Generating genetically modified mice using CRISPR/Cas-mediated genome engineering

Hui Yang[1], Haoyi Wang[1] & Rudolf Jaenisch[1,2]

[1]Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, USA. [2]Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA. Correspondence should be addressed to R.J. (jaenisch@wi.mit.edu).

Published online 24 July 2014; doi:10.1038/nprot.2014.134

Mice with specific gene modifications are valuable tools for studying development and disease. Traditional gene targeting in mice using embryonic stem (ES) cells, although suitable for generating sophisticated genetic modifications in endogenous genes, is complex and time-consuming. We have recently described CRISPR/Cas-mediated genome engineering for the generation of mice carrying mutations in multiple genes, endogenous reporters, conditional alleles or defined deletions. Here we provide a detailed protocol for embryo manipulation by piezo-driven injection of nucleic acids into the cytoplasm to create gene-modified mice. Beginning with target design, the generation of gene-modified mice can be achieved in as little as 4 weeks. We also describe the application of the CRISPR/Cas technology for the simultaneous editing of multiple genes (five genes or more) after a single transfection of ES cells. The principles described in this protocol have already been applied in rats and primates, and they are applicable to sophisticated genome engineering in species in which ES cells are not available.

INTRODUCTION

Genetically modified mice are used extensively in research for understanding gene function and for modeling human disease. In conventional gene-targeting methods[1], mutant mice are generated by introducing mutations through homologous recombination (HR) in mouse ES cells. Targeted ES cells injected into wild-type (WT) mouse blastocysts can contribute to the germ line of chimeric mice, which, when transmitted through the germ line, generate progeny containing the targeted gene[1]. The generation of mutant mice by HR in ES cells is costly and time-consuming because gene-targeted ES cell clones need to be selected and injected into blastocysts to generate chimeric mice, which then have to be bred to generate single-gene mutant offspring, a procedure that usually takes 9–12 months. The construction of mice carrying multiple alterations will add substantially more time and effort. Moreover, gene targeting by HR requires the use of ES cell technology, a method that is not available in most mammalian species.

Genetically modified mice using engineered nucleases: overview

Alternative methods have been developed in various species, which are aimed at generating DNA double-strand breaks (DSB) at specified loci; these methods involve direct injection of DNA or mRNA coding for site-specific nucleases into the one-cell embryo, a procedure that markedly accelerates the process of genome modification. By using site-specific nucleases such as zinc-finger nucleases (ZFNs)[2–5], transcription activator–like effector nucleases (TALENs[6–8]) or the RNA-guided clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) nuclease system[9–15], DNA DSBs are generated at a specified genomic locus, which are then repaired by error-prone non-homologous end joining (NHEJ), resulting in mutant alleles[2,3,6,7,10]. Alternatively, if a single-stranded DNA (ssDNA) or a donor plasmid with homology flanking the DSB is introduced into the cells, a defined modification can be inserted into the genome by high-fidelity HR[4,5,11,16].

The CRISPR/Cas system compared with other genome engineering methods

The CRISPR/Cas system functions as the RNA-based adaptive immune system in bacteria and archaea[17,18]. In the type II CRISPR system from *Streptococcus pyogenes*, CRISPR-associated protein 9 (Cas9), CRISPR RNAs (crRNAs) and trans-activating crRNA (tracrRNA) target and degrade foreign nucleic acids, guided by crRNAs[19,20]. The optimized CRISPR/Cas system consists of a fusion between the crRNA and the tracrRNA, providing a chimeric single-guide RNA (sgRNA) that can be used to produce sequence-specific DSBs[20]. For Cas9 to bind to DNA, the target sequence in the genomic DNA must be complementary to the sgRNA sequence and, in addition, it must be immediately followed by the correct protospacer adjacent motif (PAM) sequence, a 3-bp NGG motif present in the target sequence (**Fig. 1**).

Compared with ZFNs and TALENs, the main advantages of CRISPR/Cas-mediated genome engineering are the efficiency of targeting genomic loci and the ease of designing, constructing and delivering multiple sgRNAs, thus suggesting the possibility of multiplexed genome editing in mammals. By co-transfection of a plasmid expressing the *Cas9* gene and five short PCR products encoding different sgRNAs, we have generated clonally derived mES clones containing homozygous mutant alleles for all five genes with high efficiency[10]; a protocol for targeting multiple genes is provided in **Box 1**. This method will allow for interrogation of cellular phenotypes caused by disruption of multiple genes. However, as mentioned above, the generation of mice from targeted ES cells is laborious, requiring the production of chimeras and breeding for germline transmission.

Direct co-injection of RNA encoding the Cas9 protein and locus-specific guide RNAs into the one-cell embryo circumvents selection of targeted ES cell clones and chimera formation, and thus it allows for the efficient generation of mice carrying mutations in single and multiple genes, as well as of mice carrying endogenous reporters and conditional alleles[11] (**Fig. 2**, **Table 1**).

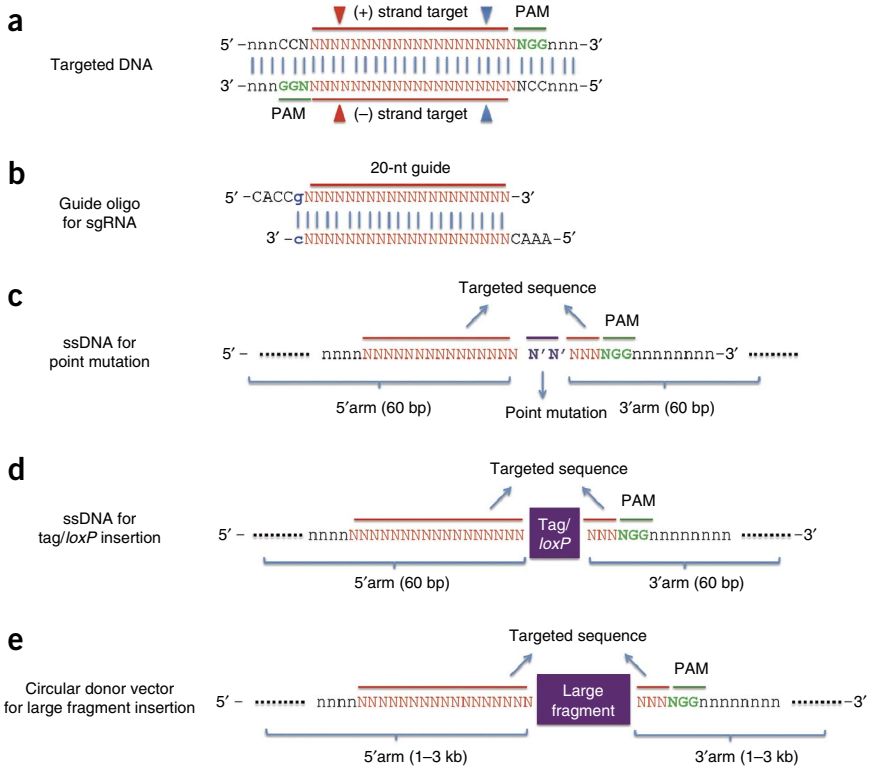


Figure 1 | sgRNA and donor DNA construction. (**a**) Targeted DNA sequence consists of the DNA target (red bar) directly upstream of a requisite 5′-NGG adjacent motif (PAM; green). Cas9 mediates a DSB ~3 bp upstream of the PAM for (+) strand (blue triangle) or (–) strand (red triangle). (**b**) The guide oligos contain overhangs for ligation into the BbsI sites in px330, a G-C base pair (blue) added at the 5′ end of the guide sequence for T7 transcription and the 20-bp sequence preceding 5′-NGG in genomic DNA. (**c**) ssDNA for point mutation consists of a point-mutation site (purple), flanked by 60 base sequences on each side adjoining the DSBs. (**d**) ssDNA for tag/*loxP* insertion consists of tag/*loxP* site (purple), flanked by 60-bp sequences on each side adjoining the DSBs. (**e**) A circular donor vector for large fragment insertion consists of a large fragment, flanked by homology arm sequences on each side adjoining the DSBs.

