CRISPR/Cas-mediated genome editing in the rat via direct injection of one-cell embryos

Yanjiao Shao[1,3], Yuting Guan[1,3], Liren Wang[1,3], Zhongwei Qiu[1], Meizhen Liu[1], Yuting Chen[1], Lijuan Wu[1], Yongmei Li[1], Xueyun Ma[1], Mingyao Liu[1,2] & Dali Li[1]

[1]Shanghai Key Laboratory of Regulatory Biology, Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai, China. [2]Center for Cancer and Stem Cell Biology, Institute of Biosciences and Technology, Texas A&M University Health Science Center, Houston, Texas, USA. [3]These authors contributed equally to this work. Correspondence should be addressed to M.L. (myliu@bio.ecnu.edu.cn) or D.L. (dlli@bio.ecnu.edu.cn).

Published online 25 September 2014; doi:10.1038/nprot.2014.171

Conventional embryonic stem cell (ESC)–based gene targeting, zinc-finger nuclease (ZFN) and transcription activator–like effector nuclease (TALEN) technologies are powerful strategies for the generation of genetically modified animals. Recently, the CRISPR/Cas system has emerged as an efficient and convenient alternative to these approaches. We have used the CRISPR/Cas system to generate rat strains that carry mutations in multiple genes through direct injection of RNAs into one-cell embryos, demonstrating the high efficiency of Cas9-mediated gene editing in rats for simultaneous generation of compound gene mutant models. Here we describe a stepwise procedure for the generation of knockout and knock-in rats. This protocol provides guidelines for the selection of genomic targets, synthesis of guide RNAs, design and construction of homologous recombination (HR) template vectors, embryo microinjection, and detection of mutations and insertions in founders or their progeny. The procedure from target design to identification of founders can take as little as 6 weeks, of which <10 d is actual hands-on working time.

INTRODUCTION

The rat was the first mammalian species domesticated for scientific research, and it has long been a valuable and widely used model organism for psychologists, physiologists and pharmacologists, as in many aspects of their biology, rats are more similar to humans than mice are[1]. The mouse has become the leading mammal used for biomedical research, however, largely owing to the development of sophisticated gene modification technologies, especially gene targeting by HR in mouse ESCs[2–4]. In the past 5 years, however, gene targeting technologies for the rat genome have developed very quickly, catching up with the pace set by mouse research.

Methods for genetically modifying rats

The laboratory rat has been genetically modified using several technologies, including pronuclear injection or lentiviral infection–mediated transgenesis[5,6], *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis–driven knockout[7] and transposon-mediated mutagenesis[8,9]. More recently, several site-specific targeting strategies have been developed using HR in ESCs[10], ZFNs[11,12], TALENs[13], engineered meganucleases[14] and CRISPR/Cas[15–17]. ENU-induced mutagenesis and transposon-based gene traps are powerful technologies for large-scale phenotypic screens. However, the cost, space and labor requirements for handling these screening projects in rats are extremely high. In addition, some genetic regions ('cold spots') are difficult to target by random mutagenesis. The identification of specific mutation sites in rats is sometimes challenging and time-consuming. Although ESC–based gene modification is a key breakthrough for precise gene editing in the rat genome[10,18], it is an expensive, time-consuming and labor-intensive technology that demands experience and skillful handling of rat ESCs. In addition, although germline-competent ESCs are available from different rat strains[19–23], the germline transmission efficiency is very low after gene targeting. These disadvantages have delayed the adaptation of this technology worldwide. The emergence of engineered DNA nucleases, ZFNs[11,24–28], TALENs[13,29–33], engineered meganucleases[14] and the CRISPR/Cas system[34–40] is greatly accelerating the development of genetic engineering technologies in the rat, and it is providing benefits to the research community. Engineered nucleases are guided to specific genomic loci to make site-specific DNA double-strand breaks (DSBs). Distinct from the other engineered nuclease-based technologies, the CRISPR/Cas system is composed of a Cas9 nuclease and a guide RNA that directs the nuclease to the target DNA through base-pairing rules[41,42] (**Fig. 1**). We have adapted the CRISPR/Cas system to modify both mouse and rat genomes and demonstrated that it is an efficient, reliable and convenient technology for multiple gene knockout in mice and rats[15].

The CRISPR/Cas system

The CRISPR/Cas system is an RNA-mediated adaptive immune system found in bacteria and archaea that protects against the invasion of viruses and plasmids[41]. On the basis of locus organization and signature *Cas* gene composition, three major types of CRISPR systems (types I–III) have been identified[43]. The type II CRISPR/Cas system derived from *Streptococcus pyogenes* has been modified and widely used for gene editing[43,44]. The main components of this system are the CRISPR RNA (crRNA) array, the *trans*-activating crRNA (tracrRNA) and the Cas9 nuclease. The crRNA array comprises direct repeats that flank different variable elements (protospacers) derived from the exogenous target genome. In the target genome, the sequence complementary to the protospacer must be directly upstream of a protospacer-adjacent motif (PAM) for cleavage to occur. The PAM sequence varies depending on the host system and Cas9 nuclease present[43]; the PAM for Cas9 in *S. pyogenes* is NGG (where N is any nucleotide)[37]. The crRNA forms a duplex with the tracRNA, and it is processed so that the protospacer sequence (20 bases at the 5′ end of the processed crRNA) can direct the Cas9 nuclease to the complementary target DNA, where Cas9 mediates site-specific cleavage[37]. Theoretically, by replacing the crRNA protospacer

