

## SHORT REPORT

## Efficient genome editing by CRISPR-Mb3Cas12a in mice

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## ABSTRACT

As an alternative and complementary approach to Cas9-based genome editing, Cas12a has not been widely used in mammalian cells largely due to its strict requirement for the TTTV protospacer adjacent motif (PAM) sequence. Here, we report that Mb3Cas12a (*Moraxella bovoculi* AAX11\_00205) can efficiently edit the mouse genome based on the TTV PAM sequence with minimal numbers of large on-target deletions or insertions. When TTTV PAM sequence-targeting CRISPR (cr)RNAs of 23 nt spacers are used, >70% of the founders obtained are edited. Moreover, the use of Mb3Cas12a tagged to monomeric streptavidin (mSA) in conjunction with biotinylated DNA donor template leads to high knock-in efficiency in two-cell mouse embryos, with 40% of founders obtained containing the desired knock-in sequences.

**KEY WORDS:** Genome editing, CRISPR, Cas9, Cas12a, Cpf1, PAM

## INTRODUCTION

The rapid advancement of CRISPR-Cas-based genome editing technologies has made their clinical applications increasingly promising. However, major obstacles still remain, including safety concerns due to both off-target effects and large on-target deletions or insertions (Adikusuma et al., 2018; Fu et al., 2013; Hsu et al., 2013; Kosicki et al., 2018; Lee and Kim, 2018), and the requirement of proper PAM sequences for efficient and precise cleavage by the commonly used endonucleases, for example, Cas9 and Cas12a/Cpf1 (Komor et al., 2017). The Cas12a endonuclease differs from Cas9 in many aspects and thus, has been considered as an alternative and complementary tool to Cas9. First, the most commonly used *Streptococcus pyogenes* (Sp)Cas9 and Cas12a [*Acidaminococcus* sp. BV3L6 (As)Cas12a and *Lachnospiraceae* bacterium ND2006 (Lb)Cas12a] utilize the NGG and TTTV PAM sequences, respectively, for efficient genome editing (Cong et al., 2013; Jinek et al., 2012; Wang et al., 2013; Zetsche et al., 2015). Second, Cas12a is guided by a single short CRISPR RNA (crRNA), and can efficiently process its own crRNAs, while Cas9 is directed by dual RNAs consisting of a crRNA and a tracrRNA or by a joint large single-guide RNA (sgRNA), and rarely processes its own crRNAs (Cong et al., 2013; Fonfara et al., 2016; Jinek et al., 2012; Zetsche

et al., 2017a). Third, Cas12a displays reduced off-target effects compared to SpCas9 due to its irreversible binding to the target region and strong discrimination against off-target sequences (Kim et al., 2017; Kleinstiver et al., 2016; Strohkendl et al., 2018). Finally, Cas9 causes large on-target deletions or insertions (Adikusuma et al., 2018; Kosicki et al., 2018; Lee and Kim, 2018), whereas Cas12a only generates staggered DNA overhangs (Zetsche et al., 2015), which lead to a much lower rate of large on-target deletions or insertions due to the preferred microhomology-mediated end joining (MMEJ) repair mechanism. Despite the advantages, practical applications of Cas12a have been severely hindered due, at least in part, to its strict requirement for the TTTV PAM sequence. Although the PAM sequence for *Francisella novicida* U112 (Fn)Cas12a has been shown to be YTV (Y stands for C/T and V for A/C/G), the actual editing efficiency of the YTV PAM sequences in mammalian cells remains rather low (Tu et al., 2017; Zetsche et al., 2015). Inspired by a recent report suggesting that Mb3Cas12a (*Moraxella bovoculi* AAX11\_00205) edits HEK-293 cells at a much higher efficiency through the TTV PAM sequences than does AsCas12a and LbCas12a (Zetsche et al., 2017b preprint), we explored whether Mb3Cas12a could be utilized for efficient genome editing to produce knockout (KO) and knock-in (KI) mouse lines.

## RESULTS AND DISCUSSION

To determine whether Mb3Cas12a is active in mouse zygotes, we first tried to use one crRNA harboring a 20 nt direct repeat (DR) sequence with a 20 nt spacer recognizing the TTTV PAM sequence of *Prps111* (Table 1; Fig. S1A,D), a testis-specific gene dispensable for spermatogenesis (Wang et al., 2018). One out of six founders obtained had an indel (insertion or deletion) (16.7%) (Table 1; Fig. S1A,D), suggesting that Mb3Cas12a indeed works in mouse zygotes. Previous studies have shown that FnCas12a, AsCas12a and LbCas12a are capable of processing their own crRNAs (Fonfara et al., 2016; Zetsche et al., 2017a), and that residues H843, K852, K869 and F873 in FnCas12a are required for crRNA processing (Fonfara et al., 2016). Given that these four residues are highly conserved among Mb3Cas12a and its three orthologs (FnCas12a, AsCas12a and LbCas12a) (Fig. S1E), we hypothesized that the Mb3Cas12a could also process its own crRNAs. To test this, we designed crRNAs harboring two 20 nt spacers recognizing two TTTV PAM sequences or two TTV PAM sequences at the *Saraf* locus (Table 1; Fig. S1B,F). The 20 nt spacers were separated by two 20 nt DRs. One out of three founders was edited by spacer 1 targeting the TTTV PAM sequence (33.3%) from the first crRNA, and one out of four founders was edited with the spacer 2 (25%) targeting the TTV PAM sequence from the second crRNA (Table 1; Fig. S1B,F). Next, we designed one single crRNA containing two spacers recognizing the TTTV and TTV PAM sequences in the *Mrv1* locus, respectively (Table 1, Fig. S1C,G). Out of five founders, one was edited with the spacer recognizing the TTTV PAM sequence (20%), but none for the TTV PAM sequence (Table 1; Fig. S1C,G).

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**Table 1. Editing efficiency of Mb3Cpf1 in murine zygotes**

Target genes	Length of spacer	No. of spacer in crRNA used	PAM sequence	Site of microinjection	No. of zygotes injected and transferred	No. of newborns (birth rate) <sup>a</sup>	No. of mutants (targeting efficiency) <sup>b</sup>	Indel/KI	Mono-allelic mutation	Bi-allelic mutation	Multi-allelic mutation
<i>Prps111</i>	20 nt	1	TTTV (Spacer 1)	Cytosol (zygote)	22	6 (27.3%)	1 (16.7%)	Indel	Y	N	N
<i>Saraf</i>		2	TTTV (Spacer 1)		22	3 (13.6%)	1 (33.3%)		Y	N	N
			TTTV (Spacer 2)				0 (0%)		N	N	N
		2	TTV (Spacer 1)		21	4 (19%)	0 (0%)		N	N	N
			TTV (Spacer 2)				1 (25%)		Y	N	N
<i>Mrv1</i>		2	TTTV (Spacer 1)		23	6 (26.1%)	1 (16.7%)		Y	N	N
			TTV (Spacer 2)				0 (0%)		N	N	N
<i>Kcnj10</i>	23 nt	2	TTTV (Spacer 1) and TTV (Spacer 2)	Pronucleus and cytosol (zygote)	39	14 (35.9%)	4 (28.6%)		Y	Y	N
<i>miR-10b</i>		3	TTV (Spacer 1)	Cytosol (zygote)	20	13 (65%)	10 (77%)		Y	N	N
			TTTV (Spacer 2)	Cytosol (zygote)	12	5 (41.7%)	1 (20%)		N	Y	N
			TTTV (Spacer 3)				5 (100%)		N	Y	N
<i>Prps1, Prps2 and Prps111</i>		3	TTTV (Spacer 1 targeting <i>Prps1</i> )		30	11 (36.7%)	11 (100%)		N	Y	Y
			TTTV (Spacer 2 targeting <i>Prps2</i> )				1 (9.1%)		N	Y	Y
			TTTV (Spacer 3 targeting <i>Prps111</i> )				11 (100%)		Y	N	N
<i>miR-547 and miR-509</i>		3	TTTV (Spacer 1 targeting <i>miR-547</i> )		38	6 (15.8%)	6 (100%)		N	Y	Y
			TTTV (Spacer 2 targeting <i>miR-509</i> )				6 (100%)		N	Y	Y
			TTV (Spacer 3 targeting <i>miR-509</i> )				1 (16.7%)		Y	N	N
<i>Slit2</i>		2	TTTV (Spacer 1)	Two-cell	23	5 (21.7%)	2 (40%)	KI	N/A		
			TTV (Spacer 2)								

<sup>a</sup>(number of newborns/number of zygotes injected and transferred)×100; <sup>b</sup>(number of mutants/number of newborns)×100.