Limitations of the CRISPR/Cas system

There are several potential limitations of the CRISPR/Cas system. First, the requirement for a NGG PAM sequence of *S. pyogenes* Cas9 limits the target space in the mouse genome. It has been shown that Cas9 homologs from other bacteria use different PAM sequences[21,22] and also induce targeted DNA cleavage in mammalian cells. Therefore, exploiting different Cas9 homologs may expand targeting to more genomic sites. Second, two recent studies reported a high off-target mutation rate in CRISPR/Cas9-transfected human cell lines[23,24]. Although our extensive off-target cleavage analysis showed that off-target mutations are rare in targeted mice derived from CRISPR/Cas zygote injection[10–12],

Box 1 | Simultaneous targeting of multiple genes in ES cells ● TIMING 3 weeks

Here we provide a brief protocol for simultaneous targeting of multiple genes in ES cells. The efficiency of targeting a given allele is 30–50% for indels[10].

MATERIALS

Cell lines

- V6.5 mESCs[27] on a 129/Sv × C57BL/6 F1 hybrid background
- DR4 MEF feeders[28] (derived from 13.5-d-old mouse embryos that are genetically engineered to contain resistance genes to neomycin, hygromycin, puromycin and 6-thioguanin)

Plasmids

- Bicistronic expression vector px330 (ref. 21; Addgene, cat. no. 42230)
- Plasmid containing a selectable cassette (PGK-puroR) that constitutively expresses the puromycin-resistant gene, such as OCT4-eGFP-PGKpuroR (Addgene, cat. no. 31937), used for temporary selection
- Gelatin solution, 0.1% (wt/vol) (Millipore, cat. no. ES-006-B)
- Standard mESC culture medium: 1× DMEM medium (Life Technologies, cat. no. 11965-118) supplemented with 2 mM glutamine (Life Technologies, cat. no. 25030-024), 1 mM sodium pyruvate (Life Technologies, cat. no. 11360-039), 1× non-essential amino acids (Life Technologies, cat. no. 11140-035), 10% (vol/vol) FBS (Life Technologies, cat. no. 16000-044), 0.1 mM β-mercaptoethanol (Sigma, cat. no. M-7522) and 500–1,000 units per ml of leukocyte inhibitory factor (LIF; Millipore, cat. no. ESG1107)
- PBS (Life Technologies, cat. no. 14190-094)
- Trypsin-EDTA, 0.25% (wt/vol) (Life Technologies, cat. no. 25200-056)
- FuGENE HD transfection reagent (Promega, cat. no. E2311)
- Puromycin (Sigma, cat. no. P9620-10ML)
- DNeasy blood and tissue kit (Qiagen, cat. no. 69581)
- Opti-MEM I reduced serum medium (Life Technologies, cat. no. 31985062)

PROCEDURE

1. Culture mESCs such as V6.5 cells[27] on gelatin-coated plates with standard mESC culture conditions[29].
2. 16 to 24 h before transfection, plate mESCs as single cells onto gelatin-coated 10-cm plates.

Box 1 | (continued)

3. Prepare nucleic acids for transfection: Mix px330-Cas9, multiple PCR products each coding for sgRNA targeting each gene and a plasmid expressing PGK-puroR at 1:1:1 Cas9:sgRNA:PGK-puroR ratio. For a 10-cm plate, use 10 μg of total DNA (in a maximum volume of 50 μl) and 30 μl of transfection reagent.
4. For each 10-cm plate, dilute the nucleic acid mix from **Box 1** step 3 (10 μg total) into 500 μl of Opti-MEM, and then add 30 μl of Fugene HD directly to the mixture. Tap the tube to mix, and let it sit for 15 min at room temperature.
5. Add 530 μl of transfection mix dropwise to each plate of cells (from **Box 1** step 2).
6. 12 h after transfection, add DR4 MEF feeders[30] to the plate, so that the transfected colonies can grow better under transient puromycin selection.
7. Add puromycin-containing medium (2 μg/ml) 12 h after feeder addition.
8. Replace puromycin-containing medium with standard medium 48 h after its addition.
9. Allow the cells to recover for 4–6 d in normal mES cell medium.

? TROUBLESHOOTING

10. Pick individual colonies and culture them in 96-well plate with feeders.
11. Collect the remaining mES cells on the plate for the Surveyor assay[25,30] to assess the total efficiency of sgRNA-mediated cleavage.

? TROUBLESHOOTING

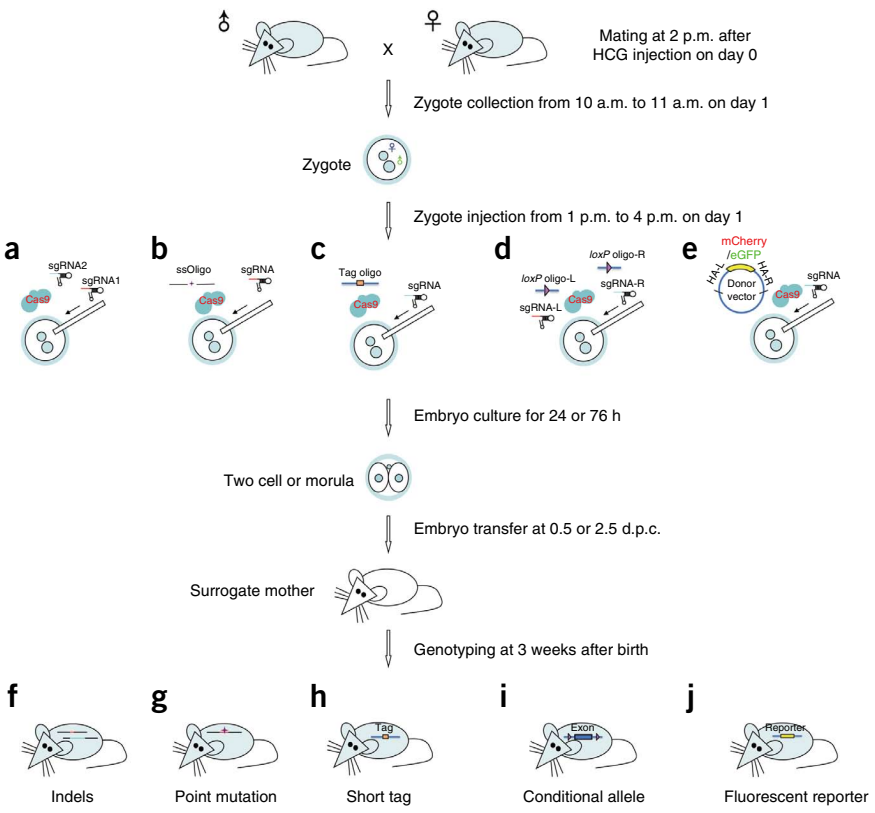
12. After most wells become confluent, trypsinize clones in each well and passage them onto two sets of 96-well plates using a multichannel pipette.
13. When cells have reached confluency, use one plate for DNA extraction using the DNeasy blood and tissue kit, according to the manufacturer's instructions.