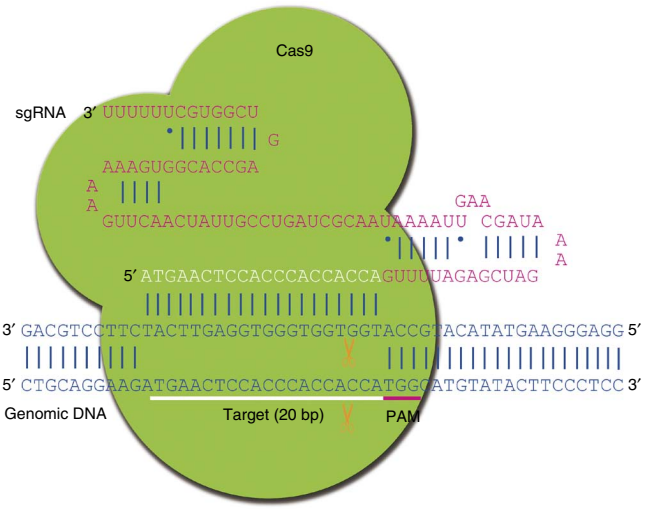


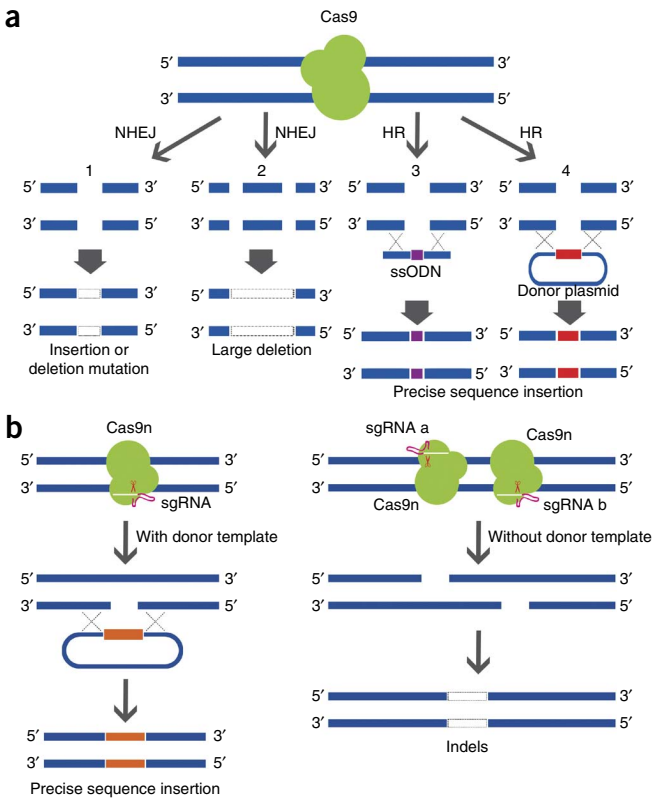
Figure 1 | Schematic of RNA-guided Cas9 nuclease. The CRISPR/Cas system is composed of a Cas9 nuclease (green) and a synthetic single-guide RNA (sgRNA) that directs the nuclease to the target DNA sequence directly upstream of a requisite adjacent 5′-NGG motif according to Watson-Crick base-pairing rules. The sgRNA consists of a universal scaffold (red) and a 20-bp targeting sequence (white), which can be designed to target any selected genomic locus.

sequence with an appropriate targeting sequence, Cas9 can be directed to any genomic locus that fulfills the requirements of the PAM in order to generate DSB-mediated site-specific mutations (**Fig. 1**). Much as in the human genome, on average every 8–10 bp of the rat genome contains a NGG PAM for Cas9 targeting.

Generation of site-specific mutations using CRISPR/Cas

The CRISPR/Cas system (such as ZFNs and TALENs) induces DSBs at precise genomic loci. As DSBs are highly cytotoxic lesions, DNA damage repair responses are initiated to join the broken ends. In mammalian cells, nuclease-induced DSBs can be repaired by two major pathways: nonhomologous end joining (NHEJ) and HR[45,46] (**Fig. 2a**). The different repair pathways will result in different types of mutations.

NHEJ promotes direct ligation of the DSB ends in an error-prone manner usually resulting in random indels (small insertions, deletions and substitutions) as well as large deletions[46]. For gene-editing purposes, indels can be induced in the desired coding region of the gene to make frameshift mutations leading to disruption of the original gene coding[11,29] or to in-frame deletions or mutations of specific amino acids. If two adjacent DSBs occur, the DNA fragment between these two breaks can be deleted via NHEJ-mediated ligation (**Fig. 2a**).



The HR pathway requires a homologous sequence to serve as a template for repair of both broken strands in a high-fidelity manner. HR-mediated gene editing can be used to generate point mutations, precise deletions, conditional knockouts or reporter knock-ins in the rat genome. Either double-stranded DNA or single-stranded oligodeoxynucleotides (ssODNs) are suitable donor templates for HR of nuclease-engineered DSBs[47]. The presence of a DSB increases the efficiency of an HR event by at least 1,000-fold (ref. 48), which makes it feasible to generate precisely gene-edited rat strains through direct injection of a Cas9–single-guide RNA (sgRNA) combination and donor template into one-cell embryos (see ANTICIPATED RESULTS section). The cleavage of the double-stranded DNA is catalyzed by two nuclease domains of Cas9, and a single mutation in either domain results in a nick (single-strand break) rather than a DSB at the target genomic locus[37]. Nicks created by mutant (DNA-nicking) nucleases also stimulate HR. Therefore, to minimize off-target mutations, DNA-nicking enzymes are used to stimulate HR in the targeted site[49] (**Fig. 2b**).