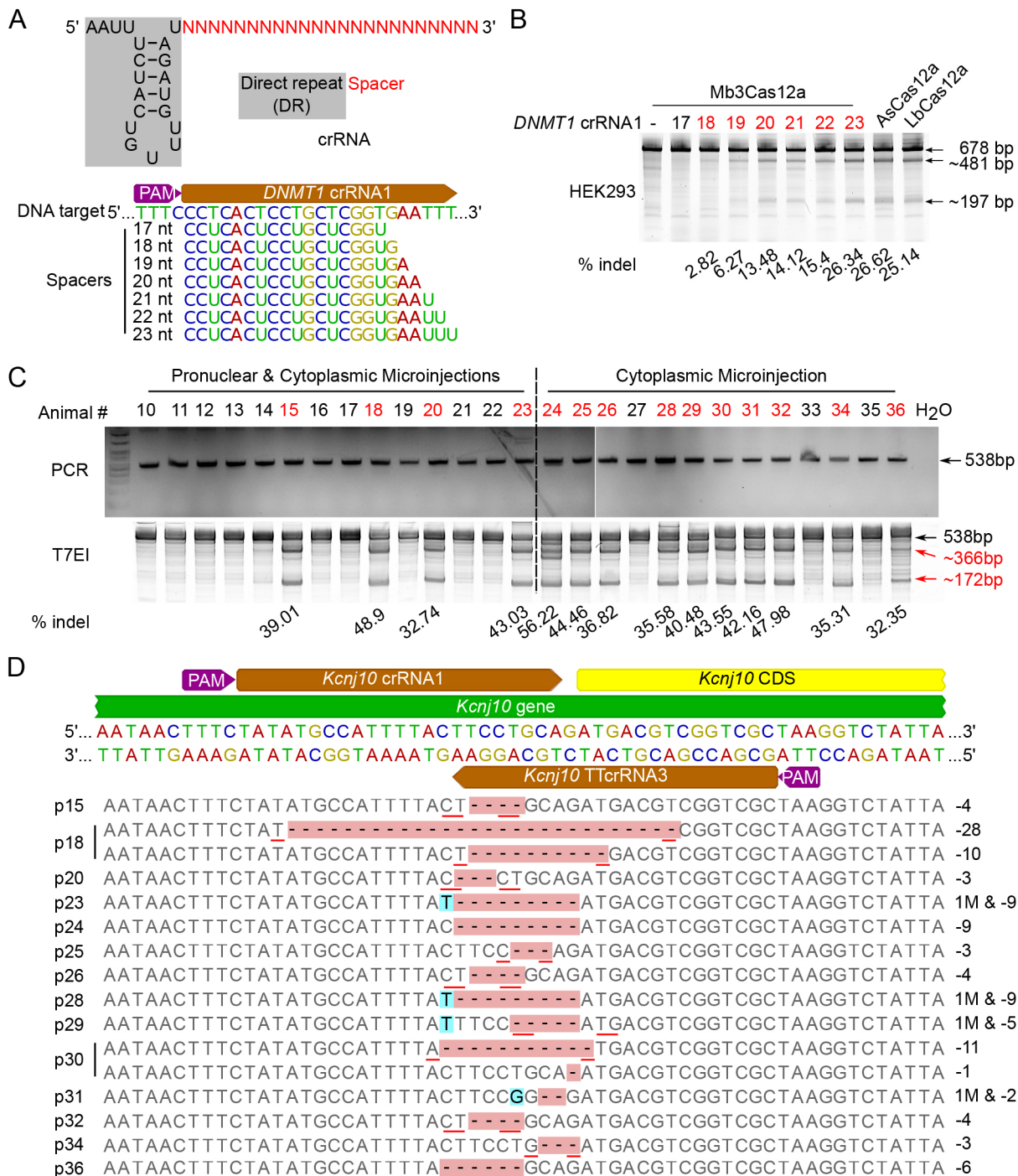
These results suggest that Mb3Cas12a can target genomic DNA with either the TTTV PAM or the TTV PAM sequence.

Although Mb3Cas12a can edit the genome in mouse zygotes, the efficiency appeared to be low. We sought to optimize the editing efficiency by adjusting either the crRNA length or microinjection sites. We first tested the effects of crRNA length on genome editing in the *DNMT1* locus in HEK-293 cells (Fig. 1A,B). No Mb3Cas12a activities were detected when no spacer or a 17 nt spacer was used, whereas the highest Mb3Cas12a activities, comparable to those for AsCas12a and LbCas12a, were observed in the crRNAs with a 23 nt spacer (Fig. 1B). Although FnCas12a uses 21 nt crRNAs more efficiently (Tu et al., 2017), Mb3Cas12a, similar to AsCas12a and LbCas12a, appears to prefer 23 nt crRNAs. We then examined the potential effects of microinjection sites (cytoplasmic versus pronuclear) on targeting efficiency by injecting Mb3Cas12a mRNA and one *Kcnj10*-targeting crRNA harboring two spacers (one recognizing the TTTV PAM sequence, and the other specific for the TTV PAM sequence) into either the cytoplasm or both the pronucleus and cytoplasm of the zygotes. Interestingly, the cytoplasmic-only microinjection yielded more edited mice (77%,  $n=20$ ) than the combined (pronuclear+cytoplasmic) microinjection (28.6%,  $n=39$ ) (Table 1, Fig. 1C,D). Taken together, our data suggest that the use of crRNAs with a 23 nt spacer in conjunction with cytoplasmic microinjection represents a highly efficient method to generate indels using Mb3Cas12a in murine zygotes.

To explore whether Mb3Cas12a could target multiple loci simultaneously, we used one crRNA harboring three 23 nt spacers recognizing the TTTV PAM sequences in three *Prps* family genes, including *Prps1*, *Prps2* and *Prps111*. Eleven founders obtained were all edited by both the *Prps1* and *Prps111* crRNAs (100%), whereas

one (9.1%) was edited only by the *Prps2* crRNA (Table 1; Fig. S2A). These results suggest that Mb3Cas12a can indeed process its own crRNAs and edit multiple loci in murine zygotes. Next, we used a crRNA containing three 23 nt spacers separated by three 20 nt DRs to target *miR-10b* (Table 1, Fig. S2B,C). Two of the spacers (spacers 2 and 3) were designed to recognize the TTTV PAM sequence, whereas the other targets the TTV PAM sequence (spacer 1) in the *miR-10b* locus. All five founders were edited by the two spacers targeting the TTTV PAM sequence (100%), and one of them was edited by the one recognizing the TTV PAM sequence (20%) (Table 1; Fig. S2B,C). Moreover, the edited alleles were successfully transmitted to the next generation (Fig. S2D). Similar results were obtained when we used one crRNA containing one 23 nt spacer targeting *miR-547* with the TTTV PAM sequence and two 23 nt spacers targeting *miR-509* with the TTTV PAM and the TTV PAM (Table 1, Fig. 2). All six founders were edited by the two spacers targeting the TTTV PAM sequence (100%), and one of them was edited by the one recognizing the TTV PAM sequence (16.7%) (Table 1, Fig. 2B,C). Although the 23 nt spacers recognizing the TTTV PAM sequence often led to bi- or multi-allelic targeting, and those recognizing the TTV PAM sequence often yielded mono-allelic targeting, the 20 nt spacers appeared to cause mostly mono-allelic mutations (Table 1, Figs 1D, 2C; Figs S1, S2). The mono-allelic targeting may reflect lower Mb3Cas12a editing efficiency when 20 nt spacers or TTV PAM sequences are used. Recent studies have shown that Cas9 with one single gRNA tends to induce large indels in genomic DNA in mouse embryonic stem cells (ESCs), progenitor cells and zygotes (Adikusuma et al., 2018; Kosicki et al., 2018), whereas Cas9 with two gRNAs often causes large deletions within the two flanking gRNA-targeting sites (Wang et al., 2018, 2020).

