods and figure legends.](#)

Reviewer 2 Advance Summary and Potential Significance to Field:

In this manuscript the authors demonstrated that the *Moraxella bovoculi* (Mb) Cas12a enzyme can

be utilized to edit the genome of murine zygotes to generate both knock-in and knockouts. The authors demonstrated >70% editing efficiency of the murine genome using an alternative TTV PAM sequence (as opposed to the TTTV PAM typically utilized for this enzyme). The authors demonstrate that MbCas12a can also utilize a streptavidin conjugated approach to generate knock-ins.

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Reviewer 2 Comments for the Author:

Major Points:

1. The authors indeed demonstrate editing in vitro in HEK293 cells in figure 1b, 1c, 2b and 3c via T7E1 endonuclease assay and show sanger sequencing tracks for their in vivo editing in figure 1d, 2c and 3c. However, they do not quantify editing at the genetic level. Editing should be quantified via TIDE or other means of quantifying indels of at least 3 independent replicates as editing efficiency can be vary with transfection/injection efficiency in the above-mentioned figures.

Reply: Agreed. We performed quantification using a previously described method (Cong et al., 2013), and the percentage of indels is now marked in all of the figures.

2. The authors mention that the advantage of utilizing enzyme is decreased off-target editing however they show no off-target analyses to substantiate this claim. Off-target analyses should be performed for the in vivo studies at a minimum.

Reply: Agreed. We performed off-target analyses, and found off-target effects of Mb3Cas12a. The data were added (Page 6, lines 16-27).

3. In order to truly establish that mice generated in Figure 1 and Figure 3 are knockouts and knockins respectively, analyses at the protein level is required i.e. western blot or immunohistochemistry.

Reply: Agreed. We run a genome editing core and have to transfer the mice to the Pls' labs as soon as positive founders are identified. Unfortunately, we do not have any of these mice in hands and thus, cannot perform Western blots. (FYI-We attempted to contact the Pls, but they seemed quite reluctant to collaborate as if we are trying to "steal" something from their mice. We hope that this reviewer understands our situation.)

4. What is the knock-in efficiency utilizing this enzyme without the biotinylation conjugation? This is an important control that is missing from the study.

Reply: Agreed. We performed microinjections of 2-cell embryos using the non-biotinylated donor together with Mb3Cas12a-mSA and the crRNA, and obtained no positive knock-ins among 36 mice generated although the T7E1 assays clearly indicated that the enzyme did work. The new data were added (Page 6, lines 11-14).

Minor Points:

1. Why did the authors utilize two different sites in Figure 1a and Figure 1b? The authors should demonstrate editing in a mouse fibroblast cell line in vitro of the same locus as they edited in Figure 1b in-vivo.

Reply: These two panels were meant to address two different questions. Figure 1a is to show effects of different lengths of crRNA on the efficiency of Mb3Cas12a, whereas Figure 1b is to demonstrate how microinjection site affect the efficiency of Mb3Cas12a. We rarely pre-test efficiency using mouse fibroblast cells because we have found that success in mouse fibroblast cells does not grantee good targeting in mouse zygotes. Also, with the increased efficiency, we can

always obtain edited founders from even one injection attempt.

2. Was there a non-target control utilized for these studies? Editing using the non-target control at the target locus should also be shown in the figures.

Reply: Yes. We used no-crRNA as the non-target control, as shown in Fig. 1B. Non-target control may not be necessary because of the following: 1) When 17nt crRNAs are used, this method generated no editing, as shown in Fig. 1A, B. 2) Even with the optimized combination of Mb3Cas12a and 23nt crRNAs, many showed no editing (e.g., #27, #33 and #35 in Fig. 1C).

3. The authors seem to define editing efficiency as the number of animals successfully edited. This is slightly misleading in the CRISPR field as editing efficiency implies quantification of genomic editing. The authors should consider revising this throughout the manuscript to “% of animals successfully edited.”

Reply: Agreed. The reason why we did our way was that people tend to care more about positive animals rather than editing efficiency in individual mice. Nevertheless, we revised the text throughout by stating “% of animals successfully edited.”

4. Can the authors speculate why the TTV PAM sequenced targeting was mono-allelic?

Reply: Mb3Cas12a displays lower editing efficiency in general when TTV PAM is used instead of TTTV PAM. Thus, we believe that the TTV PAM targeting tend to yield mono-allelic mutations because of lower targeting efficiency. We added this into discussion in the main text (Page 5, lines 23-24).

5. How large were the large deletions identified in this study? The large deletions identified should also be validated via PCR.

Reply: As discussed in the main text, Mb3Cas12a mostly induces indels rather than large deletions, which may be due to the stagger ends generated by Mb3Cas12a endonuclease in conjunction with the MMEJ DNA repair mechanism. The largest deletions that we observed is 43 bp, which occurred in P1 and P4 in the miR-10b locus targeted by 3 crRNAs (Fig. S2C).

6. The authors identify four residues that they claim are responsible for conferring Mb3Cas12a with the ability to process its own crRNAs and elegantly demonstrate that the enzyme can indeed process its own crRNAs. In order to fully substantiate the claim that these residues are responsible in this enzyme, the authors should mutate these residues in order to abolish that function.

Reply: The four residues responsible for crRNA processing were identified by others (Fonfara et al., 2016). By alignment analyses, we found that these four residues were conserved among Mb3Cas12a and its three orthologs (FnCas12a, AsCas12a and LbCas12a). We revised the sentence to clarify this point (Page 4, lines 4-9).

References:

Cong, L., Ran, F. A., Cox, D., Lin, S. L., Barretto, R., Habib, N., Hsu, P. D., Wu, X. B., Jiang, W. Y., Marraffini, L. A. et al. (2013). Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* 339, 819-823.

Fonfara, I., Richter, H., Bratovic, M., Le Rhun, A. and Charpentier, E. (2016). The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA. *Nature* 532, 517-21.

Second decision letter

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ARTICLE TYPE: Short Report

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

Thank you. No further comments.

Comments for the author

Thank you.

Reviewer 2

Advance summary and potential significance to field

In this manuscript the authors demonstrated that the *Moraxella bovoculli* (Mb) Cas12a enzyme can be utilized to edit the genome of murine zygotes to generate both knock-in and knockouts. The authors demonstrated >70% editing efficiency of the murine genome using an alternative TTV PAM sequence (as opposed to the TTTV PAM typically utilized for this enzyme). The authors demonstrate that MbCas12a can also utilize a streptavidin conjugated approach to generate knock-ins. Although previous reports by the Zhang group demonstrate that this Cas12a enzyme can efficiently edit the genome in HEK293 cells, the authors do advance the field by demonstrating that direct injection of the Mb3Cas12a mRNA into murine zygotes can indeed edit the genome utilizing a more flexible PAM sequence.

Comments for the author

The authors have satisfactorily addressed this reviewers comments.