Overview of the procedure

For Cas9-mediated gene editing, the crRNA-tracrRNA duplex is linked together as a single chimeric RNA, termed an sgRNA[37] (**Fig. 1**), which simplifies the system and increases the mutation efficiency. To generate mutant rats, a 20-nt guide sequence 5′

Figure 2 | DSB repair promotes gene editing. (**a**) Nuclease-induced DSBs can be repaired by two major pathways: nonhomologous end joining (NHEJ) and homologous recombination (HR). NHEJ promotes direct ligation of the DSB ends in an error-prone manner, usually leading to indels (small insertions and deletions). HR requires a homologous sequence, either from a plasmid or from single-stranded oligonucleotides (ssODN), to serve as a donor template for repair of both broken strands in a high-fidelity manner. (1) NHEJ-mediated production of indels; (2) induction of large deletions through NHEJ using two adjacent DSBs; (3) HR-mediated precise modification using ssODNs as donor template; and (4) HR-mediated precise large insertions using plasmid donor template. (**b**) Highly specific gene editing by Cas9 nickase. Left, strand-specific nicking induced by Cas9 D10A nickases (Cas9-D10A) guided by an sgRNA stimulates HR in the presence of homology templates. Nicked DNA can be repaired error free if not repaired through HR. Right, with two paired sgRNAs targeting two sites offset with a 4- to 20-bp spacer, the Cas9-D10A induces DSB with sticky ends, which are repaired through error-prone NHEJ to produce indels.

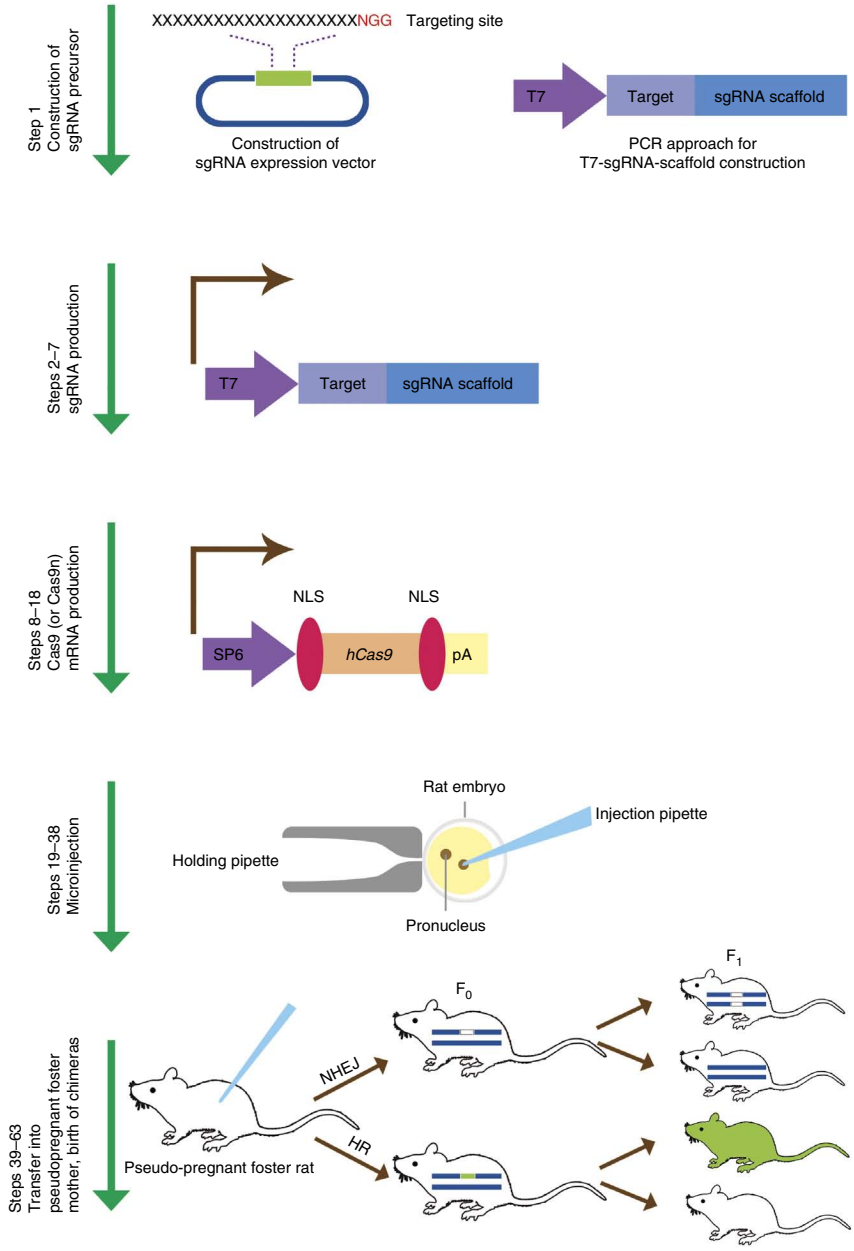


Figure 3 | Workflow for production of mutant rats using the CRISPR/Cas system. Steps for target design, homology template design and construction, sgRNA and Cas9 mRNA synthesis, microinjection, founder identification and germline transmission test. T7 and SP6 indicate the respective promoters. *hCas9*, human codon-optimized Cas9; pA, polyadenylation signal; NLS, nuclear localization signal.

to a NGG PAM is selected to synthesize sgRNA template. For NHEJ-induced mutations, *in vitro*–synthesized Cas9 mRNA and sgRNA are co-injected into the cytoplasm of one-cell-stage rat embryos. Pronuclear injection of a single plasmid containing both Cas9 and the sgRNA sequence is also possible[15,50], but the efficiency is lower than with RNA injection. For the generation of rat strains with precisely edited genomes, donor templates should be co-injected with CRISPR/Cas RNAs into the pronuclei. The injected embryos are transferred into pseudopregnant rats, and the genotype of individual pups is determined by sequencing. In our laboratory, the desired founders are usually identified by initial genotyping ~6 weeks after target design. After sequencing, the desired founders are crossed to wild-type rats to establish mutant strains through germline transmission. In this protocol, we describe the full procedures step by step for the production of gene knockout or knock-in rat strains (**Fig. 3**). Here, the humanized Cas9 from *S. pyogenes* is used as previously described[15]. All plasmids used in this protocol are available to researchers upon request.