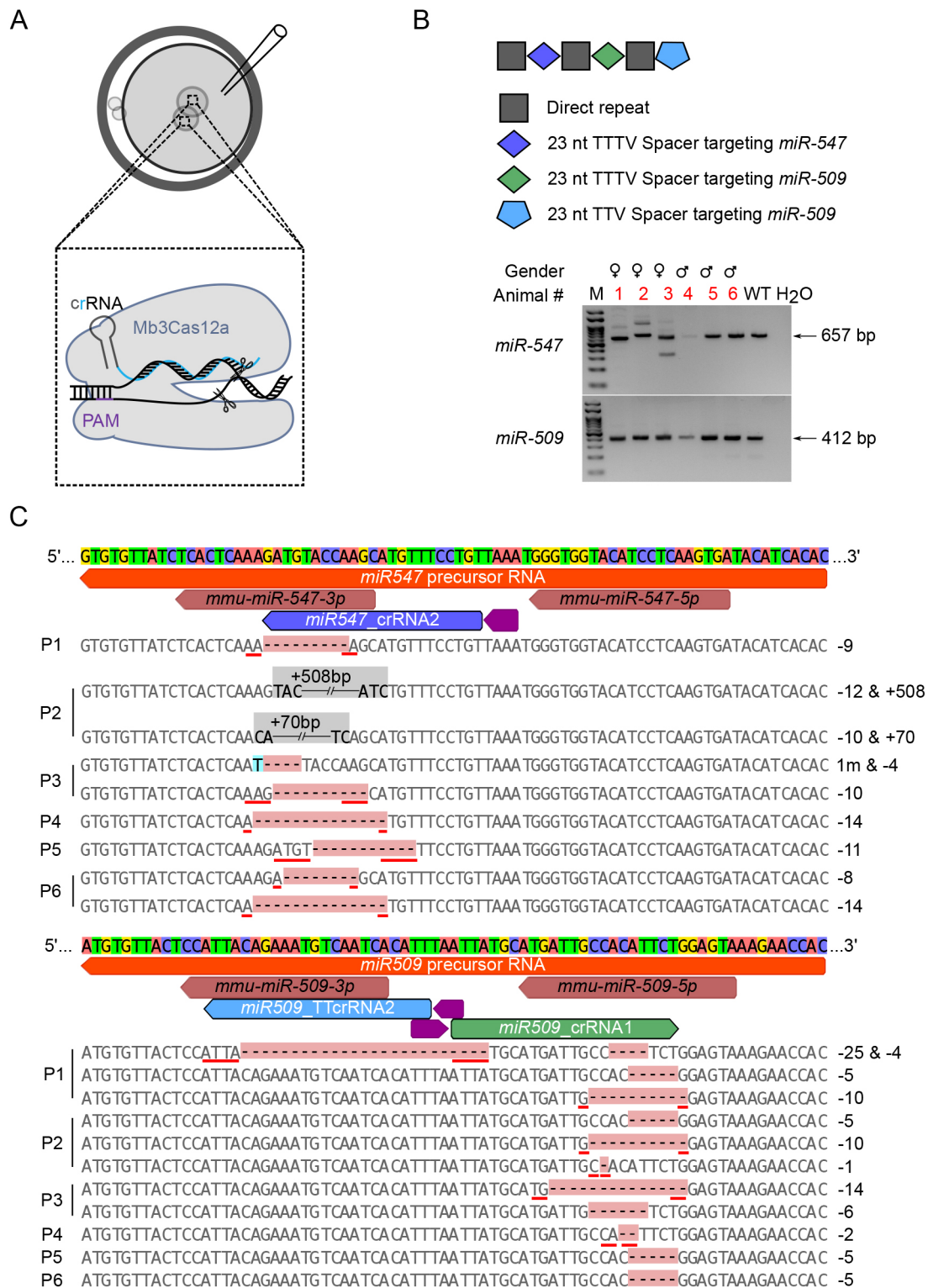




**Fig. 1. Optimization of Mb3Cas12a-based genome editing through adjusting crRNA length and microinjection sites.** (A) Structure and sequences of the crRNAs used to target the *DNMT1* locus. Upper panel, structure of the crRNAs used, which contain both the 20 nt DR (highlighted in gray) and the 23 nt spacer (red). Lower panel, crRNAs with spacers of variable length, which recognize the TTTC PAM sequences (purple) and were used for targeting *DNMT1* in HEK-293 cells. (B) T7EI assay results of the Mb3Cas12a-edited *DNMT1* locus in HEK-293 cells. The edited samples are shown in red, and AsCas12a and LbCas12a served as positive controls. The sizes of the expected bands after T7EI assays are indicated on the right side of the gel. The percentage of indels are indicated at the bottom of the gel. (C) PCR (upper panel) and PCR-T7EI (lower panel) to identify the efficiency of Mb3Cas12a-based genome editing in the *Kcnj10* locus after either combined pronuclear and cytoplasmic microinjection or cytoplasmic microinjection only in murine zygotes. The edited samples are shown in red. Sizes of the expected bands after T7EI assays are indicated on the right side of the gel. The percentage of indels are indicated at the bottom of the gel. (D) crRNAs used to target *Kcnj10* locus (upper panel) and Sanger sequencing results of Mb3Cas12a-based genome editing in *Kcnj10* locus in murine zygotes (lower panel). Upper panel, PAM sequence is marked in purple, whereas crRNAs are shown in brown. Lower panel, red underlines represent microhomology sequences; the pup number (p) and the total number of edited bases are indicated on the left and the right sides of the Sanger sequencing results, respectively. M, mismatch.

However, Mb3Cas12a-based genome editing appears to generate predominantly indels, because 39 out of the 42 pups derived from Mb3Cas12a-based editing all displayed indels, and only three

contained large insertions (7.1%) (Table 1, Figs 1D, 2C; Figs S1, S2). The lack of large on-target insertions or deletions in Mb3Cas12a-based genome editing suggests that the MMEJ mechanism may



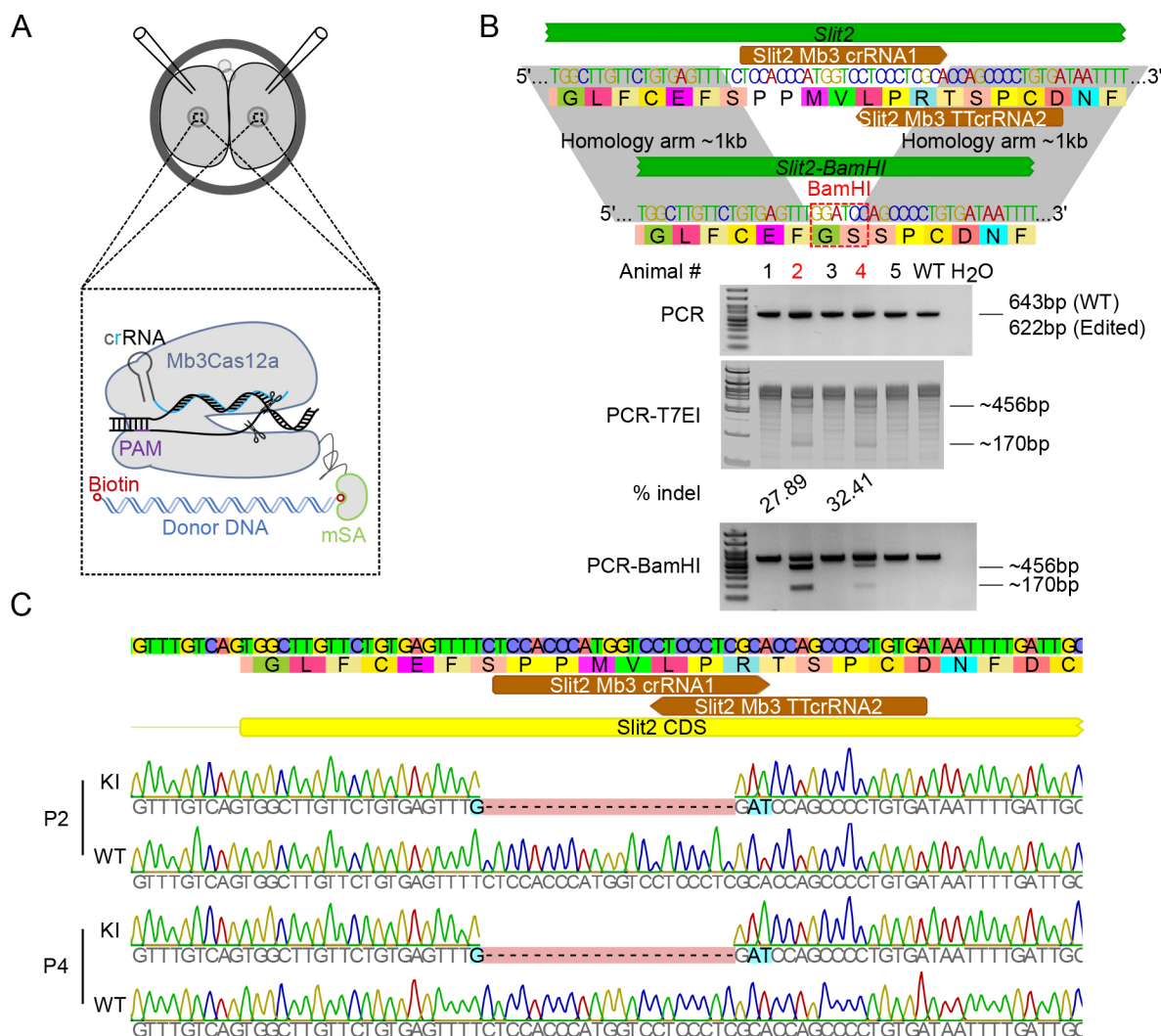
**Fig. 2. Multiplex targeting efficiency of Mb3Cas12a-based genome editing in *miR-547* and *miR-509* loci with the TTV PAM sequence in murine zygotes.** (A) Schematics showing the strategy used to generate indels in mouse zygotes. Mb3Cas12a mRNA and 23 nt crRNA are microinjected into the cytoplasm of mouse zygotes. The black line represents genomic DNA, purple and cyan lines indicate the PAM sequence and spacer, respectively. (B) PCR genotyping results of Mb3Cas12a-edited *miR-547* and *miR-509* founders. One crRNA with one 23 nt spacer targeting *miR-547* with a TTV PAM (diamond) sequence and two 23 nt spacers targeting *miR-509* with one TTV PAM (pentagon) and one TTV PAM (diamond) sequences were used to target the *miR-547* and *miR-509* loci. Squares in gray represent the DR. The edited founders are marked in red, and the corresponding genes and expected band sizes are indicated on the left and right sides of the gels, respectively. (C) Sanger sequencing results of Mb3Cas12a-edited pups 1–6 (P1–P6) at the *miR-547* and *miR-509* loci. Red underlines represent microhomology sequences, whereas the sizes of large insertions are highlighted in gray. Spacers used are marked using the same color as those in B. The PAM sequence is shown in purple. The 508 bp and 70 bp insertions in P2 match a retrotransposon (ERV1-MaLR) and a fragment from the X chromosome (chrX, 67987576–67987641), respectively.

prefer staggered DNA ends for repair. Interestingly, the large insertions detected in the three founders appeared to be endogenous retroviruses type-L (ERVL) and mammalian apparent LTR retrotransposon (ERVL-MaLR) sequences. A similar phenomenon has been reported in SpCas9-induced double-stranded breaks (DSBs) (Ono et al., 2015), suggesting these retrotransposons may hijack the DSBs induced by either SpCas9 or Mb3Cas12a.

