Generation of knockout mice by Cpf1-mediated gene targeting

To the Editor:

Engineered nucleases, including transcription activator-like effector nucleases (TALENs) and CRISPR-Cas9, are under active development as versatile genome-editing tools in various organisms including mice[1–3]. Recently, diverse Cpf1 orthologs were identified as representatives of a class 2 CRISPR-Cas system *in vitro* and showed target-specific endonuclease activities in human cells comparable to that of *Streptococcus pyogenes* Cas9 (SpCas9)[4]. In contrast to Cas9, Cpf1 is guided by a single CRISPR RNA (crRNA) without an additional trans-activating crRNA, requires a short thymidine-rich protospacer-adjacent motif (PAM) preceding the target sequence, and introduces a staggered double-stranded break[4]. These simple characteristics of Cpf1 should be of great use for the generation of knockout mice.

To examine the gene-targeting potential of Cpf1 in mice, we selected two target DNA sequences in exon 7 of the gene encoding the mouse *transformation related protein 53* (*Trp53*) and used Cpf1 orthologs from *Acidaminococcus sp. BV3L6* (AsCpf1) and *Lachnospiraceae bacterium N D2006* (LbCpf1) (**Fig. 1a** and **Supplementary Table 1**). Each *Cpf1* mRNA and a cognate crRNA were microinjected into the cytoplasm of pronuclear-stage mouse embryos from C57BL/6J mice, after which the embryos were transferred into the oviduct of foster mothers (**Supplementary Methods**). Embryos did not show any signs of toxicity immediately after microinjection of *AsCpf1* and *LbCpf1* mRNAs, but the percentages of injected embryos that developed at least until embryonic day (E)12.5~E14.5 were variable (11.9–37.1%; **Table 1**). A PAGE-based genotyping method showed that both Cpf1 nucleases with their cognate crRNAs could induce mutations in mouse embryos (7.7–75.0% of isolated embryos; **Table 1** and **Supplementary Figs. 1** and **2**). These results suggest that the mutation rates induced by both Cpf1 orthologs in mouse embryos are high enough to generate mutant mice.

To generate *Trp53* knockout mice, we simultaneously microinjected crRNAs with their cognate *Cpf1* mRNAs into fertilized eggs, and obtained live mice. Neither Cpf1 ortholog induced acute toxicity; AsCpf1 was associated with a higher birth rate (40.7%) than LbCpf1 (28.6%, **Table 1**). Although fertilized eggs from C57BL/6J mice are relatively sensitive to microinjection of engineered nuclease mRNAs[5], these birth rates are suitable for gene targeting in C57BL/6J mice and similar to those observed

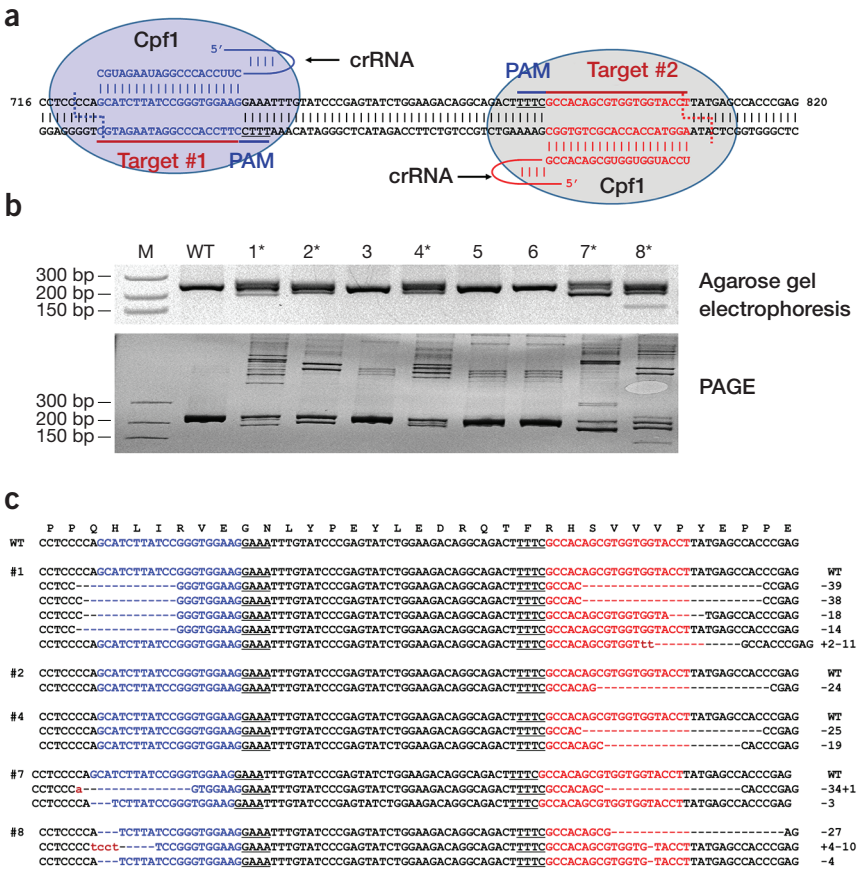


Figure 1 Generation of Cpf1-mediated *Trp53* mutant mice. (**a**) Two target DNA sequences in the mouse *Trp53* locus are denoted in blue and red, respectively. This region corresponds to nucleotides 716–820 of the *Trp53* mRNA (NM_00127233) encoding amino acids 187–221 of the mouse Trp53 protein (NP_001120705). Each dotted line marks the potential staggered cut possibly generated by the Cpf1-crRNA pairs. The underlined sequences represent the PAM. (**b**) Agarose gel electrophoresis- and PAGE-based genotyping assays identifying founder mice derived from pronuclear-stage embryos intra-cytoplasmically injected with *LbCpf1* mRNA and both of the crRNAs targeting the mouse *Trp53* locus. The numbers denote each newborn mouse; M, a molecular size marker; WT, wild-type mouse; *, founder mice. (**c**) Mutated *Trp53* sequences observed in the founder mice in **b**. -, denotes deleted nucleotides; sequences in lower case represent nucleotide insertions.