Limitations of the protocol

Off-target digestion is a common disadvantage for engineered nucleases. Indeed, as its targeting specificity is determined by a single sgRNA, the CRISPR/Cas system has much greater levels of off-target cleavage than ZFNs and TALENs[51,52]. However, several studies have suggested that off-target effects of the CRISPR/Cas system are much lower in animals than in cultured cells, partially because the Cas9 and sgRNA are injected as short-lived RNAs in embryos instead of as the longer-expressing plasmids used in cell lines[53]. In addition, off-target mutations may be gradually eliminated after a couple of generations of breeding with selection for on-target mutations. A recent publication suggests that a 17- to 18-nt guide sequence greatly decreases undesired mutations at some off-target sites in cell lines[54]. In addition, a double-nicking strategy has been determined to effectively reduce the off-target activity[55] (see Experimental design section). A detailed description of the double-nicking strategy is discussed in the protocol by Ran *et al.*[56]. For precise gene editing, HR efficiency is relatively low (usually <20% in rat embryos) even at the site of nuclease-induced DSB. It remains a challenge to improve the HR activity for precise gene-editing efficiency and fidelity.

Experimental design

Target-site selection. The first step in target selection is to determine which part of the gene should be mutated. For example, for conventional knockout, the first coding exon of the gene of interest is usually chosen. When an outbred strain is used, it is advisable to sequence the chosen gene fragment from several wild-type rats to check for single-nucleotide polymorphisms; any variant sequences should be eliminated before target selection. The sequence of the selected gene fragment can then be submitted to an online design tool (e.g., CRISPR Design Tool, http://tools.genome-engineering.org, created by F. Zhang's laboratory at the Broad Institute of Harvard and MIT) to generate information about potential targets, likely sites of off-target cleavage and targets

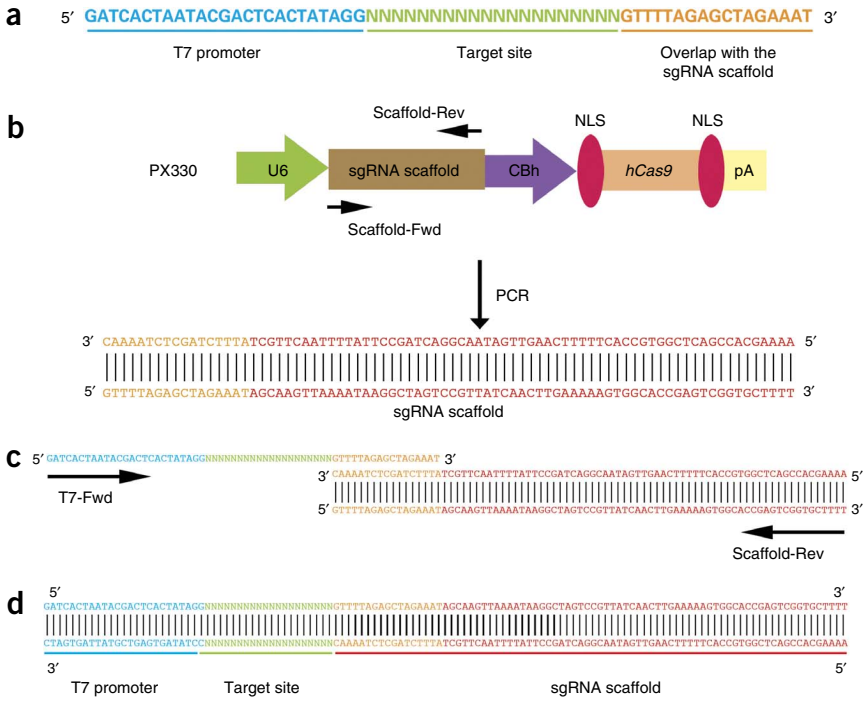


Figure 4 | PCR approach for sgRNA template production. (**a**) Synthesis of an oligo (T7-target oligo) containing the T7 promoter, target site and a 17-nt oligodeoxynucleotide sequence corresponding to the 5′ end of the sgRNA scaffold. (**b**) Preparation of the DNA template of the sgRNA scaffold through PCR using the PX330 plasmid as template. The product is universal for any sgRNA preparation. (**c**) Amplifying the DNA template for the sgRNA through overlapping PCR using ssDNA oligo and sgRNA scaffold from **b** as the template. (**d**) Purification of the DNA template of sgRNA for *in vitro* transcription. U6 and CBh indicate the respective promoter. NLS, nuclear localization signal; *hCas9*, human codon-optimized Cas9; pA, polyadenylation signal.

for double nickase cleavage[56]. Alternatively, the target can be designed manually; once a PAM (5′-NGG) has been identified in the selected gene fragment, the consecutive 20 bp immediately 5′ to the PAM is the target sequence. The potential off-target sites of selected sgRNAs can be predicted using a genome-wide sgRNA off-target searching tool (e.g., CasOT, http://eendb.zfgenetics.org/casot/)[57]. Previous reports have suggested that some targets may not work for unknown reasons[58]. We therefore suggest designing two sgRNAs at a 100-bp interval to increase the mutation efficiency. Furthermore, if the DNA fragments between two target sites are deleted in founders, it will facilitate genotyping by PCR. The double-nicking strategy can greatly reduce off-target effects: here, a pair of sgRNAs are used to target mutant Cas9 (Cas9n) to opposite single strands at adjacent sites separated by a gap of <20 bp (ref. 56).