Two-cell homologous recombination (2C-HR)-CRISPR, where Cas9 is tethered with monomeric streptavidin (mSA), which binds to biotinylated DNA donor template, has been shown to have higher KI efficiency (Gu et al., 2018). Given that, we tested whether Mb3Cas12a-mSA could do the same in generating KI mice (Fig. 3A,B). We microinjected two-cell embryos with Mb3Cas12a-mSA mRNA, the *Slit2*-targeting crRNA and the biotinylated DNA donor template (Fig. 3A). Two out of five founders obtained (40%) contained the KI alleles (Fig. 3B,C). In contrast, microinjection of

the non-biotinylated donor together with Mb3Cas12a-mSA mRNA and the *Slit2*-targeting crRNA generated no positive founders among 36 pups, although T7EI assays verified that Mb3Cas12a worked (Fig. S3A). These results indicate the Mb3Cas12a-mSA indeed can generate KI mice efficiently.

To test whether Mb3Cas12a could induce off-target effects, we predicted the off-targets using Cas-OFFinder (Bae et al., 2014) and performed T7EI assays on those predicted off-targets. T7EI assays revealed that Mb3Cas12a, just like other Cas12a endonucleases (Kleinstiver et al., 2019; Kocak et al., 2019), also has off-target effects (Fig. S3B–D). Further work is needed to improve its specificity, which may be achieved by either modifying the endonuclease (Kleinstiver et al., 2019) or by using hairpin gRNAs (Kocak et al., 2019). For example, a mutation in enhanced (en)AsCas12a (N282A) can turn it to a high-fidelity endonuclease, whereas a mutation in AsCas12a (R1226A) renders it the nickase that cleaves the non-target DNA



**Fig. 3. Generation of a KI in *Slit2* locus using Mb3Cas12a-mSA in mouse two-cell embryos.** (A) Schematics showing the strategy used to generate a KI in two-cell mouse embryos. Mb3Cas12a-mSA mRNA, crRNA and biotinylated donor DNA template are microinjected into two-cell mouse embryos. Purple and cyan lines indicate the PAM sequence and spacer, respectively. The blue double-stranded helix indicates the donor DNA with biotin (red open circle). (B) The efficiency of Mb3Cas12a-mSA-mediated KI at the *Slit2* locus in mouse. One crRNA harboring two 23 nt spacers recognizing one TTTV PAM and one TTV PAM sequence was used to target the *Slit2* locus. Upper panel, the strategy used to generate *Slit2*-BamHI KI. The colored characters represent the DNA sequence, and the black characters in colored background indicate corresponding amino acids. Spacers used are shown in brown. The edited BamHI site is shown in red dashed square. Lower panel, PCR, PCR-T7EI (T7 endonuclease I assay) and PCR-BamHI digestion results showing the KI efficiency. The edited founders are indicated in red. The expected band sizes and the percentage of indels are indicated on the right and bottom of the gels, respectively. (C) Sanger sequencing results of Mb3Cas12a-mSA-mediated KI in pups #2 (P2) and #4 (P4) in *Slit2* locus. Spacers used are shown in brown.



strand (Kleinstiver et al., 2019; Yamano et al., 2016). Given that these two residues are conserved between AsCas12a and Mb3Cas12a (Fig. S3E), similar modifications in Mb3Cas12a may also enhance its fidelity, and also lead to the Cas12a version of prime editing, which utilizes the combined Cas9 H840A nickase and MMLV reverse transcriptase activities to minimize off-target effects (Anzalone et al., 2019). In summary, our data demonstrate that Mb3Cas12a can edit the murine genome in a manner that is independent of the TTTV PAM sequence and with minimal on-target mutations and high targeting efficiency. Mb3Cas12a-mediated genome editing expands the toolkit for efficient production of mutant mouse lines.

## MATERIALS AND METHODS

### Animal use and generation of KO and KI mice

The animal protocol for this study was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Nevada, Reno (protocol #00494).

All mice were housed and maintained under specific pathogen-free conditions with a temperature- and humidity-controlled animal facility at the University of Nevada, Reno. Generation of KO and KI mice was performed as previously described with modifications (Gu et al., 2018; Wang et al., 2019). Briefly, 4–6-week-old FVB/NJ or C57BL/6J female mice were super-ovulated and mated with C57BL/6J stud males; zygotes and two-cell stage embryos were collected from the oviducts for KO and KI, respectively. For KO, Mb3Cas12a mRNA (200 ng/μl) and crRNA (100 ng/μl) were mixed and injected into the cytoplasm or pronucleus of the zygotes in M2 medium (cat. #MR-051-F, Millipore, Burlington, MA). For KI, Mb3Cas12a-mSA mRNA (75 ng/μl), crRNA (50 ng/μl) and biotinylated donor DNA template (20 ng/μl) were mixed and injected into the cytoplasm or pronucleus of the two-cell stage embryos in M2 medium. After injection, all embryos were cultured for 1 h in KSOM+AA medium (cat. #MR-121-D, Millipore) at 37°C under 5% CO<sub>2</sub> in the air before being transferred into 7–10-week-old female CD1 recipients.

### Plasmid construction

To prepare pcDNA3.1-Mb3Cas12a-mSA plasmid, the monomeric streptavidin (mSA) DNA fragment was amplified from PCS2+Cas9-mSA plasmid (Addgene #103882) using Q5<sup>®</sup> Hot Start High-Fidelity 2X Master Mix (Cat. #M0494S, NEB). The PCR conditions are as follows: initial denaturation at 98°C for 30 s followed by 35 cycles of amplification (denaturation at 98°C for 10 s, annealing at 65°C for 20 s, and elongation at 72°C for 1 min) with the final elongation at 72°C for 2 min. After digestion with BamHI (Cat. #R0136S, NEB, Ipswich, MA) and EcoRI (Cat. #R3101S, NEB) at 37°C for at least 2 h, the digested pY117 plasmid (pcDNA3.1-huMb3Cpf1) (Addgene #92293) and PCR products were purified using Ampure beads and ligated using T4 DNA ligase (Cat. #M0202L, NEB) at 4°C overnight. An aliquot (2 μl) of the ligation products was introduced into 16.7 μl of Mix & Go competent cells (DH5 alpha strain, Cat. #T3007, Zymo Research) for transformation. The transformed cells were spread onto agar plates containing ampicillin (Cat. #BP1760-5, Thermo Fisher Scientific) (100 μg/ml), followed by culture overnight at 37°C. The colonies were picked and genotyped, and the potential positive colonies were cultured overnight in culture medium containing NZCYM broth (Cat. #BP2464-2, Thermo Fisher Scientific) (20 mg/ml) and ampicillin (100 μg/ml). The plasmids were extracted using ZR Plasmid Miniprep (Cat. #D4054, Zymo Research) followed by Sanger sequencing to verify successful cloning. To prepare the pUC-Slit2-BamHI plasmid, two homology arms (~1 kb) flanking the crRNAs cutting sites of *Slit2* locus and pUC empty vector were amplified by Q5<sup>®</sup> Hot Start High-Fidelity 2X Master Mix (Cat. #M0494S, NEB) from mouse tail genomic DNA and pX330 plasmid (Addgene #42230), respectively. A BamHI restriction site was introduced between the two homology arms through PCR amplification. After purification with Ampure beads, these three DNA fragments were assembled using the NEBuilder<sup>®</sup> HiFi DNA Assembly Master Mix (Cat. #E2621L, NEB) at 50°C for 1 h. Transformation and verification of ligation were conducted as described above. The primers used for plasmid construction are listed in Table S1.