■ **PAUSE POINT** Freeze the other plate at –80 °C for up to 3 months or in liquid nitrogen for up to 2 years.

14. Genotype the extracted DNA as described in Step 38 of the main PROCEDURE.

? TROUBLESHOOTING

it is possible that other mutations were induced according to as yet unidentified rules. Clearly, deep-sequencing analyses for a large number of sgRNAs are needed to provide more definitive information about the potential off-target cleavage of the CRISPR/Cas system and to allow for better prediction of potential off-target sites. Finally, mice generated by CRISPR/Cas injection into the zygote are frequently mosaics, which are similar to conventional transgenic animals produced by pronuclear injection, and they require breeding and genetic transmission for the production of mutant mice. Therefore, gene targeting in zygotes of large animals or nonhuman primates[15] may require further optimization of the injection method to reduce the mosaic rate and thus cut the cost and time required for the production of mutant animals.



Experimental design

sgRNA, ssDNA and plasmid DNA design and construction. For sgRNA design and construction (**Figs. 1a,b** and **2**, **Table 1**), follow the protocol described by Zhang's laboratory[25].

- For multiple-gene knockout by indels, two or more sgRNAs are designed to target each gene. For large fragment deletion (**Fig. 2a**, **Table 1**), two sgRNAs are designed, each of which generates a DSB at either the start or end point of the sequence to be deleted.
- To introduce a point mutation, in addition to the sgRNA targeting the site of interest, an ssDNA oligo is designed that contains the desired alteration flanked on each side by 40–60 bases homologous to the sequence adjoining the DSB (**Figs. 1c** and **2b**, **Table 1**).

Figure 2 | Procedure for the generation of gene-modified mice by CRISPR/Cas. (**a–j**) By co-injection of Cas9 mRNA and sgRNAs targeting specific genes independently (**a**) or with ssDNA harboring a point mutation (**b**), tag (**c**), *loxP* (**d**) or plasmid DNA (**e**), gene-modified mice can be generated with indels (**f**), precise mutation (**g**), tag (**h**), conditional allele (**i**) or a fluorescent reporter (**j**).

TABLE 1 | Genetically modified mice generated by CRISPR/Cas-mediated genome engineering[13,14].

Type of gene modification	Components	Repair	Targeting efficiency (%)	Application
Indels	Cas9 and sgRNA	NHEJ	80–90	Gene disruption by frame shift
Point mutation	Cas9, sgRNA and ssDNA	HDR	50–80	Precise gene modification
Small tag insertion	Cas9, sgRNA and ssDNA	HDR	30–50	Gene labeling
Conditional allele generation	Cas9, sgRNA and ssDNA	HDR	10–20	Conditional gene knockout
Large fragment deletion	Cas9, sgRNA	NHEJ	30[a]	Gene disruption of specific domain
Large fragment insertion	Cas9, sgRNA and plasmid DNA	HR	10–20	Gene reporter or gene expression

[a]The efficiency of a 700-bp deletion is ~30%. The larger the fragment to be deleted, the lower the efficiency.

- To introduce a short fusion tag into an endogenous gene, an sgRNA is designed to generate a DSB at the start (for an N-terminal fusion) or stop (for a C-terminal fusion) codon of the endogenous gene. In addition, an ssDNA oligo is designed that encodes the corresponding N- or C-terminal fusion tag, which is flanked on each side by 40–60 bases homologous to the sequence surrounding the sgRNA-mediated DSB (**Figs. 1d** and **2c**, **Table 1**).
- For conditional allele generation, two sgRNAs are designed to elicit DSBs that flank the sequence to be deleted. In addition, an ssDNA oligo is designed that contains the corresponding *loxP* site flanked by two 40- to 60-base homology arms that correspond to the sequence surrounding each sgRNA-mediated DSB (**Figs. 1d** and **2d**, **Table 1**).
- To construct a double-stranded circular DNA donor vector for the introduction of large fragments (**Figs. 1e** and **2e**, **Table 1**), isogenic genomic fragments (3–5 kb) flanking the targeted site are amplified by PCR using primers containing unique restriction sites (**Fig. 2e**). The homologous fragments are then cloned into targeting vector backbone to flank the transgene. Neither side of the homology arm should contain the intact sgRNA-targeting sequences and the PAM sequence to avoid cleavage of the donor plasmid by Cas9. A circular donor vector is used to minimize random integrations.

When introducing a point mutation, a fused tag/*loxP* or a large fragment, a potential problem should be considered. After induction of the DSB, the insertion site of the oligo or vector can vary, as integration is driven by homology and is therefore dependent on oligo or vector design; thus, integration may occur within the target sequence (20-nt guide sequence + PAM) or outside of the target sequence. In the latter case, a second cut could occur in the target site of the modified allele (possibly because the Cas9 and sgRNA may still be active), and thus it would mutate the desired allele by introducing NHEJ-mediated mutations (**Fig. 3**). Sequencing should be performed to exclude this possibility and to select the desired mutant allele.