Construction of templates for *in vitro* transcription of sgRNA

For highly efficient gene targeting in rats, we suggest using RNA injection instead of DNA constructs, although we and others have shown that injection of Cas9 plasmids is also possible[15,50]. *In vitro* synthesis of the sgRNA is usually driven by T7 or T3 promoters. Three common strategies for sgRNA synthesis work well for sgRNA production. The simplest strategy is to directly anneal two complementary oligonucleotides containing the T7 promoter and sgRNA targets[39]. The second strategy is to insert the 20-bp target sequence between the T7 promoter and the sgRNA scaffold through a BbsI restriction site into the pGS3-T7-sgRNA vector, as previously described[15]. In this protocol, we use a ligation-free PCR approach for sgRNA template synthesis (**Fig. 4**). Through synthesis of a 60-bp target-specific oligo containing the T7 promoter sequence followed by one PCR with universal primers, the sgRNA template can be easily produced for *in vitro* transcription (see PROCEDURE section).

Construction of the repair template. Precise gene editing by the HR pathway requires a donor template, supplied either as a plasmid-based donor template (for large insertions) or as an ssODN (for short sequence changes). The donor template (both plasmid and ssODN) always contains two homology arms, which are homologous to the sequence flanking the site of alteration (**Figs. 2a** and **3**). The target site itself should not be included in the repair template DNA. The length of the homology arms can vary. For plasmid-based donors, longer homology arms give higher repair efficiency[59]. Mutant F_0 knock-in rats were obtained with an average mutation rate of 10% by using arms of ~700 bp on each side through nuclease-mediated HR by supplying with plasmid-based donor template[60]. This method can be used to generate large insertions containing reporter genes such as GFP and larger fragments. Our unpublished data suggest that 700-bp homology arms on both sides of a 3-kb exogenous fragment can be inserted into the desired site with efficiency of ~6% of total F_0 pups (Y.S., Y.G., L.W., Mi.L. and D.L., unpublished data). We usually use circular donor plasmids to minimize random insertions, as suggested by others[53,61]; however, in our experience, this method also results in random insertions, which need to be carefully characterized when genotyping.

A few different strategies can be used to construct donor plasmids (**Fig. 5**). The conventional approach independent amplification of two homology arms followed by sequential ligation steps to place the homology arms flanking exogenous DNA templates[18]. Alternatively, overlapping PCR can be used to construct donor plasmids (**Fig. 5a**). Another way to insert the DNA cassette into the homology arm is an overlap extension PCR with a circular plasmid as the template[62]. After the homologous genomic sequence is inserted into the destination plasmid, the gene of interest can be integrated into the homology DNA fragment through overlap extension PCR strategy (**Fig. 5b**). Another approach is to directly synthesize the donor template.

For a short sequence change, an ssODN is used instead of a plasmid-based donor template. It has been suggested that the homology sequence should be at least 40 bp for precise editing in cell lines to achieve high repair efficiency[56]. We have successfully inserted a LoxP-EcoRI (40 bp) cassette flanked by 30-bp homology arms on each side into the rat genome with good efficiency (see ANTICIPATED RESULTS section).

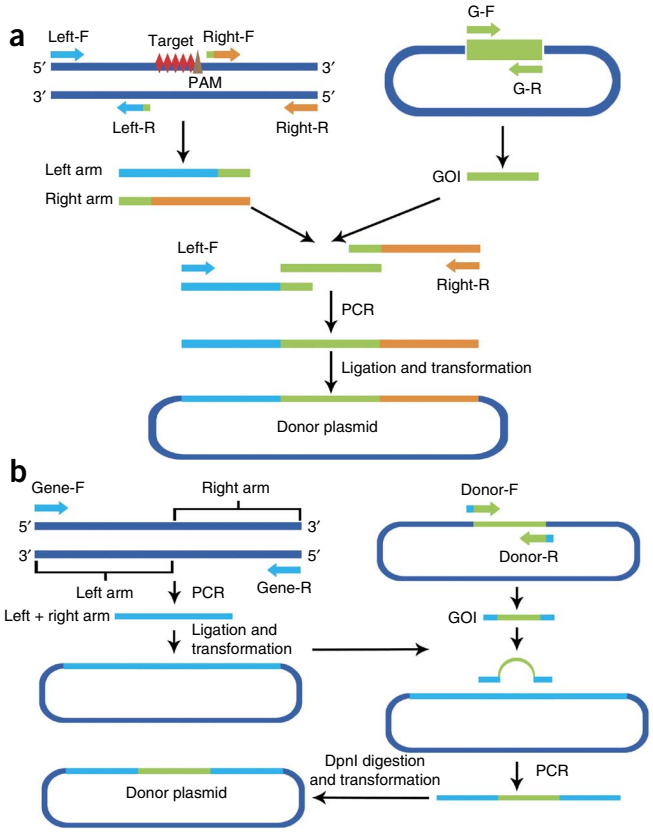


Figure 5 | Schematic diagrams showing the strategy for constructing a rat gene-targeting vector. (**a**) Construction of a rat gene-targeting vector through overlapping PCR. The left and right homology arms are amplified with chimeric primers containing 25 bp of sequence (green in primers) overlapping with the gene of interest (GOI) that is intended to be inserted. The GOI is amplified by PCR using G-F (GOI-forward) and G-R (GOI-reverse) primers. The donor DNA template is amplified by PCR using the left-F and right-R primer pair in the presence of the left arm, the right arm and the GOI fragments as templates. The donor DNA template is then inserted into the chosen vector to prepare the circular donor plasmid. (**b**) Insertion of GOI into the targeting vector by the overlap extension PCR cloning strategy. The homology arms are subcloned into a vector. The donor fragment is amplified by PCR using the chimeric primers donor-F and donor-R, each containing a sequence overlapping with the GOI fragment. The GOI fragment is inserted into the targeting vector by PCR.