### Generation of Mb3Cas12a and Mb3Cas12a-mSA mRNA, crRNAs and donor DNA template

To synthesize Mb3Cas12a and Mb3Cas12a-mSA mRNAs, the pY117 plasmid (pcDNA3.1-huMb3Cpf1) (Addgene #92293) and pcDNA3.1-Mb3Cas12a-mSA were digested with EcoRI (Cat. #R3101S, NEB) overnight at 37°C, followed by purification with Ampure beads and mRNA synthesis with the HiScribe<sup>™</sup> T7 ARCA mRNA kit (Cat. #E2065S, NEB) with addition of a RNase inhibitor (Cat. #M0314L, NEB) at 37°C for at least 2 h. Then the *in vitro* transcribed mRNAs were treated with DNase I (NEB, Cat. #M0303S) to remove the plasmid DNA template, followed by poly(A) tailing using *E. coli* poly(A) polymerase (Cat. #M0276S, NEB) at 37°C for 30 min. The poly(A)-tailed mRNAs were purified using the RNA Clean & Concentrator<sup>™</sup>-5 (Cat. #R1016, Zymo Research, Irvine, CA) and eluted in a Tris-EDTA solution (Cat. #11-01-02-02, IDT, Coralville, IA).

crRNAs were designed using Benchling (<https://benchling.com/>). DNA oligonucleotides for making each crRNA were synthesized by the IDT Inc. and are listed in Table S1. To prepare crRNAs for microinjection, the T7 Top strand primer and antisense oligonucleotides specific for each crRNA were mixed in 1× T4 DNA ligase buffer and heated to 95°C for 5 min, and then allowed to cool down to room temperature on the bench. The annealed oligonucleotides were used as the templates for *in vitro* transcription (IVT) using the HiScribe<sup>™</sup> T7 High Yield RNA Synthesis Kit (Cat. #E2040S, NEB) at 37°C overnight. After IVT, crRNAs were purified using the RNA Clean & Concentrator<sup>™</sup>-5 (Cat. #R1016, Zymo Research) and eluted in Tris-EDTA solution (Cat. #11-01-02-02, IDT). To prepare crRNAs for transfection of HEK-293 cells, PCR products corresponding to each crRNA were amplified with U6 forward primer and corresponding antisense oligonucleotides (as listed in Table S1) from the pX330 plasmid (Addgene #42230). After digestion with DpnI (Cat. #R0176S, NEB), the PCR products were purified using Ampure beads. The biotinylated donor DNA template was amplified from the pUC-Slit2-BamHI plasmid with biotinylated primers (as listed in Table S1), followed by DpnI digestion to remove the plasmid and purification with Ampure beads.

### HEK-293 cell transfection

HEK-293 cells (Cat. #ATCC<sup>®</sup> CRL-1573<sup>™</sup>, ATCC) were maintained in DMEM (Cat. #11995, Thermo Fisher Scientific) containing 10% FBS (Cat. #S12450, R&D Systems) and 1× antibiotic-antimycotic (Cat. #15240062, Thermo Fisher Scientific). At 1 day before transfection, the culture medium was changed to DMEM containing 10% FBS. At ~60% confluence, the HEK-293 cells were co-transfected with 400 ng of pY117 (pcDNA3.1-huMb3Cpf1) (Addgene #92293) and 100 ng of crRNA PCR product using 2.5 μl Lipofectamine 2000 (Cat. #11668, Thermo Fisher Scientific, Waltham, MA) in a 24-well cell culture plate (Cat. #3524, Corning, Corning, NY). The culture medium was changed back to DMEM containing 10% FBS and 1× antibiotic-antimycotic at 12 h after transfection. The cells were collected 48 h after transfection for analyses.

### Mouse genotyping and Sanger sequencing

Mouse tail or ear snips were lysed in a lysis buffer (40 mM NaOH, 0.2 mM EDTA, pH 12.0) for 1 h at 95°C, followed by neutralization using the same volume of neutralizing buffer (40 mM Tris-HCl, pH 5.0). PCR was conducted using Platinum<sup>™</sup> SuperFi<sup>™</sup> Green PCR Master Mix (Cat. #12359010, Thermo Fisher Scientific). The 20 μl PCR mix contained 10 μl of 2× Platinum<sup>™</sup> SuperFi<sup>™</sup> Green PCR Master Mix, 4 μl of 5× SuperFi<sup>™</sup> GC Enhancer, 1 μl of 10 μM forward/reverse primers, 5–50 ng of genomic DNA and nuclease-free water. The PCR conditions were as follows: initial denaturation at 98°C for 30 s, followed by 35 cycles of amplification (denaturation at 98°C for 10 s, annealing at 68°C for 10 s, and elongation 72°C for 1 min) with a final elongation at 72°C for 5 min. Positive samples were subjected to single-A tailing using GoTaq<sup>®</sup> Green Master Mix (Cat. #M7123, Promega, Madison, WI) for 5 min at 95°C, followed by 15 min at 72°C. The single A-tailed PCR products were then ligated to pGEM<sup>®</sup>-T Easy Vector using T4 DNA ligase from the pGEM<sup>®</sup>-T Easy Vector Systems (Cat. #A1360, Promega) at 4°C overnight before transformation. Transformation and verification of ligation were conducted as described in the 'Plasmid construction' section. Data were analyzed using Geneious software (Biomatters, Inc.). The primers used for genotyping are listed in Table S1.

## T7EI assay

The T7EI assay (Cat. #M0302L, NEB) was used to detect mutations. PCR products from genotyping were resuspended in 1× buffer 2 and then underwent an initial denaturation at 95°C for 5 min. The denatured PCR products were allowed to anneal by decreasing temperature from 95°C to 85°C with a ramp rate of −2°C/s, followed by a second annealing period from 85–25°C with a ramp rate of −0.1°C/s. The annealed PCR products were then treated with 0.5 µl of T7EI at 37°C for 30 min. The reaction was stopped by adding 0.75 µl of 0.25 M EDTA, as well as 1 µl of Proteinase K (Cat. #P8107S, NEB), followed by incubation at 37°C for 30 min. The T7EI-digested samples (10 µl) were mixed with 2 µl of 6× Purple Gel Loading Dye (Cat. #B7024S, NEB), and loaded into each well of the 10% Novex™ TBE Gels (Cat. #EC62755BOX, Thermo Fisher Scientific) in 1× TBE Buffer (Tris-borate EDTA; Cat. #B52, Thermo Fisher Scientific). The gels were run at 180 V for 30 min, then incubated with 1× TBE buffer containing SYBR Gold (Cat. #S11494, Thermo Fisher Scientific) or ethidium bromide (Cat. #X328, Amresco) for 10 min. The gels were then imaged and analyzed based on the gray density by ImageJ (NIH) and the percentage of indels was quantified as described previously (Cong et al., 2013).

## MiSeq library construction and sequencing

Genomic fragments of the *Prps1*, *Prps2* and *Prps11* loci were amplified from DNA isolated from mouse tail or ear snips using Platinum™ SuperFi™ Green PCR Master Mix (Cat. #12359010, Thermo Fisher Scientific). The PCR products were then tagged using Nextera XT DNA Library Preparation Kit (Cat. #15032354, Illumina, San Diego, CA) and indexed using Nextera XT Index Kit (Cat. #15055294, Illumina) according to the manufacturer's instructions. Briefly, 5 µl of the PCR product (total 1 ng) were mixed with 10 µl of Tagment DNA (TD) Buffer and 5 µl of Amplicon Tagment Mix (ATM), followed by incubation at 55°C for 5 min. The tagging reaction was then stopped by adding 5 µl of Neutralize Tagment (NT) buffer and incubation at room temperature for 5 min. For indexing, the reaction was mixed with 15 µl of Nextera PCR Master Mix (NPM), 5 µl Index i7 adapters and 5 µl Index i5 adapters, then incubated at 72°C for 3 min. After initial denaturation at 95°C for 30 s, 12 cycles of amplification were performed with the following conditions: denaturation at 95°C for 10 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s, ending with a final elongation at 72°C for 5 min. After purification with Ampure beads, the DNA library was sequenced using MiSeq Reagent Kit v2 (500-cycles) (Cat. #MS-102-2003, Illumina). Data were analyzed using Geneious software. The primers used for *Prps1*, *Prps2* and *Prps11* are listed in Table S1.

## Competing interests

The authors declare no competing or financial interests.

## Author contributions

Conceptualization: Z.W., W.Y.; Methodology: Z.W., Y.W., S.W., A.J.G., H.M., T.Y., K.C.-G., B.P., H.W., H.Z.; Software: Z.W.; Validation: Z.W., S.W.; Investigation: Z.W., W.Y.; Resources: Y.W., A.J.G., H.Z., W.Y.; Data curation: Z.W., Y.W.; Writing - original draft: Z.W., W.Y.; Writing - review & editing: Z.W., W.Y.; Visualization: Z.W., S.W.; Supervision: W.Y.; Project administration: W.Y.; Funding acquisition: W.Y.

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## Data availability

The datasets generated and/or analyzed during the current study are available in the Sequence Read Archive (SRA) under accession no. PRJNA556550.