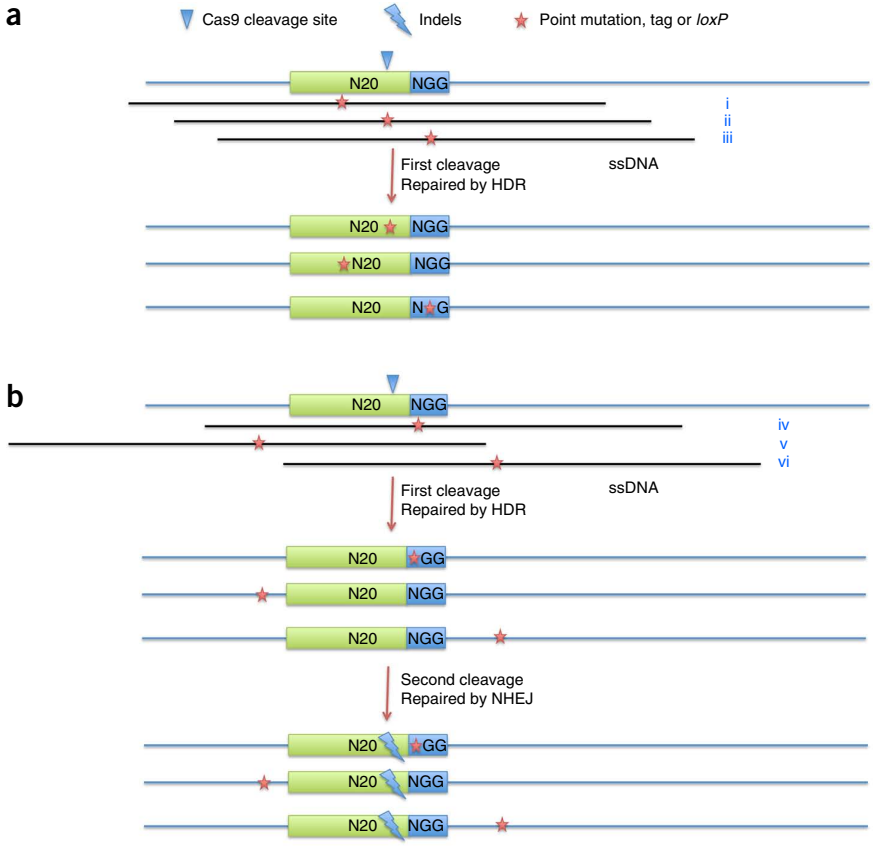


Figure 3 | Schematic illustrating HDR-mediated gene editing by an ssDNA template at a DSB created by Cas9. After the DSB, the insertion site determined by the oligo or vector can vary and it may occur within the target sequence: 20-nt guide sequence (i–ii) + PAM (iii–iv) or outside of the target sequence (v–vi). (**a**) In one case (i–iii, iv if the insertion is larger than 1 bp), the target sequence is disrupted in the modified allele. (**b**) In another case (iv for 1 bp insertion, v–vi), a second cut could occur in the target site of the modified allele, and thus it would mutate the desired allele by introducing NHEJ-mediated mutations. Green box, 20-nt guide sequence; blue box, PAM.

Zygote injection. Besides piezo-based cytoplasmic injection, the nucleic acids can be introduced into the cytoplasm of embryos by using a microinjector (FemtoJet; Eppendorf). Alternatively, the microinjector can also be used to introduce nucleic acids into the nucleus of embryos by

TABLE 2 | Primers for cloning and *in vitro* transcription.

Primer	Sequence (5′–3′)	Purpose
T7-sgRNA_F	ttaatacgactcactataggNNNNNNNNNNNNNNNNNN	*In vitro* transcription of sgRNA: Step 8
T7-sgRNA_R	AAAAGCACCGACTCGGTGCC	
T7-Cas9_F	ttaatacgactcactatagGGAGAATGGACTATAAGGACCACGAC	*In vitro* transcription of Cas9: Step 1
T7-Cas9_R	GCGAGCTCTAGGAATTCTTAC	

pronuclear injection, which is traditionally used for the production of transgenic mice[26]. However, because of toxicity, the concentrations of DNA and RNA need to be substantially lowered. Both modes of injection have comparable efficiencies of gene targeting[14], but cytoplasmic injection is more convenient and simpler.

Functional testing. The desired mutations, including gene disruption, point mutation, small tag insertion or conditional allele generation, can be verified by PCR amplification of the sequence around the target site, by cloning the product into the original TA Cloning vector and by subsequently validating the mutation by sequencing. To facilitate genotyping, it is convenient if the sgRNA target locus contains a restriction enzyme recognition site that can be used to verify correct targeting by a restriction fragment length polymorphism (RFLP) assay. To identify large insertions or deletions, flanking PCR and Southern blot analysis are usually used.

MATERIALS

REAGENTS

Mice

- B6D2F1 (C57BL/6 × DBA/2) or C57BL/6, age ~2–3 months, for zygote collection (Charles River Laboratories)
- ICR (CD-1) mice for pseudopregnant mother and vasectomized males (Charles River Laboratories) ! CAUTION Experimental procedures involving animals must be carried out according to all relevant institutional and governmental regulations.

Plasmids

- Bicistronic expression vector px330 (ref. 21; Addgene, cat. no. 42230) containing individual sgRNAs, used for sgRNA *in vitro* transcription ▲ CRITICAL For sgRNA design and construction, follow the protocol described by Zhang's lab[25].
- px330-Cas9 (Addgene, cat. no. 42230) containing *Cas9* coding region, used for Cas9 mRNA *in vitro* transcription
- Oligos for *in vitro* transcription (Integrated DNA Technologies; **Table 2**)
- Custom-designed ssDNA (if performing point mutation, small fragment insertion or conditional allele), designed as outlined in Experimental design (Integrated DNA Technologies)
- Circular DNA donor vector (if performing large fragment insertion), designed as outlined in Experimental design and prepared in-house
- Gene-specific primers for genotyping, designed according to standard methods (Integrated DNA Technologies)
- Pregnant mare serum gonadotropin (PMSG; Sigma, cat. no. G4527)
- Human chorionic gonadotropin (hCG; Sigma, cat. no. C8554)
- KSOM medium (Millipore, cat. no. MR-106-D) ▲ CRITICAL Store the medium at −20 °C. After thawing, keep it at 4 °C and use it within 2 weeks.
- M2 + hyaluronidase (M2 + Hy; Millipore, cat. no. MR-051-F)
- M2 medium (Millipore, cat. no. MR-015-D)
- Polyvinylpyrrolidone (PVP; 360 kDa, Sigma, cat. no. PVP-360)
- Mercury (Fisher Scientific, cat. no. M-140) ! CAUTION Mercury is hazardous. Handle it with gloves and dispense waste according to local institutional guidelines.
- Cytochalasin B (CB; Sigma, cat. no. C6762)
- Mineral oil (Sigma, cat. no. M8410)
- Nuclease-free water (Life Technologies, cat. no. AM9932)
- MEGAclear kit (Life Technologies, cat. no. AM1908)
- mMESSAGE mMACHINE T7 ultra kit (Life Technologies, cat. no. AM1345)
- MEGAshortscript T7 kit (Life Technologies, cat. no. AM1354)
- QIAGEN Plasmid Plus midi kit (100; Qiagen, cat. no. 12945)
- QIAQuick PCR purification kit (Qiagen, cat. no. 28106)
- TA Cloning kit (Invitrogen, cat. no. 450641)
- Phusion high-fidelity DNA polymerase (NEB, cat. no. M0530)
- AccuPrime Taq DNA polymerase high fidelity (Life Technologies, cat. no. 12346086)
- Tris-acetate-EDTA (TAE) buffer (Thermo Scientific, cat. no. B49)
- Prim-It II random primer labeling kit (Agilent, cat. no. 300385)
- SSC buffer, 20× (Sigma-Aldrich, cat. no. S3017)
- SDS (Sigma-Aldrich, cat. no. 4390)

EQUIPMENT

- Inverted microscope with Hoffman optics (Olympus, cat. no. IX73)
- Micromanipulator set (Narishige, cat. no. MMO-202ND)
- Piezo impact drive system (Prime Tech, cat. no. PMM-150FU) or microinjector (FemtoJet; Eppendorf) as an alternative
- Piezo drill micropipets (Origio, cat. no. PIEZO-8-15) for piezo impact drive system or microinjection capillaries (Femtotip; 5242952.008; Eppendorf) for microinjector
- Holding pipette (Origio, cat. no. MPH-SM-20)
- Transfer pipette (WPI, cat. no. TW100F-6)
- CO_2 incubator (Thermo, cat. no. BB15)
- Stereomicroscope, such as the SZX7 (Olympus, cat. no. SZX7)
- Falcon 10-cm (100 × 20 mm) dishes; bottoms are suitable for oocyte/embryo collection and lids are suitable for micromanipulation (Becton Dickinson, cat. no. 353003)
- Falcon 6-cm (60 × 15 mm) dishes, used for longer culture (Becton Dickinson, cat. no. 351007)
- 26-G needle (Becton Dickinson, cat. no. 309625)
- Clear multiple-well plates, 24 wells (Corning, cat. no. 3337)
- Clear multiple-well plates, 96 wells (Corning, cat. no. 3080)

REAGENT SETUP

CB 50× stock, 500 µg/ml Add 1 mg of CB to 2 ml of DMSO, and then divide it into 100 × 20-µl tubes. Store the stock at −80 °C indefinitely.