***In vitro* sgRNA validation.** Injection of active sgRNAs into embryos is crucial for successful gene editing in the rat. Validation of the designed target in rat cells is the best way to obtain active sgRNA. However, transfection efficiency in rat cell lines is very low. The ability of recombinant Cas9 protein (**Supplementary Method 1**) to induce DSBs in a PCR fragment containing the target in the presence of sgRNA can be used as an alternative method to validate sgRNA activity *in vitro*[37] (**Supplementary Method 2** and **Supplementary Fig. 1**). Although the system works well in our experience, we do not have enough information to demonstrate whether the *in vitro* system is 100% reliable for predicting sgRNA activity *in vivo*.

Generation of pseudopregnant recipient rats. Before starting the procedures for genetically modified rat production, at least 20 vasectomized male rats should be generated through surgery (**Box 1**) and mated with female rats to produce pseudopregnant embryo recipients for oviduct transfer. 8-week-old Sprague-Dawley (SD) male rats are generally used for vasectomy, as this strain is widely available and it shows excellent reproductive performance. The vasectomized male rats should be tested for infertility before use. For pseudopregnant female rats, 10-week-old (body weight over 250 g) SD female rats are used because of their docile behavior and good postnatal care of pups. It is best to determine the stage of the estrous cycle: female rats in proestrus are suitable for mating with vasectomized male rats for pseudopregnancy. However, it takes time and labor to determine the estrous cycle by vaginal smears. Without determining the estrous cycle, setting up 40 crossing pairs will generally obtain 4–12 pseudopregnant female rats (those with copulatory plugs) the following morning.

Embryo donor rats. Before microinjection, it is important to choose an appropriate strain as the embryo donor for subsequent studies. A few inbred or outbred rat stains are widely used around the world. Different strains show different characteristics for modeling distinct human diseases. Many spontaneously arisen strains have been identified as being susceptible to cardiovascular, metabolic, behavioral or oncologic disorders[63]. We usually use the SD rat strain for its calmness, ease of handling and high reproduction rate. For SD rats, 15 hormone-primed female rats are crossed with healthy young fertile male rats, and usually more than 80% of the female rats develop plugs.

Embryo collection. One-cell embryos are obtained from the ampulla oviducts of superovulated female rats the morning after mating. On average, 20–30 embryos are collected per rat. Injecting 150 embryos is generally sufficient to generate a few total-knockout founders. For knock-in studies, we suggest injecting 250 embryos to produce more than one founder because of the low efficiency of site-specific integrations. The embryos are washed and incubated in KSOM embryo culture medium for 3–4 h at 37 °C, 5% CO_2. This short period in culture helps embryonic development, and it also allows for clear visibility of the pronuclei, which is important for pronuclear injection to generate precise knock-in strains.

Cytoplasmic microinjection for NHEJ-mediated indel production. A mixture of Cas9 (or Cas9-D10A for the double-nicking strategy) mRNA (25 ng μl^{-1}) and sgRNA (12.5 ng $\mu l^{-1}$of each target) are introduced by microinjection into the cytoplasm of one-cell embryos. Injection with a higher concentration of Cas9 mRNA (100 ng μl^{-1}) and sgRNA (50 ng μl^{-1}) can yield mostly healthy embryos. Varying concentrations of RNA and the corresponding mutant efficiency from three different groups are presented in **Supplementary Table 1** for reference.

Pronuclear microinjection for HR-mediated repair. In this approach, donor template (either ssODNs or plasmids) must be introduced into the pronucleus. The recommended concentration of the injection solution is 10 ng μl^{-1} Cas9, 5 ng μl^{-1} sgRNA and 10 ng μl^{-1} plasmid-based donor template DNA similar to the optimal concentrations used in mice[53]. We have tried cytoplasmic injection with higher concentrations of RNA (100 ng μl^{-1} Cas9 and 50 ng μl^{-1} sgRNA) and repair template plasmid DNA (200 ng μl^{-1}), but no pups carrying the desired mutations were identified. Compared with pronuclear injection in mice, it is more difficult to penetrate the pronuclear membrane in rat embryos because the pronucleus is extremely elastic, smaller in size and

Box 1 | Generation of vasectomized sterile male rats ● TIMING 3 h

Male SD rats are usually used for the generation of vasectomized male rats. The male rats should be older than 8 weeks and should weigh at least 250 g. Leave the vasectomized male rats for at least 2 weeks to allow them to recover after the surgical procedures. At least two breeding tests should be done before use. There are two different surgical procedures depending on the incision site.

1. Anesthetize a male rat with 10% chloral hydrate by i.p. injection (3 ml kg^{-1}). Use an electric shaver to remove the fur on the posterior 1/3 of the abdomen (optional).
2. Place the rat on its back and wipe the abdominal skin with 70% (vol/vol) ethanol.
3. Make an incision on the abdomen using either options A or B. Option B results in less bleeding and enhanced recovery, but the smaller incision makes it technically more difficult.

(A) Transverse incision

(i) Make a 2.5-cm transverse abdominal incision with fine dissection scissors at a point level with the top of the legs. Make a similar incision in the body wall.

(B) Scrotal midline incision

(i) Make a 10-mm incision along the midline of the scrotal sac. Usually, blood vessels are less abundant in the scrotal sac than in the abdomen.