## Supplementary information

Supplementary information available online at <http://jcs.biologists.org/lookup/doi/10.1242/jcs.240705.supplemental>

## Peer review history

The peer review history is available online at <https://jcs.biologists.org/lookup/doi/10.1242/jcs.240705.reviewer-comments.pdf>

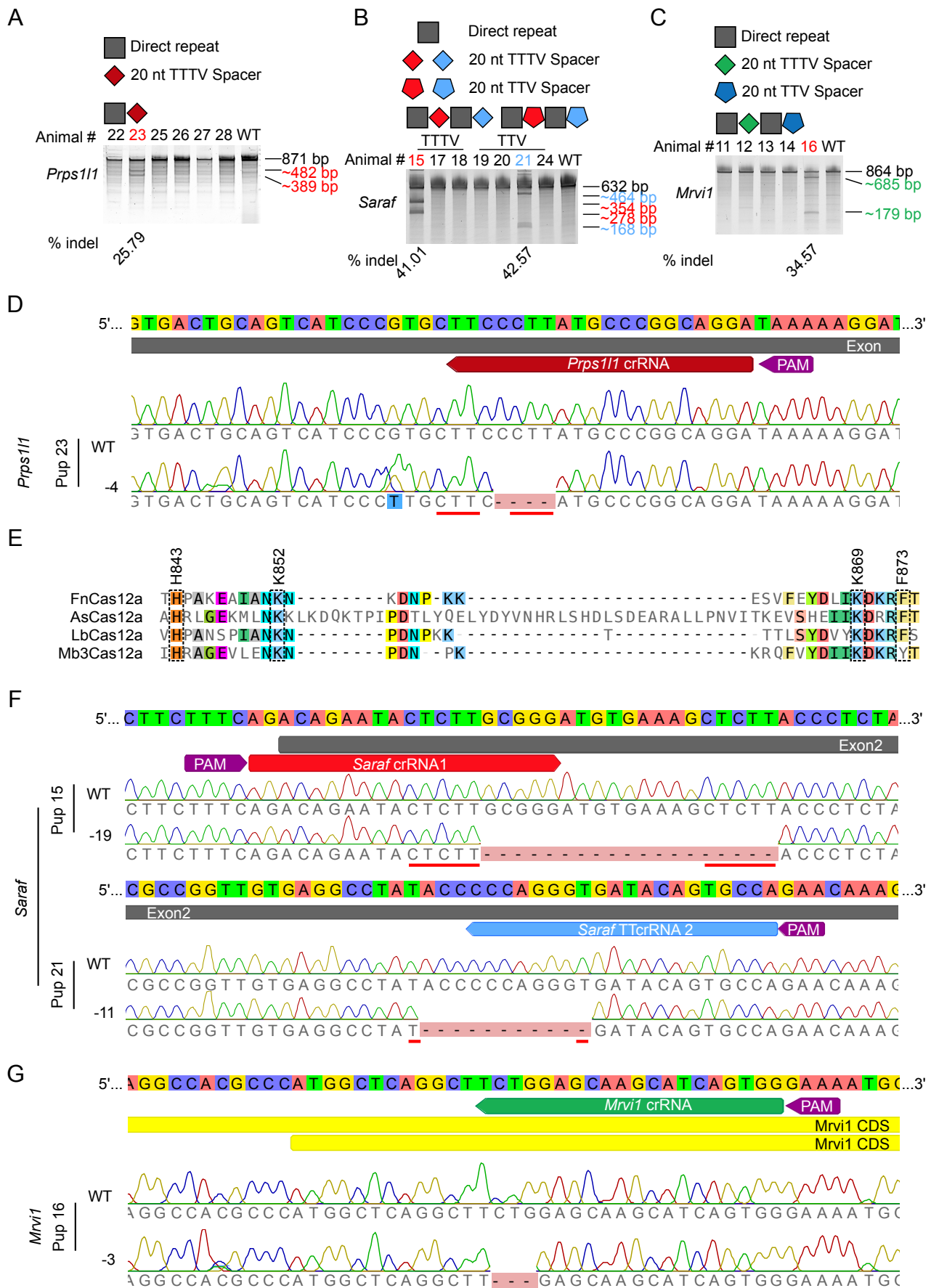
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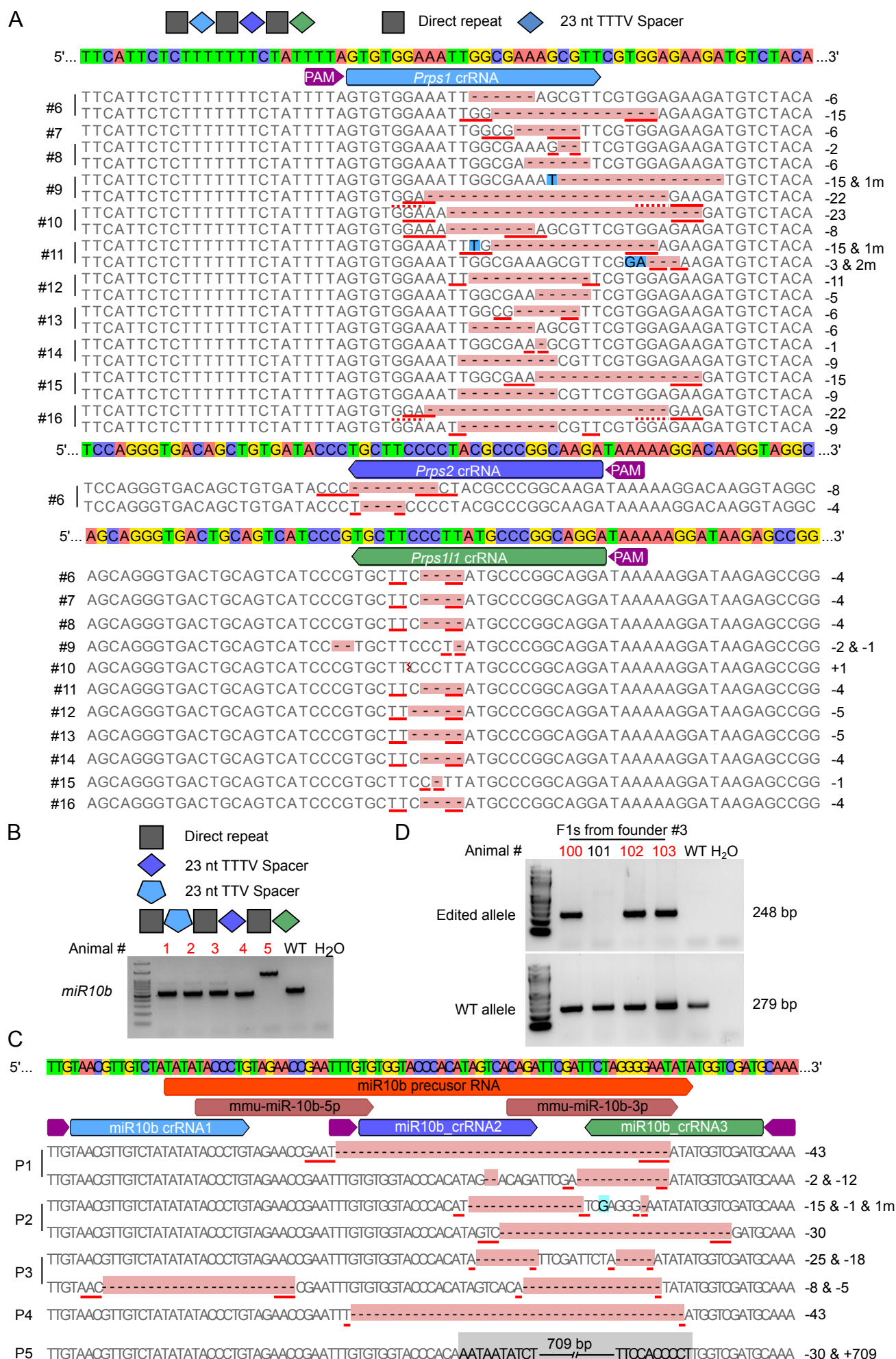
Figure S1



**Figure S1. Efficiencies of Mb3Cas12a-based genome editing using 20nt spacers to target *Prps11l*, *Saraf* and *Mrvi1* loci in mouse zygotes.**

A. T7EI assay results of Mb3Cas12a-edited *Prps11l* locus. One crRNA with one 20nt DR (square) and one 20nt spacer (diamond) recognizing a TTTV PAM sequence was used to target the *Prps11l* locus. The expected band sizes and the percentage of indels are indicated on the right and bottom of the gel, respectively. B. T7EI assay results of Mb3Cas12a-edited *Saraf* locus. Two crRNAs harboring two 20nt spacers either recognizing two TTTV (diamond) or two TTV (pentagon) PAM sequences were used to target the *Saraf* locus. The expected bands corresponding to the pups after T7EI assays are indicated with the same color. The grey square represents DR. The indel percentage is indicated at the bottom of the gel. C. T7EI assay results of Mb3Cas12a-edited *Mrvi1* locus. One single crRNA containing two spacers recognizing both TTTV (diamond) and TTV PAM (pentagon) sequences was used to target the *Mrvi1* locus. The grey square represents DR. The indel percentage is indicated at the bottom of the gel. D. Sanger sequencing results of Mb3Cas12a-edited pup #23 in the *Prps11l* locus. Spacers used are indicated in the same color as panel A. PAM sequence is shown in purple. Red underlines represent microhomology sequences. E. RNase-active domain alignment of Mb3Cas12a orthologues. The conserved residues responsible for crRNA processing are indicated. F. Sanger sequencing results of Mb3Cas12a-edited pups #15 and #21 in the *Saraf* locus. Spacers used were indicated as the same color as panel B. PAM sequence is shown in purple. Red underlines represent microhomology sequences. G. Sanger sequencing results of Mb3Cas12a-edited pup #16 in *Mrvi1* locus. Red underlines represent microhomology sequences. Spacers used are indicated as the same color as panel C. PAM sequence is shown in purple.