M2 + CB, 10 µg/ml Add 2 µl of CB stock solution to 98 µl of M2 medium. Mix it just before use.

M2 + PVP, 10% (wt/vol) PVP Add 1.0 g of PVP (360 kDa) to 9.0 ml of M2 medium; keep it in the refrigerator overnight (4 °C), and then filter it using a 0.45-µm filter and divide it into aliquots in 1-ml tubes. Store the aliquots at 4 °C for up to 3 months.

ssDNA stock, 1 µg/µl Resuspend and dilute ssDNA in RNase-free water at a final concentration of 1 µg/µl. Store the stock at −20 °C for at least 3 months.

Circular DNA donor vector, 1 µg/µl The plasmid is purified using the QIAGEN Plasmid Plus midi kit according to the kit protocol. Store it at −20 °C for at least 3 months.

^{32}P-labeled probes for Southern analysis Southern probes are usually 400 bp–1 kb long. Suitable PCR products or restriction fragments can be labeled with ^{32}P by random priming using the Prim-It II random primer labeling kit, according to the manufacturer's instructions.

PROCEDURE

In vitro transcription and purification of Cas9 mRNA ● TIMING 1 d

▲ CRITICAL Steps 1–7 and 8–14 can be carried out in parallel.

1| For Cas9 mRNA preparation, add the *T7* promoter sequence to the *Cas9* coding region by PCR amplification using the appropriate primer pair listed in **Table 2**:

Component	Amount (per reaction, μl)	Final
px330-Cas9 (5 ng/μl)	2	10 ng
5× GC buffer (NEB)	10	1×
DMSO	1.5	
Forward primer (5 μM)	2	0.2 μM
Reverse primer (5 μM)	2	0.2 μM
Phusion high-fidelity DNA polymerase (NEB)	0.5	
ddH_2O	30	
Total	50	

2| Perform PCR using the following cycling conditions:

Cycle number	Denature	Anneal	Extend
1	98 °C, 5 min		
2–36	98 °C, 30 s	60 °C, 30 s	72 °C, 4 min
37			72 °C, 5 min

3| Run the PCR product on a 1% (wt/vol) agarose gel in TAE buffer to estimate its concentration and to verify that the product is unique and of the expected size (~4.3 kb for *T7-Cas9*).

4| Gel-purify the *T7-Cas*9 PCR product using the QIAQuick PCR purification kit according to the manufacturer's instructions.
▲ CRITICAL STEP The minimum required concentration of PCR products after gel purification is ~40 ng/μl. To increase the yield, the template concentration in the PCR (Step 1) can be increased.

5| Use the gel-purified PCR product as the template for *in vitro* transcription of Cas9 mRNA using the mMESSAGE mMACHINE T7 kit according to the kit protocol.

6| Purify the Cas9 mRNA using the MEGAclear kit according to the manufacturer's instructions and elute it with 50 μl of elution buffer.

7| After purification, dilute the Cas9 mRNA to 500 ng/μl in RNase-free water and check its quality on a 2% (wt/vol) agarose gel in TAE buffer; smeared bands indicate degradation. Samples should be discarded if degradation is observed.
▲ CRITICAL STEP If the RNA appears degraded (i.e., smeared), remove residual RNase from the template DNA before *in vitro* transcription.
? TROUBLESHOOTING
■ PAUSE POINT Store the samples at –80 °C for up to 3 months.

In vitro transcription and purification of sgRNA ● TIMING 1 d

8| For sgRNA preparation, add the T7 promoter sequence to the sgRNA template by PCR amplification using the appropriate primer pair listed in **Table 2**.

▲ CRITICAL STEP For sgRNA design and construction, follow the protocol described by Zhang's laboratory[25].

Component	Amount (per reaction, μl)	Final
px330-sgRNA (5 ng/μl)	2	10 ng
5× GC buffer (NEB)	10	1×
DMSO	1.5	
Forward primer (5 μM)	2	0.2 μM
Reverse primer (5 μM)	2	0.2 μM
Phusion high-fidelity DNA polymerase (NEB)	0.5	—
ddH_2O	30	—
Total	50	

9| Perform PCR using the following cycling conditions:

Cycle number	Denature	Anneal	Extend
1	98 °C, 5 min		
2–36	98 °C, 30 s	60 °C, 30 s	72 °C, 30 s
37			72 °C, 5 min

10| Run the PCR product on a 2% (wt/vol) agarose gel in TAE buffer to estimate its concentration and to verify that the product is unique and of the expected size. The size of the PCR product is ~100 bp for sgRNA.

11| Gel-purify the T7-sgRNA PCR product using the QIAQuick PCR purification kit according to the manufacturer's instructions.

12| Use the gel-purified T7-sgRNA PCR product as the template for *in vitro* transcription of sgRNA using the MEGAshortscript T7 kit according to the kit protocol.

13| Purify the sgRNA using the MEGAclear kit and elute with elution buffer according to the kit protocol.

14| Dilute the sgRNA to 500 ng/μl in RNase-free water and check its quality on a 2% (wt/vol) agarose gel in TAE buffer; smeared bands indicate degradation. Samples should be discarded if degradation is observed.
▲ CRITICAL STEP If the RNA appears degraded (i.e., smeared), remove residual RNase from the DNA template preparation before *in vitro* transcription.
? TROUBLESHOOTING
■ PAUSE POINT Store the samples at –80 °C for up to 3 months.

Zygote preparation ● TIMING **3 d**
15| Inject 12–15 female B6D2F1 (8 weeks old) or C57BL/6 (3–4 weeks old) mice with PMSG (5 IU) at 1:00–2:00 p.m. on day 1.
! CAUTION Experimenters must comply with national regulations concerning animals and their use.
▲ CRITICAL STEP In our experience, these ages give maximal numbers of eggs for the two donor-strain females.

16| After 48 h (i.e., on day 3), inject female mice with hCG (5 IU). After the hCG injection, house female mice with B6D2F1 or C57BL/6 male mice overnight.

17| On the morning of day 4, collect female mice with plugs for zygote preparation and eliminate the female mice without plugs.

18| Prepare the medium for embryo culture. Place several drops (30–50 µl for each drop) of KSOM medium on a 6-cm dish, and cover it with mineral oil, and then place the dish into a 37 °C incubator for at least 30 min before use.

19| At 20–21 h after hCG injection, euthanize the mice and collect zygote-cumulus complexes from the oviduct where it is most swollen.
▲ **CRITICAL STEP** The time of zygote collection is crucial. If the zygotes are collected too early, some embryos will not be fertilized. If they are collected too late, some embryos will enter metaphase during injection.