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Table 1 Nuclease activities of Cpf1 orthologs in C57BL/6J mouse embryos

Trp53-targeting crRNAs	*Cpf1* mRNA	No. of injected zygotes	No. of surviving and transferred (%)[a]	No. of E12.5~E14.5 embryos (%)[b]	No. of newborns (%)[b]	No. of mutants (%[b], %[c])
#1	*AsCpf1*	55	55 (100)	16 (29.1)	–	12 (21.8, 75.0)
	LbCpf1	56	56 (100)	13 (23.2)	–	1 (1.8, 7.7)
#2	*AsCpf1*	59	59 (100)	7 (11.9)	–	3 (5.1, 42.9)
	LbCpf1	62	62 (100)	23 (37.1)	–	11 (17.7, 47.8)
#1 + #2	*AsCpf1*	60	59 (98.3)	–	24 (40.7)	19 (32.2, 79.2)
	LbCpf1	63	63 (100)	–	18 (28.6)	13 (20.6, 72.2)

Percentages were calculated using the number in each column as the numerator; the denominators are indicated in the footnotes. [a]Injected zygotes. [b]Surviving and transferred zygotes. [c]Newborns (or E12.5~E14.5 embryos). –, not tested.

with Cas9. After microinjection of *AsCpf1* mRNA, 19 mutant mice were obtained from 24 newborns (79.2%; **Supplementary Fig. 3a** and **Table 1**). Similarly, 72.2% of newborns from *LbCpf1* mRNA-injected embryos were identified as mutant founders (13 mutants/18 newborns; **Fig. 1b**, **Supplementary Fig. 4a** and **Table 1**). Sanger sequencing analyses showed that most mutations were indels (**Fig. 1c** and **Supplementary Figs. 3b** and **4b**). Notably, mosaicism was frequently observed (12 out of 19 founders for AsCpf1, 63.2%; 6 out of 13 founders for LbCpf1, 46.2%; **Fig. 1c** and **Supplementary Figs. 3b** and **4b**). Sperm genotyping suggests that most, if not all, mutant alleles are expected to be germline transmitted (**Supplementary Fig. 5a**). Indeed, germline transmission of mutant alleles identified in the founder mice was also demonstrated in both embryos and pups (**Supplementary Fig. 5b**,**c**).

To examine the functional consequences of *Trp53* gene targeting, we compared the levels of Trp53 protein from the tissues of an X-irradiated wild-type mouse and a founder (#13) with only mutant alleles generated with LbCpf1. As expected, the Trp53 protein was detected in wild-type tissues only, but not in founder tissues (**Supplementary Fig. 6**). We also engineered NOD/ShiLtJ mice by targeting the gene encoding *protein kinase, DNA activated, catalytic polypeptide* (*Prkdc*) (**Supplementary Fig. 7**). Similar to what we observed in the *Trp53* targeting experiment, a considerable proportion of the newborns possessed mutations in the *Prkdc* locus (18.2% for AsCpf1 and 72.7% for LbCpf1; **Supplementary Fig. 7** and **Supplementary Table 2**). On the basis of the high mutation rates associated with LbCpf1, we tried to target both *Trp53* and *Prkdc* genes simultaneously using *LbCpf1* mRNA. Even though the mutation rate at each gene was lower owing to the smaller amount of each crRNA available (**Supplementary Methods**), 42.9% of embryos possessed mutations in both genes (15 out of 35 embryos; **Supplementary Fig. 8** and **Supplementary Table 3**). Although more detailed studies should be conducted to determine the best experimental conditions, these results suggest that Cpf1-based gene targeting will be a useful tool for generating gene knockout mice.

To determine the specificity of the Cpf1 orthologs in gene targeting *in vivo*, we examined potential off-target mutations in the founder mice as we previously described[6]. Possible off-target sites in the mouse genome, which had similarity to the target sequences in *Trp53* and *Prkdc*, were selected (**Supplementary Tables 4** and **5**) and analyzed by deep sequencing of pooled genomic DNA samples from five mutant founders that were generated with AsCpf1 and LbCpf1. We found no notable off-target effects at 17 potential off-target sites with 2- to 4-bp mismatches to target sequences #1 and #2; we did identify one off-target site harboring a 1-bp mismatch to target sequence #2, however, which was mutated at frequencies of 18.6% (AsCpf1) and 16.3% (LbCpf1) by the Cpf1 orthologs (**Supplementary Fig. 9**). Consistently, no substantial off-target effects were detected at potential off-target sites with 2- to 4-bp mismatches with the two target sequences in *Prkdc* (**Supplementary Fig. 10**). These results suggest that both AsCpf1–crRNA complexes and LbCpf1–crRNA complexes may exhibit minimal off-target effects comparable to results with the Cas9–short guide RNA (sgRNA) complex. Such off-target effects could be avoided by carefully selecting target sequences.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper (doi:10.1038/nbt.3614).

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AUTHOR CONTRIBUTIONS

S.-W.L., M.S.L., I.-J.B. and Y.H.S. conceived and supervised the project. I.-J.B. contributed to microinjection. Y.H.S contributed to the design and production of *Cpf1* mRNAs and crRNAs. S.-A.C. contributed to the screening of founder mice and management of mouse colonies. J.G.L. contributed to the immunoblot analyses. Y.K contributed to the off-target analyses. Y.K., S.-W.L., M.S.L., I.-J.B and Y.H.S. analyzed the data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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