Figure S2

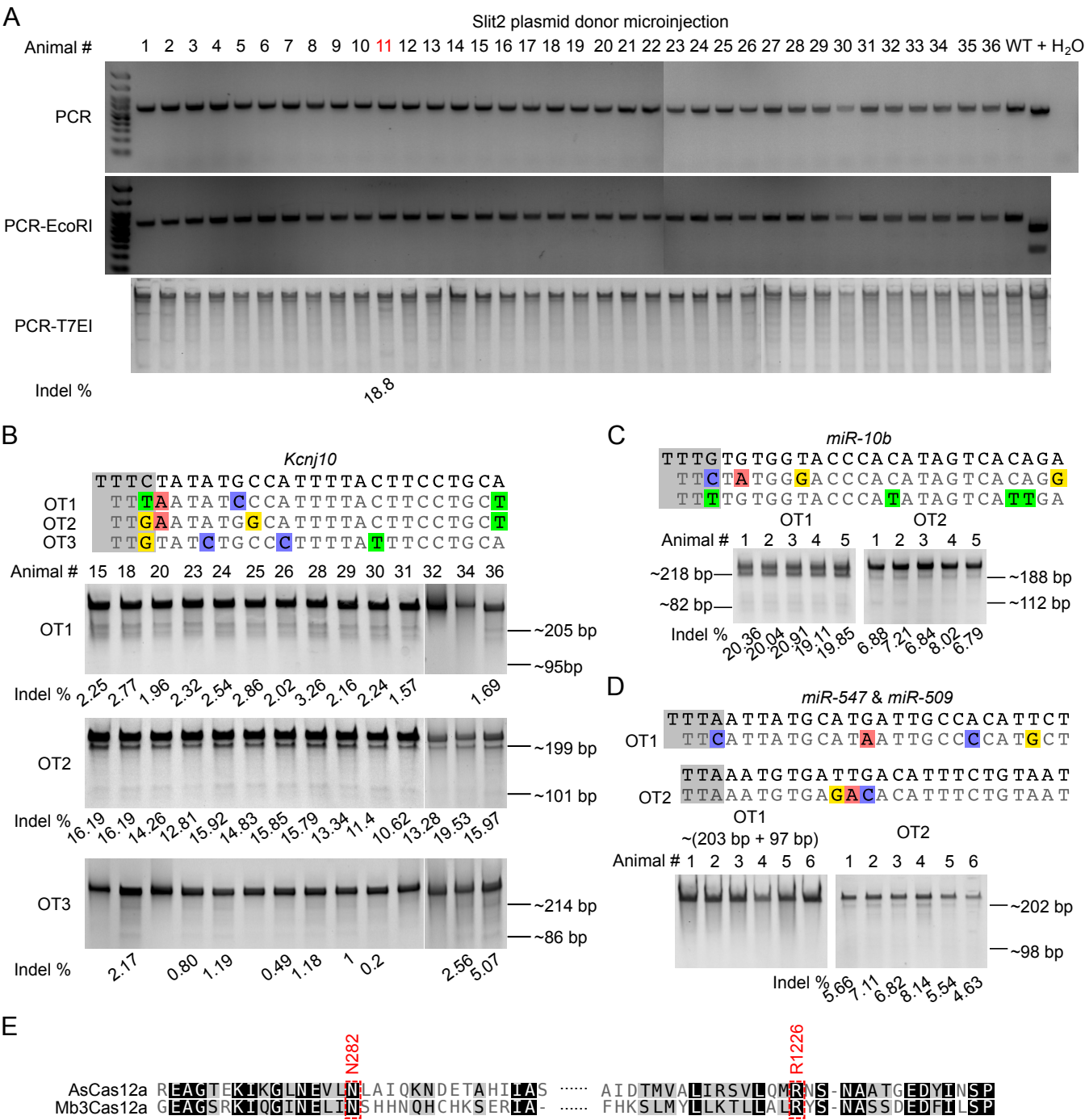




**Figure S2. Multiplex targeting efficiency of Mb3Cas12a-based genome editing in mouse zygotes.**

A. MiSeq results of Mb3Cas12a targeting efficiency in *Prps1*, *Prps2*, and *Prps111* with one crRNA, which contains three 20nt DR (grey square) and three 23nt spacers (diamond) targeting *Prps1*, *Prps2* and *Prps111* with TTTV PAM sequences. Red underlines represent microhomology sequences. The edited pup number and the summary of edited bases are indicated on the left and right sides of the sequencing results, respectively. B. PCR genotyping results of Mb3Cas12a-edited *miR-10b* founders. One crRNA with three 20nt DR (grey square) and three 23nt spacers recognizing two TTTV PAM (diamond) and one TTV PAM (pentagon) sequences was used to target *miR-10b* locus. The edited founders are shown in red. C. Sanger sequencing results of Mb3Cas12a-edited pups #1-5 in *miR-10b* locus. The 709 bp insertion in #5 matches to a retrotransposon (ERV1). D. Genotyping results of F1s from *miR-10b* founder #3. The edited mice are marked in red. The expected sizes are indicated on the right of the gel.

Figure S3



**Figure S3. The efficiency of 2C-HR using Mb3Cas12a-based genome editing with unbiotinylated *Slit2* donor, detection of off-target effects, and high-fidelity and nickase residues alignment of AsCas12a and Mb3Cas12a.**

A. PCR, PCR-EcoRI and PCR-T7EI assays on the *Slit2* samples that microinjected with Mb3Cas12a-mSA mRNA, crRNA and non-biotinylated plasmid donor DNA. The animals shown in red (#11) was edited by Mb3Cas12a (as shown in PCR-T7EI) but without knock-in (as shown in PCR-EcoRI). B. Off-target analyses of Mb3Cas12a-edited *Kcnj10* animals. The sequences of predicted off-targets (OT) are shown on the top. The PAM sequences are highlighted in grey. The expected size and indel percentage are indicated on the right and bottom of the gels, respectively. C. Off-target analyses of Mb3Cas12a-edited *miR-10b* animals. The PAM sequences are highlighted in grey. The expected sizes for OT1 and OT2 are indicated on the left and right of the gels, respectively. Indel percentage is indicated on the bottom of the gels. D. Off-target analyses of Mb3Cas12a-edited *miR-547 & 509* animals. The PAM sequences are highlighted in grey. The expected size and indel percentage are indicated on the right and bottom of the gels, respectively. E. Protein sequence alignment of Mb3Cas12a and AsCas12a. The mutations to Ala in the residues N282 and R1226 (indicated in the red dot squares) that made enAsCas12a a high-fidelity endonuclease and AsCas12a a nickase cleaving non-target DNA strand are conserved between these two orthologues.

**Table S1. Primer sequences used in this study.**

Name	Sequence	Usage
T7 Top strand primer	GAAATTAATACGACTCACTATAGGG TTCCCTTATGCCCCGGCAGGAATCTACAAA CAGTAGAAATTCCCTATAGTGAGTCGTAT	In vitro transcription of crRNA
T7 Mb3 Prps1l1 R	TAATTTC GTGCGCATGCAGAAGCTGACATCTACAAA CAGTAGAAATTTCTGGAGCAAGCATCAGT	In vitro transcription of crRNA
T7 Mb3 Mrvi1 cr(+TT) R	GGATCTACAAACAGTAGAAATTCCCTATA GTGAGTCGTATTAATTTC AGGGTGATACAGTGCCAGAAATCTACAA ACAGTAGAAATTTCCGCAAGAGTATTCTG TCTATCTACAAACAGTAGAAATTCCCTAT	In vitro transcription of crRNA
T7 Mb3 Saraf 2cr R	AGTGAGTCGTATTAATTTC CCCAGGGTGATACAGTGCCAATCTACAAA CAGTAGAAATTCAAGAGTATTCTGTCTGA AAATCTACAAACAGTAGAAATTCCCTATA	In vitro transcription of crRNA
T7 Mb3 Saraf 2tcr R	GTGAGTCGTATTAATTTC TCTAGGGGAATATATGGTTCGATGATCTAC AAACAGTAGAAATTTCTGTGACTATGTGG GTACCACAATCTACAAACAGTAGAAATTC AGGGTATATATAGACAACGTTAATCTACA AACAGTAGAAATTCCCTATAGTGAGTCGT	In vitro transcription of crRNA
T7 Mb3 miR10b R	ATTAATTTC TGCTTCCCTTATGCCCCGGCAGGAATCTAC AAACAGTAGAAATTTGCTTCCCCTACGCC CGGCAAGAATCTACAAACAGTAGAAATT AACGCTTTCGCCAATTTCCACACATCTAC AAACAGTAGAAATTCCCTATAGTGAGTCG	In vitro transcription of crRNA
T7 Mb3 prps fam new R	TATTAATTTC ATTACAGAAATGTCAATCACATTATCTAC AAACAGTAGAAATTAGAATGTGGCAATC ATGCATAATATCTACAAACAGTAGAAATT GATGTACCAAGCATGTTTCCTGTCCCTAT	In vitro transcription of crRNA
T7 Mb3 547 509 R	AGTGAGTCGTATTAATTTC TTCCTGCAGATGACGTCGGTCGCATCTAC AAACAGTAGAAATTTGCAGGAAGTAAAA TGGCATATAATCTACAAACAGTAGAAATT	In vitro transcription of crRNA
T7 Mb3 Kcnj10 crRNA1 &3 R	CCCTATAGTGAGTCGTATTAATTTC CTCCCTCGCACCAGCCCCCTGTGAATCTAC AAACAGTAGAAATTTGCGAGGGAGGACC ATGGGTGGAATCTACAAACAGTAGAAATT	In vitro transcription of crRNA
T7 Mb3 Slit2 crRNA	CCCTATAGTGAGTCGTATTAATTTC	In vitro transcription of crRNA
Prps1l1 Ext F	GGAAGGGACAGTAACGGCTTT	Genotyping
Prps1l1 qPCR R	GATGCCATGAGTCAAGATGGCATAAACTC	Genotyping
Mrvi1 ext F2	CTTTCAGAATGTCTCCAAAGGGCTAGC	Genotyping
Mrvi1 ext R	GTGGCTAACTTCTCTTGAAGTGTGGTCAC CACGCTTTGTGTAGAACCATCAAAATGTC	Genotyping
Saraf ext F3	TA	Genotyping