20| Move the zygote-cumulus complexes into M2 + Hy medium, pipette up and down several times with yellow tips, pick them up with a transfer pipette, wash several times in M2 medium and place the embryos into KSOM medium at 37 °C in a 5% CO_2 incubator.

Preparation for microinjection ● TIMING 1 h
21| Prepare the appropriate injection mix depending on the aim of the experiment, as outlined in the table below. Cas9 stock from Step 7 and sgRNA stock from Step 14 are both at concentrations of 500 ng/µl. Circular DNA donor stock should be at a concentration of 1 µg/µl (see Reagent Setup).

Injection mix	Components	Purpose
Cas9 (100 ng/µl) + sgRNA (50 ng/µl)	Add 1 µl of Cas9 stock, 0.5 µl of each sgRNA stock and nuclease-free water up to 5 µl	Gene disruption by NHEJ (**Fig. 2a**)
Cas9 (100 ng/µl) + sgRNA (50 ng/µl) + ssDNA (100 ng/µl)	Add 1 µl of Cas9 stock, 0.5 µl of each sgRNA stock, 0.5 µl of each ssDNA stock and nuclease-free water up to 5 µl	Point mutation, small tag insertion or conditional allele generation by HDR (**Fig. 2b–d**)
Cas9 (100 ng/µl) + sgRNA (50 ng/µl) + circular DNA donor (200 ng/µl)	Add 1 µl of Cas9 stock, 0.5 µl of each sgRNA stock, 1 µl of circular DNA donor stock and nuclease-free water up to 5 µl	Large fragment insertion by HR (**Fig. 2e**)

▲ **CRITICAL STEP** Mix all the injection components just before use.

22| Place the droplets of three different media (5 µl of injection mix, 15 µl of M2 + CB, 15 µl of M2 + PVP) on top of a 10-cm dish, and then cover it with mineral oil (**Fig. 4**). Place the dish under a stereoscopic microscope.

23| Introduce ~2–3 mm of mercury (Hg) into the injection pipette from a 1-ml syringe fitted with a 26-G needle.
! CAUTION Mercury is a cumulative neurotoxin that is absorbed through the skin. Handle it with appropriate gloves and use it in a chemical fume hood.
▲ **CRITICAL STEP** Hg allows efficient transmission of the piezo pulse to the injection pipette.

24| Attach the injection pipette to the pipette holder of the piezo unit and attach the piezo unit to the micromanipulator.

25| Expel the mercury to the tip of the pipette and lower the assembly into a droplet containing PVP solution. Wash the pipette with PVP solution by expelling some mercury and by applying power from the piezo unit.
▲ **CRITICAL STEP** While washing the pipette in the PVP solution, the piezo unit must be applied with high power (>6 units) and high speed (>6 units) for at least 30 s continuously.

26| Adapt the position of the pipette and set the power and the speed to 3–4 units.

27| Attach the holding pipette to the other side of the micromanipulator.

Injection of zygotes ● TIMING 4 h
28| Transfer 30–50 zygotes, depending on the experimenter's skill level, into M2 + CB medium. Wait for at least 5 min before starting the injection. Each group of zygotes should be injected within 30 min.

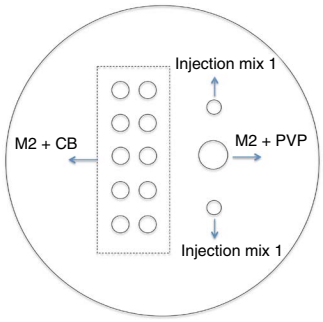


Figure 4 | Suggested layout of droplets in the manipulation chamber. The drops in the dotted box are the M2 + CB medium for zygote injection. Right middle is the M2 + PVP solution for pipette washing. Right top and bottom contain the injection mix consisting of sgRNA, Cas9 and donor DNA.

▲ **CRITICAL STEP** CB makes the walls of the zygotes smoother, and thus it makes micromanipulation easier. Accordingly, wait for at least 5 min before starting injection; otherwise, most of the zygotes will lyse after injection.

29| Aspirate the injection mix into the injection pipette.
▲ **CRITICAL STEP** It is difficult to control the injection pipette smoothly if too much medium is aspirated into the injection pipette.

30| Hold a zygote using a holding pipette. Apply a few piezo pulses to break the zona pellucida (**Fig. 5a**).
▲ **CRITICAL STEP** To avoid damaging the zygote, ensure that there is a large space between the zona and the oolemma.
▲ **CRITICAL STEP** Accurate focus is crucial for several steps in zygote micromanipulation. The injection pipette, zygote and the holding pipette must be in the same horizontal plane.

31| Push the injection mix forward to the tip of the pipette (**Fig. 5b**), and then insert the injection pipette into the zygote without breaking the oolemma and advance the pipette until it almost reaches the opposite side of the zygote's cortex (**Fig. 5c**).

32| Push the mix forward again until it forms a droplet outside the oolemma (**Fig. 5d**). Puncture the oolemma by applying one weak piezo pulse at the pipette tip (as indicated by a rapid relaxation of the zygote membrane; **Fig. 5e**), and immediately withdraw the injection pipette from the zygote to the zona pellucida (**Fig. 5f**).
▲ **CRITICAL STEP** Do not apply the piezo pulse until the pipette reaches the opposite side. The zygote will die after injection if the piezo pulse is applied with the tip of the pipette in the middle of the zygote.
▲ **CRITICAL STEP** It is recommended that beginners use rhodamine B as an indicator of successful injection.
? TROUBLESHOOTING

33| Slowly withdraw the injection pipette to suck the cytoplasm into the pipette, allowing closure of the zygote's oolemma (**Fig. 5g**,**h**, see also **Supplementary Video 1**).
▲ **CRITICAL STEP** The injection pipette must be kept clean outside; otherwise, the oolemma will be pulled out and the zygote will die after injection.
? TROUBLESHOOTING

34| Culture the injected zygotes in KSOM medium at 37 °C in a 5% CO_2 incubator until the two-cell stage after 24 h (for oviduct transfer), or the blastocyst stage after 72 h (for uterus transfer).
? TROUBLESHOOTING

Embryo transfer and production of mice ● **TIMING 3 weeks**
35| Prepare pseudopregnant foster mothers by mating estrous ICR female mice with vasectomized male mice on the same day as injection.

36| Transfer the two-cell embryos into oviducts of 0.5 days post coitum (d.p.c.) recipient or blastocysts into uteri of 2.5 d.p.c. recipient, respectively. Recipient mothers deliver pups at 19.5 d.p.c.
▲ **CRITICAL STEP** Do not transfer more than 30 two-cell embryos or 20 blastocyst embryos into each foster mother.
▲ **CRITICAL STEP** Cesarean section is necessary if recipient mice fail to deliver naturally. Recipient mothers are euthanized at 19.5 d.p.c. of gestation, and the pups are quickly removed from the uteri. After cleaning fluid from their air passages, the pups are kept in a warm box supplied with oxygen. Surviving pups are raised by lactating mothers.