4. Gently push the testes into the abdomen near the incision with fingers. Use the blunt forceps to pull out one of the testicular fat pads gently. The vas deferens, epididymis and testis will be exposed outside the body cavity. The vas deferens is tightly connected to the epididymis with a blood vessel running alongside it.
5. Hold the vas deferens with a forceps and cut it with fine scissors or cauterize it directly with the red-hot tips of a heated second pair of forceps or with a cauterizer. A small portion (~1 cm) of the vas deferens should be removed. We recommend the cauterization procedure to limit the bleeding and to prevent the cauterized ends from joining back together.
6. Put the tissues back into the body cavity gently with blunt forceps.
7. Repeat the procedure to remove part of the vas deferens from the other testis.
8. Sew up the abdominal wall and the skin separately.
9. Transfer the anesthetized rat to a warm plate for recovery. When the rats are fully recovered, return them to breeding cages.

15 d after vasectomy, the male rats should be tested for sterility before use.

hard to distinguish. With the positive pressure generated by the microinjector, the nucleic acids are injected into the pronuclei, and injection success is confirmed by observing nuclear membrane swelling (**Supplementary Video 1**). For successful pronuclear injection, the angle of the injection pipette in the *z* axis is very important. If the angle is too large, injection causes more damage to the embryo membrane, which will kill the embryo. We usually bend the pipette tip before loading the injection buffer to make it parallel with the injection chamber, which makes it easier to focus both on the pronucleus and the pipette tip at the same plane and reduces damage to the membrane. It has been suggested that foreign DNA delivered into a male pronucleus may be slightly more efficient for random insertional transgenesis in the mouse[64]. It is unclear whether this rule also applies to Cas9-mediated site-specific integration in rat.

Embryo culture. The embryos are generally injected in groups of 50 embryos. Injected embryos are cultured in suitable medium, such as M16, mR1ECM or KSOM, in the incubator overnight. When culturing in KSOM, rat embryos can grow to the two-cell stage by the next morning, but most of them will not develop further. If longer culture is necessary, 18- to 22-h culture of one-cell rat embryos in KSOM (or M16) followed by culture in mR1ECM medium for the development of blastocyst-stage embryos can be performed.

Transfer of embryos to a recipient rat. Injected embryos can be transferred to the oviduct of 0.5-d pseudopregnant female rats on the day of injection or on the following day. The embryos can be transferred through the infundibulum or through a small incision near the opening of the oviduct. One-cell embryos to blastocysts can be transferred to the oviduct of 0.5-d pseudopregnant female rats. Only blastocysts can be transferred into the uterine horns of 3.5-d pseudopregnant female rats. Usually, 20 embryos are transferred to each recipient through bilateral oviducts or through the uterus for cytoplasmic injection. For pronuclear-injected embryos, 30 embryos are divided into two groups and transferred into recipients bilaterally, as pronuclear injection usually causes a lower embryo survival rate compared with cytoplasmic injection.

Determination of founder and progeny genotypes. Rats produced from the embryos injected with Cas9 mRNA and sgRNA for NHEJ-mediated mutation are first detected by digestion of PCR products with the mismatch-sensitive T7 endonuclease 1 (T7E1). Primers are designed to amplify a 600- to 800-bp genomic DNA sequence centered on the target site; this setup generates distinct bands on an agarose gel after nuclease digestion. It is necessary to verify the specificity of the primers before endonuclease treatment. Only primers that produce a single PCR product from genomic template can be used, because nonspecific products will interfere with the results of digestion. The primer pairs that are used in the sgRNA validation step can be reused here. Annealed PCR products can be treated with T7E1 directly, without purification. Usually the founders are chimeras bearing multiple mutations. The PCR products containing mutations can either be directly sequenced to confirm the mutation or be ligated into vectors for sequencing to determine the precise sequence. For the F_1 generation, the genotype can be determined by direct sequencing of the PCR products.

For the identification of founders generated by pronuclear injection and HR repair, the primers should be located outside of the homology arms (**Supplementary Fig. 2**). If the insertion size is small (ssODNs), a pair of primers outside of both sides of

the homology arms can be used. If a large fragment was inserted, two pairs of primers are used. One pair is used to amplify the 5′ part of the insertion. The forward primer is located 5′ outside of the homology arm, and the reverse primer is located on the sequence inserted. The same principle should be followed for the design of primers at the insertion 3′ end. Usually, a positive control needs to be generated for PCR amplification to make sure that the primers work well. To generate a positive control for testing the genotyping primers, amplify a DNA fragment containing the homology arm with a 400-bp extension each side, and ligate it with the fragment to be inserted. After identifying possible founders, it is highly recommended to amplify and sequence the insertion fragment using two primers outside of the donor vector to determine whether concatemers of the donor DNA are present. Southern blotting is the most reliable technique to determine whether ectopic gene targeting has occurred[65].

MATERIALS

REAGENTS

Animals

Sprague-Dawley (SD) rats (SLACCAS, Shanghai) ! **CAUTION** All experimental procedures should be approved by the committees for animal usage. If the anesthesia agents listed in this protocol are prohibited by local regulations, use agents that are permitted or inhalation anesthetic instruments. ▲ **CRITICAL** Rats must be kept in a specific pathogen–free facility and provided with sufficient food and water with a 12-h light cycle.