Saraf ext R5	GCATCACTGCATGGGAGGCC	Genotyping
miR10b ext F	TTAAGAAGAAGAAGGTCCTGGCTGCTCA	Genotyping
miR10b ext R	AATACCCTAAAACCTGGCTCTCTGGC	Genotyping
	CTTCTAGTATACAGGTTTCCTTTAGGTGCTC	
Prps1 ext F	TTTCTCTA	Genotyping
Prps1 ext R	GCTGTCCGTTACAATGCCATTTACAGTA	Genotyping
	ACTCTGCTTTCCGATTAAGAGTGAAGTGT	
Prps2 ext F2	G	Genotyping
Prps2 ext R2	TTTGACTGCCTTTGAAACATACATTGCCTG	Genotyping
miR201 ext F2	GAGACTCTTAGTTTCTAGATCTGGTTCCTT	Genotyping
	AAGAAGGACAGGGGTGGTAAAAGAGAAG	
miR547 ext R	TA	Genotyping
	AGATTGTGGTCAGGACAGAGATAGAGGA	
miR509 ext F	AA	Genotyping
	CACTGGTTGGGTGCAAATATCTACATCTG	
miR509 ext R	A	Genotyping
Kcnj10 geno F	TTCATGTTTGACTTATAGGACCTCACGCTG	Genotyping
	TCGTCCATAGATCCTTGAGGTAGAGGAAA	
Kcnj10 geno R	C	Genotyping
	AAGAAGGGATCCAGCGGTTACAGAGACCC	pcDNA3.1-Mb3Cas12a-
BamHI opti linker F	CAGGA	mSA construct
	ACTGTGCTGGATATCTGCAGAATTCTCAT	pcDNA3.1-Mb3Cas12a-
mSA GBS R	TTAACTTTGGTGAAGGTGTCCTGACC	mSA construct
pUC F	TGATGCGGTATTTTCTCCTTACGCATCTG	pUC-Slit2-BamHI plasmid
		construct
pUC R	ACATGTGAGCAAAAGGCCAGCAAAAG	pUC-Slit2-BamHI plasmid
	CTGGCCTTTTGCTCACATGTTAAGTACATG	construct
Slit2 up GBS F	TCACTGGATAAAGTCTTGAGC	pUC-Slit2-BamHI plasmid
	GGCTGGATCCAAACTCACAGAACAAGCC	construct
Slit2 up GBS R	ACTGACA	pUC-Slit2-BamHI plasmid
	CTGTGAGTTTGGATCCAGCCCCTGTGATA	construct
Slit2 down GBS F	ATTTTGATTGCCAGAATG	pUC-Slit2-BamHI plasmid
	AAGGAGAAAATACCGCATCAAAGAATGT	construct
Slit2 down GBS R	TCCTTGAAATTGTGCTTAAAGGC	pUC-Slit2-BamHI plasmid
	/5Biosg/TAAGTACATGTCACTGGATAAAGT	construct
Slit2 up bio F	CTTGAGC	PCR for Biotin donor DNA
	/5Biosg/AAGAATGTTTCCTTGAAATTGTGCT	
Slit2 down bio R	TAAAGGC	PCR for Biotin donor DNA
	TAACCCTTCTGACTTCACCTTAGTAAGCC	
Slit2 ext F	A	Genotyping
	CTTGGCTGAAGGAATCTGAAGATAGGACT	
Slit2 ext R	C	Genotyping
U6 forward	GAGGGCCTATTTCCCATGATTCCTT	PCR for Dnmt1 crRNA
	ACCGAGCAGGAGTGAGGATCTACAAACA	
U6 Mb3 Dnmt1 17nt	GTAGAAATTTCGGTGTTCGTCTTTCCAC	PCR for Dnmt1 crRNA
	CACCGAGCAGGAGTGAGGATCTACAAAC	
U6 Mb3 Dnmt1 18nt	AGTAGAAATTTCGGTGTTCGTCTTTCCAC	PCR for Dnmt1 crRNA

	TCACCGAGCAGGAGTGAGGATCTACAAA	
	CAGTAGAAATTCGGTGTTTCGTCCTTTCCA	
U6 Mb3 Dnmt1 19nt	C	PCR for Dnmt1 crRNA
	TTCACCGAGCAGGAGTGAGGATCTACAAA	
	CAGTAGAAATTCGGTGTTTCGTCCTTTCCA	
U6 Mb3 Dnmt1 20nt	C	PCR for Dnmt1 crRNA
	ATTCACCGAGCAGGAGTGAGGATCTACAA	
	ACAGTAGAAATTCGGTGTTTCGTCCTTTCC	
U6 Mb3 Dnmt1 21nt	AC	PCR for Dnmt1 crRNA
	AATTCACCGAGCAGGAGTGAGGATCTACA	
	AACAGTAGAAATTCGGTGTTTCGTCCTTT	
U6 Mb3 Dnmt1 22nt	CCAC	PCR for Dnmt1 crRNA
	AAATTCACCGAGCAGGAGTGAGGATCTAC	
	AAACAGTAGAAATTCGGTGTTTCGTCCTT	
U6 Mb3 Dnmt1 23nt	TCCAC	PCR for Dnmt1 crRNA
Dnmt1 F2	CCGTTTTGGGCTCTGGGACTCA	Genotyping
Dnmt1 R2	AATCCAGAATGCACAAAGTACTGCACA	Genotyping
	TCGTCGGCAGCGTCAGGGATGCATTTTCT	
Kcnj10 OF1 R	CAATCGAGGTTCTT	Kcnj10 off-target 1 PCR
	GTCTCGTGGGCTCGGNNNNGGCCACACCT	
Kcnj10 OF1 F	CCAGAATATACAAACCAT	Kcnj10 off-target 1 PCR
	TCGTCGGCAGCGTCCCTGCAATCCTTAAC	
Kcnj10 OF2 R	TCGCTCTTGTTAGT	Kcnj10 off-target 2 PCR
	GTCTCGTGGGCTCGGNNNNAATGCCTGGT	
Kcnj10 OF2 F	TTAGAGTCTGTCTGAAGA	Kcnj10 off-target 2 PCR
	TCGTCGGCAGCGTCTGTAGTTAACTTGAT	
Kcnj10 OF3 R	AACAGGCATGCTG	Kcnj10 off-target 3 PCR
	GTCTCGTGGGCTCGGNNNNCGAAAGAACT	
Kcnj10 OF3 F	CGACTGTGAGCTCTCAG	Kcnj10 off-target 3 PCR
	TCGTCGGCAGCGTCAGGCCAAAACACCTA	
miR10b OF1 R	TAAAAGTAAATGCA	miR-10b off-target 1 PCR
	GTCTCGTGGGCTCGGNNNNCATTCTTCC	
miR10b OF1 F	TCTCCTAACATGGACCTG	miR-10b off-target 1 PCR
	TCGTCGGCAGCGTCCAGCCACAAGTTGAC	
miR10b OF2 R	TGCTTTGGTTAAT	miR-10b off-target 2 PCR
	GTCTCGTGGGCTCGGNNNNATGTCCCTTT	
miR10b OF2 F	ATCCAGCCTTGTTACTGC	miR-10b off-target 2 PCR
	TCGTCGGCAGCGTCTGCTTTCTTAGGGTGT	miR547 & 509 off-target 1 PCR
miR547 & 509 OF1 R	ATCAACGATCCT	PCR
	GTCTCGTGGGCTCGGNNNNAGCTTTGAT	miR547 & 509 off-target 1 PCR
miR547 & 509 OF1 F	CACAACATGCACAGCTGA	PCR
	TCGTCGGCAGCGTCATGTATTATTCTGTGT	miR547 & 509 off-target 2 PCR
miR547 & 509 OF2 R	TGTAGAGACGAGGTC	PCR
	GTCTCGTGGGCTCGGNNNTGGTCTACAG	miR547 & 509 off-target 2 PCR
miR547 & 509 OF2 F	AGTGAGTTTCAGGACAGC	PCR