37| Separate male and female offspring into individual cages at 3 weeks after birth.
? TROUBLESHOOTING

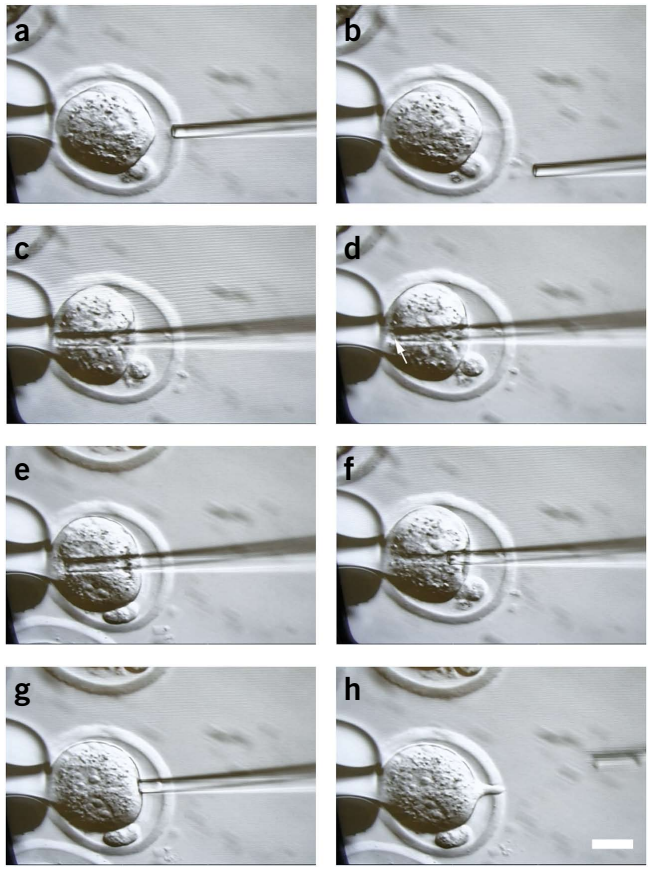


Figure 5 | Injection of zygotes. (**a**) Hold the zygote and cut the zona pellucida using piezo pulses. (**b**) Expel the zona fragment and cytoplasm from the pipette, and push the injection mix to the tip of the pipette. (**c**) Insert the injection pipette into the zygote until it almost reaches the opposite side of the zygote's cortex. (**d**) Push the mix forward again until it forms a droplet outside the oolemma. (**e**) Apply one weak piezo pulse to puncture the oolemma at the pipette tip. (**f**–**h**) Immediately withdraw the injection pipette from the zygote to the zona pellucida and then slowly suck the cytoplasm into the pipette, allowing closure of the zygote's oolemma. All injections are performed with an 20× object lens at room temperature (25–30 °C); scale bar, 25 µm. (See also **Supplementary Video 1**).

Genotyping ● TIMING 2 d

38| Extract genomic DNA from tail biopsies of the 3-week-old mice by using a DNeasy blood and tissue kit according to the manufacturer's protocol. Follow option A for genotyping small genomic modifications (gene disruption, point mutation, small tag insertion or conditional allele generation). Large deletions or insertions can be genotyped using flanking PCR (option B) and Southern blots (option C).

▲ **CRITICAL STEP** For genotyping large insertions or deletions, Southern blot analysis is crucial and should be mandatory, as it gives confidence in the correct insertion, detects random integrations and avoids PCR contamination.

? TROUBLESHOOTING

(A) Genotyping for gene disruption, point mutation, small-tag insertion or conditional allele generation

(i) PCR-amplify the extracted DNA using gene-specific primers under the following conditions:

Component	Amount (per reaction, µl)	Final
Tail DNA (4 ng/µl)	1	4 ng
10× buffer	2	1×
ddH_2O	14.5	
Forward primer (4 µM)	1	0.2 µM
Reverse primer (4 µM)	1	0.2 µM
0.5 µl AccuPrime Taq	0.5	
Total	20	

Cycle number	Denature	Anneal	Extend
1	95 °C, 5 min		
2–36	95 °C, 30 s	60 °C, 30 s	68 °C, 40 s
37			68 °C, 5 min

(ii) Purify the PCRs by using the QIAQuick PCR purification kit according to the manufacturer's instructions.

(iii) (Optional) If the sgRNA target locus contains a restriction enzyme recognition site, digest the PCR products with the corresponding enzyme. By using this RFLP assay, the targeted allele will lose the restriction site, which can be detected by failure to cleave upon enzyme treatment.

(iv) Clone the PCR products using the TA Cloning kit according to the manufacturer's instructions, and verify the mutations by sequencing.

(B) Genotyping large insertions or deletions by flanking PCR

(i) PCR-amplify the extracted DNA using gene-specific primers using the conditions tabulated below. For flanking PCR, two primers flanking the targeted domain are used, resulting in the amplification of both the targeted knockout allele (KO) and the untargeted WT allele.

Component	Amount (per reaction, μl)	Final
Tail DNA (4 ng/μl)	1	4 ng
10× buffer	2	1×
ddH_2O	14.5	
Forward primer (4 μM)	1	0.2 μM
Reverse primer (4 μM)	1	0.2 μM
0.5 μl AccuPrime Taq	0.5	
Total	20	

Cycle number	Denature	Anneal	Extend
1	95 °C, 5 min		
2–36	95 °C, 30 s	60 °C, 30 s	68 °C, 40 s
37			68 °C, 5 min

(ii) Run the PCR product on a 2% (wt/vol) agarose gel in TAE buffer to verify that the product is unique and of the expected size. The PCR product can be detected in WT but not in targeted alleles after large insertions, or in targeted alleles but not in WT alleles after large deletions.

(C) Genotyping large insertions or deletions by Southern blotting

(i) Digest 10–20 μg of genomic DNA with 20–50 units of appropriate enzyme. The selected restriction enzyme should allow for the detection of targeted and WT alleles with different sizes.

(ii) Run the digested DNA at 36 V on an 0.8% (wt/vol) agarose gel overnight or until the dye reaches the bottom of the gel.

(iii) Transfer it to a nylon membrane using standard methods.

(iv) Hybridize the DNA with ^{32}P-labeled random primed probes (see Reagent Setup) at 65 °C overnight.

(v) Wash the membrane with 2× SSC and 0.2% (wt/vol) SDS for 15 min at 65 °C.

(vi) Wash the membrane with 0.2× SSC and 0.2% (wt/vol) SDS for 30 min at 65 °C.

