Targeted mutagenesis in mice by electroporation of Cpf1 ribonucleoproteins

To the Editor:

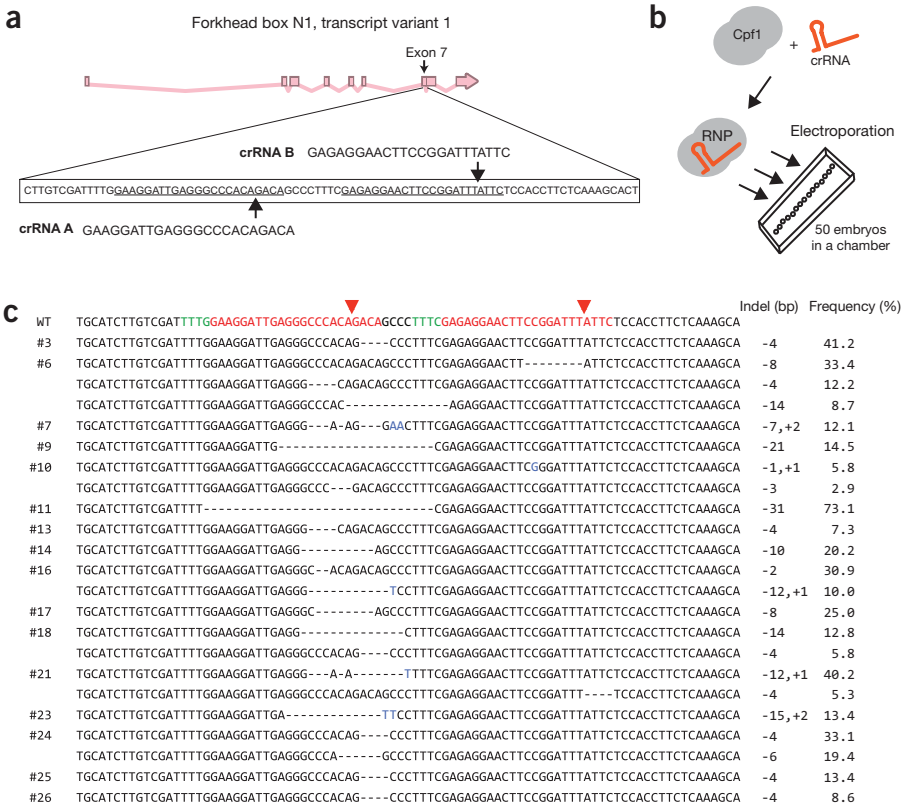
Cpf1 represents a class 2/type V CRISPR RNA-guided endonuclease[1] that is distinct from the type II CRISPR-Cas9 nuclease, widely used for genome editing[2–5]. It recognizes thymidine-rich protospacer-adjacent motif (PAM) sequences, expanding the range of RNA-guided genome editing beyond guanosine-rich PAM sequences recognized by *Streptococcus pyogenes* Cas9 (SpCas9). Yet, the potential of Cpf1 for targeted mutagenesis in whole organisms has not been demonstrated. Here, we report generation of mutant mice using preassembled recombinant CRISPR-Cpf1 ribonucleoproteins (RNPs).

First, we expressed *Acidaminococcus* sp. BV3L6 Cpf1 (AsCpf1) in *Escherichia coli* and purified the resulting recombinant Cpf1 protein (**Supplementary Table 1** and **Supplementary Methods**). We tested the activity of the purified Cpf1 protein *in vitro* (**Supplementary Fig. 1a**) and in mouse NIH3T3 cells. Preassembled Cpf1 RNPs cleaved target DNA efficiently *in vitro* and induced small insertions and deletions (indels) in NIH3T3 cells, with frequencies of up to 45% (**Supplementary Fig. 1b**), at the target sites in *Foxn1* (**Fig. 1a**), which encodes Forkhead box protein N1, a transcription factor.

Having confirmed that Cpf1 RNPs were highly active *in vitro* and in cultured cells, we carried out Cpf1 RNP-mediated genome editing in mouse embryos. We microinjected the AsCpf1 RNPs into 36 one-cell-stage embryos, cultured them *in vitro*, and obtained 12 blastocysts (**Supplementary Fig. 2a**). T7 endonuclease 1 (T7E1) assay and targeted deep sequencing showed that 10 out of 12 (83%) blastocysts contained Cpf1-mediated mutations in *Foxn1* (**Supplementary Fig. 2b,c** and **Supplementary Table 2,3**). SpCas9 RNPs targeted to the same region in the gene showed a comparable efficiency (90%) (**Supplementary Fig. 3**). No off-target mutations were detected at homologous sites with up to 4-nucleotide mismatches using the T7E1 assay and targeted deep sequencing (**Supplementary Table 4** and **Supplementary Fig. 4**), suggesting that Cpf1 RNPs were highly specific.

Next, we investigated whether both Cas9 RNPs and Cpf1 RNPs could be delivered into animal embryos by electroporation rather than microinjection. Although microinjection is a widely adopted method for making transgenic or gene knockout animals, microinjecting single-cell embryos individually is technically difficult, requiring extensive training, and is also labor-intensive. A skilled researcher can handle hundreds of embryos per day at best. Using the electroporation method, we simultaneously delivered RNP to multiple embryos, up to 50 at once, within 5 minutes. We electroporated SpCas9 RNPs targeting the *Vegfa* or *Foxn1* locus in the mouse genome into 50 embryos for each locus in a chamber electrode[6], which were then cultured to blastocysts. 12 of 15 (80%) or 11 of 11 (100%) blastocysts obtained had mutations at the *Vegfa* or *Foxn1* target site, respectively (**Supplementary Fig. 5**), suggesting that electroporation is an efficient method for delivering Cas9 RNPs into animal embryos.

We then electroporated AsCpf1 RNPs, targeting two sites in the *Foxn1* exon 7, into mouse embryos (**Fig. 1b**). Analysis of the 25 blastocysts revealed that 16 (64%) contained mutations at the target sites (**Fig. 1c** and **Supplementary Table 2**). No off-target mutations were observed at 14 homologous sites that differed from the two *Foxn1* on-



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Target gene	No. of examined embryos	No. of 2-cell stage embryos (%)	No. of blastocysts (%)	No. of transferred embryos	No. of offspring (%)	Mutant ratio (%) (no. of mutant/total blastocysts or offsprings)
Foxn1	62	49 (79)	25 (51)	NA	NA	64 (16/25)
	38	30 (79)	NA	30	7 (23)	43 (3/7)

NA, not applicable.

Figure 1 Cpf1 RNP-mediated genome editing in mouse embryos by electroporation. (**a**) Two CRISPR RNAs (crRNAs) that target sequences in *Foxn1* exon 7. (**b**) Schematic of Cpf1 RNP delivery to a mouse embryo by electroporation. (**c**) Targeted deep sequencing assay to analyze mutations at the target site. The target sequence and PAM are indicated by red and green, respectively. Predicted Cpf1 cleavage sites are marked by red arrowheads. (**d**) Summary of the numbers of embryos used and mutants generated in Cpf1 RNP electroporation with crRNAs targeting *Foxn1* sites.

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target sites by four mismatches. We also electroporated an AsCpf1 RNP targeting *Tyr*, the gene encoding tyrosinase, into mouse embryos and observed mutations in 4 out of 12 blastocysts (33%) (**Supplementary Fig. 6**). Taken together, these results show that Cas9 and Cpf1 RNPs can be delivered efficiently by electroporation into animal embryos, resulting in high mutation frequencies.

We next transplanted mouse embryos after Cpf1 microinjection or electroporation into surrogate mothers and obtained mice with targeted mutations in *Foxn1* (**Fig. 1d** and **Supplementary Fig. 7a**) or *Tyr* (**Supplementary Table 2** and **Supplementary Fig. 8a**). Three out of seven mice carried mutations at the Cpf1 cleavage site in the *Foxn1* gene. One *Tyr* mutant mouse showed a partial coat color change, consistent with its mosaic genotype (**Supplementary Fig. 8b**). To investigate whether Cpf1 had off-target effects, we performed whole genome sequencing using genomic DNA isolated from one *Foxn1* mutant mouse and its wild-type sibling (**Supplementary Note**). The sequence analysis showed that no off-target mutations were introduced at homologous sites with up to 7-nucleotide mismatches. Notably, the mutant allele in a female *Foxn1* mutant mouse was transmitted to embryos (**Supplementary Fig. 7b**,**c**).

In summary, our results show that electroporation of AsCpf1 RNPs resulted in efficient and specific genome editing in mouse embryos. RNPs[7] are as effective as mRNA[8] or plasmids[9], but are degraded rapidly by endogenous proteases and RNases in cells, and have been previously shown to reduce off-target effects[9] and mosaicism[10]. Unlike microinjection, electroporation is easy to carry out, fast, and scalable. Up to 50 embryos can be electroporated simultaneously. We propose that electroporation of Cpf1 RNPs is a potentially useful new method for genome editing in animals.

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AUTHOR CONTRIBUTIONS

J.K.H., K.M.K., Y.S.L., and J.-S.K. designed the research, and J.K.H., K.M.K., K.W.B., G.Y.B., S.H.Y., J.W.H., and S.M.R. performed the experiments. J.-S.K. supervised the research. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper (doi:10.1038/nbt.3596).

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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 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

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