(vii) Expose the membrane to the film. The mutant alleles containing large insertions or deletions should appear as bands with sizes different from the WT allele.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
7, 14	Low concentration of sgRNA or Cas9 mRNA	The amount and quality of the template is low	Improve the amount of template by increasing DNA concentration for *in vitro* transcription and improve the quality of template by avoiding RNase contamination of mRNA
		Low yield	Extend the reaction time and elute RNA with less elution solution

(continued)

TABLE 3 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
32, 33	Zygotes lyse after injection	Incubation time before starting injection is too short	Wait at least 5 min before starting injection
		Pipette is not inserted deeply enough	The injection pipette must be inserted very deeply into the oocyte before applying the piezo
		The injection pipette and zygote are not in the same horizontal plane	Adjust the injection pipette to the same horizontal plane with the zygote
		Too much injection mix is injected into the zygote	Immediately withdraw the injection pipette once the oolemma has been punctured with the pipette tip
	It is difficult to control the injection pipette	The inside of the injection pipette is not clean	Clean the pipette with M2 + PVP medium
		Too much medium is aspirated into the pipette	Aspirate appropriate medium into the pipette, about 2–3 mm away from the tip
32	It is difficult to estimate the volume of injection mix injected into the zygote	Lack of injection experience	Add rhodamine B as an indicator at Step 21
34	Low embryo survival rate	Concentration of sgRNA, Cas9 mRNA or DNA is too high	Use a lower RNA/DNA concentration (see **Table 1**)
		DNA contains toxic compounds	Use an endotoxin-free kit to isolate DNA
37	Low birth rate	Concentration of sgRNA, Cas9 mRNA or DNA is too high	Use a lower RNA/DNA concentration (see **Table 1**)
		Low survival rate to blastocyst stage	Transfer the embryo at the two-cell stage
38	Low targeting rate	Wrong design of sgRNA or donor DNA.	Assess the cutting efficiency on cultured cell lines on the basis of the Surveyor assay.
		Wrong design of donor DNA	Assess the targeting efficiency on cultured cell lines
		Low success rate of injection	Use rhodamine B as an indicator at Step 21
		Low concentration of sgRNA, Cas9 mRNA or DNA	Increase the concentration of sgRNA, Cas9 mRNA or DNA
Box 1	Low targeting efficiency	Low transfection efficiency	Optimize the transfection protocol using a plasmid constitutively expressing GFP
	Only a few mES colonies survive after transient puromycin selection	Puromycin concentration in the medium is too high	Reduce the puromycin selection from 48 to 24 h
		Targeted gene is essential for cell survival or maintenance of pluripotency	Exclude this sgRNA or reduce the concentration of the sgRNA to generate a heterozygote

● TIMING

Steps 1–14, *in vitro* transcription and purification: 1 d
Steps 15–20, zygote preparation: 3 d
Steps 21–27, preparation for microinjection: 1 h
Steps 28–34, injection of zygotes: 4 h

Steps 35–37, embryo transfer and production of mice: 3 weeks
Step 38, genotyping: 2 d
Box 1, simultaneous targeting of multiple genes in ES cells: 3 weeks

ANTICIPATED RESULTS

Survival rates after injection should be >90%. On the basis of the targeting experiments that we have done, almost all injections with only Cas9 mRNA and sgRNAs resulted in mutant alleles containing indels with high efficiency (>80% for one allele). With co-injection of single-stranded oligo donor DNA, the efficiency of homology-directed repair (HDR) varies from 10 to 80%. By using double-stranded plasmid donor DNA, the efficiency of HR varies from 10 to 30% (**Table 1**). However, the results may vary substantially depending on the specific genomic loci. It may be helpful to determine the targeting efficiency at the blastocyst stage by genotyping PCR using a genome amplification kit (Sigma-Aldrich, cat. no. WGA4-50RXN) as a preliminary test. For larger fragment insertion, such as reporter insertion, it may help to use the Oct4-IRES-eGFP-PGKNeo (Addgene, cat. no. 48681) vector to optimize the system[11]: successfully targeted embryos will give a GFP signal in the inner cell mass (ICM) at the blastocyst stage.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS This work was supported by US National Institutes of Health (NIH) grants R37-HD045022 and R01-CA084198. This work was also supported by a grant from the Simons Foundation to the Simons Center for the Social Brain at the Massachusetts Institute of Technology.

AUTHOR CONTRIBUTIONS H.Y., H.W. and R.J. wrote the manuscript.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

1. Capecchi, M.R. Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. *Nat. Rev. Genet.* **6**, 507–512 (2005).
2. Carbery, I.D. *et al.* Targeted genome modification in mice using zinc-finger nucleases. *Genetics* **186**, 451–459 (2010).
3. Geurts, A.M. *et al.* Knockout rats via embryo microinjection of zinc-finger nucleases. *Science* **325**, 433 (2009).
4. Brown, A.J. *et al.* Whole-rat conditional gene knockout via genome editing. *Nat. Methods* **10**, 638–640 (2013).
5. Cui, X. *et al.* Targeted integration in rat and mouse embryos with zinc-finger nucleases. *Nat. Biotechnol.* **29**, 64–67 (2011).
6. Sung, Y.H. *et al.* Knockout mice created by TALEN-mediated gene targeting. *Nat. Biotechnol.* **31**, 23–24 (2013).
7. Tesson, L. *et al.* Knockout rats generated by embryo microinjection of TALENs. *Nat. Biotechnol.* **29**, 695–696 (2011).
8. Carlson, D.F. *et al.* Efficient TALEN-mediated gene knockout in livestock. *Proc. Natl. Acad. Sci. USA* **109**, 17382–17387 (2012).
9. Shen, B. *et al.* Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. *Cell Res.* **23**, 720–723 (2013).
10. Wang, H. *et al.* One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* **153** 910–918 (2013).
11. Yang, H. *et al.* One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* **154**, 1370–1379 (2013).
12. Li, W., Teng, F., Li, T. & Zhou, Q. Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. *Nat. Biotechnol.* **31**, 684–686 (2013).
13. Li, D. *et al.* Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. *Nat. Biotechnol.* **31**, 681–683 (2013).
14. Hwang, W.Y. *et al.* Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotechnol.* **31**, 227–229 (2013).
15. Niu, Y. *et al.* Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell* **156**, 836–843 (2014).
16. Meyer, M., de Angelis, M.H., Wurst, W. & Kuhn, R. Gene targeting by homologous recombination in mouse zygotes mediated by zinc-finger nucleases. *Proc. Natl. Acad. Sci. USA* **107**, 15022–15026 (2010).
17. Horvath, P. & Barrangou, R. CRISPR/Cas, the immune system of bacteria and archaea. *Science* **327**, 167–170 (2010).
18. Wiedenheft, B., Sternberg, S.H. & Doudna, J.A. RNA-guided genetic silencing systems in bacteria and archaea. *Nature* **482**, 331–338 (2012).
19. Gasiunas, G., Barrangou, R., Horvath, P. & Siksnys, V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl. Acad. Sci. USA* **109**, E2579–E2586 (2012).
20. Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821 (2012).
21. Cong, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823 (2013).
22. Esvelt, K.M. *et al.* Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. *Nat. Methods* **10**, 1116–1121 (2013).
23. Fu, Y. *et al.* High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* **31**, 822–826 (2013).
24. Hsu, P.D. *et al.* DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol.* **31**, 827–832 (2013).
25. Ran, F.A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281–2308 (2013).
26. Ittner, L.M. & Gotz, J. Pronuclear injection for the production of transgenic mice. *Nat. Protoc.* **2**, 1206–1215 (2007).
27. Eggan, K. *et al.* Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *Proc. Natl. Acad. Sci. USA* **98**, 6209–6214 (2001).
28. Tucker, K.L., Wang, Y., Dausman, J. & Jaenisch, R. A transgenic mouse strain expressing four drug-selectable marker genes. *Nucleic Acids Res.* **25**, 3745–3746 (1997).
29. Markoulaki, S., Meissner, A. & Jaenisch, R. Somatic cell nuclear transfer and derivation of embryonic stem cells in the mouse. *Methods* **45**, 101–114 (2008).
30. Guschin, D.Y. *et al.* A rapid and general assay for monitoring endogenous gene modification. *Methods Mol. Biol.* **649**, 247–256 (2010).