Cas9 and sgRNA scaffold construction

- Plasmids: PX260 (Addgene, no. 42229, a kind gift of F. Zhang, Broad Institute of MIT and Harvard) contains three expression cassettes, the humanized Cas9, crRNA and tracrRNA. PGS3-T7-sgRNA (synthesized from Taihe Biotechnology Co.LTD) contains the T7 promoter driving an sgRNA scaffold for *in vitro* transcription; the target sequence is easily inserted using the BbsI site (**Supplementary Data 1**). PX330 (Addgene, no. 42230, a kind gift from F. Zhang) is similar to PX260 except that the crRNA and tracrRNA are fused into a single chimeric guide RNA
- KOD-plus-Neo polymerase (Toyobo, cat. no. KOD-401)
- Pfu DNA polymerase (Agilent, cat. no. 600140)
- Platinum Pfx DNA polymerase (Invitrogen, cat. no. 11708-039)
- PCR primers or oligos for sgRNA construction (listed in **Supplementary Table 2**): use the oligos for Step 1A if you are using the PCR-based approach to make sgRNA, or the oligos for Step 1B if you are using the plasmid-based approach
- $MgSO_4$, 25 mM (Toyobo, cat. no. KOD-401)
- Restriction enzymes: Fast Digest BbsI (New England Biolabs, cat. no. R0539), DraI (New England Biolabs, cat. no. R0129), NotI (New England Biolabs, cat. no. R0189), NcoI (New England Biolabs, cat. no. R0193)
- T4 DNA ligase (New England Biolabs, cat. no. M0202)
- dNTP solution mix, 25 mM each (Enzymatics, cat. no. N205)
- GeneRuler DNA ladder mix (Fermentas/Thermo Scientific, cat. no. SM0311)
- DNA loading dye, 6× (Fermentas/Thermo Scientific, cat. no. R0611)
- Ethidium bromide (TianGen, cat. no. RT203)
- E.Z.N.A Plasmid Mini Kit (Omega Bio-tek, cat. no. D6943)

Cas9 mRNA and sgRNA production

- Plasmids: pSP6-Cas9, the humanized Cas9 is inserted following the SP6 promoter for *in vitro* mRNA synthesis (**Supplementary Data 2**). pSP6-Cas9n encodes an aspartate-to-alanine (D10A) mutation in the RuvC catalytic domain of WT Cas9 to produce a Cas9 nickase
- Phenol:chloroform:isoamyl alcohol (25:24:1; Amresco, cat. no. 0883)
- Ethanol, 90% (vol/vol) or higher (Ling Feng Chemical Reagent)
- QIAquick PCR purification kit (Qiagen, cat.no. 28104)
- Sodium acetate (Sigma, cat. no. S5636)
- Nuclease-free water (Promega, cat. no. P1195)
- mMessage mMachine SP6 kit (Life Technologies, cat. no. AM1340)
- *In vitro* Transcription T7 Kit (Takara, cat. no. 6140)

Preparation of donor template for precise genome editing

- ssODN or plasmid donor containing appropriate sequences. See Experimental design section. ssODNs should be synthesized by an appropriate biotech company, for example, Life Technologies
- pEASY-Blunt Cloning Vector (TransGen Biotech, cat. no. CB101) or other appropriate cloning vectors

Embryo microinjections

- Gonadotropin, Pregnant Mare Serum (Millipore, cat. no. 367222)
- Chorionic gonadotropin, human urine (hCG; Millipore, cat. no. 230734)
- M2 medium (Millipore, cat. no. MR-015-D)
- Hyaluronidase (Sigma, cat. no. H4272)
- KSOM medium (Millipore, cat. no. MR-020P-5F)
- Embryo-tested mineral oil (Sigma, cat. no. M5310) ▲ **CRITICAL** The mineral oil must be suitable for embryo culture. It is best to test for toxicity before use.
- Chloral hydrate (SanGon, cat. no. CB0288)
- Trizma hydrochloride solution (for embryo microinjections; Sigma, cat. no. T2663)
- EDTA (for embryo microinjections; Sigma, cat. no. E7889)
- Water (for embryo microinjections; Sigma, cat. no. W3500)
- DPBS (Gibco, cat. no. 14190144)
- M16 medium (Millipore, cat. no. MR-016-D)

Genotyping

- Tris base (Amresco, cat. no. 0497)
- SDS (SanGon, cat. no. SB0485)
- EDTA (SanGon, cat. no. EB0185)
- NaCl (Sigma, cat. no. S5886)
- Proteinase K (Calbiochem, cat. no. 539480)
- Sodium acetate (Sigma, cat. no. S5636)
- Q5 high-fidelity DNA polymerase (New England Biolabs, cat. no. M0491)
- Gene-specific PCR primers (see Experimental design section)
- T7 endonuclease I (New England Biolabs, cat. no. M0302)
- QIAquick Nucleotide Removal Kit (Qiagen, cat. no. 28304)
- pEASY-Blunt Cloning Vector (TransGen Biotech, cat. no. CB101)
- DH5α competent bacterial cells (TianGen, cat. no. CB101)
- Tryptone (Oxoid, cat. no. LP0042)
- Yeast extract (Oxoid, cat. no. LP0021)
- Agar A (SanGon, cat. no. FB0010)
- Ampicillin (SanGon, cat. no. AB0028)
- Kanamycin (SanGon, cat. no. KT6635)

Protein purification

- pMJ806 Plasmid for Cas9 recombinant protein production (Addgene plasmid, #39312)
- KCl (Sigma, cat. no. P5405)
- HEPES (Sigma, cat. no. H4034)
- Imidazole (Aladdin, cat. no. 288-32-4)
- Glycerol (SanGon, cat. no. G0854)
- NuPAGE Novex 4–12% Bis-Tris Gel (Invitrogen. cat. no.NP0335BOX)
- NuPAGE MOPS SDS Buffer Kit (for Bis-Tris Gels; Invitrogen. cat. no. NP0050)
- Prestained Protein Ladder (Fermentas/Thermo Scientific, cat. no. SM0671)

EQUIPMENT

- Flaming/Brown Micropipette Puller (Sutter P-97)
- Shaker (IKA, cat. no. 0003510000)
- Microforge (Narishige MF-830)
- Stereo microscope (Olympus SZX7)
- Stereo microscope (Olympus SZX10)
- Stereo microscope (Olympus IX71)
- TransferMan NK 2 micromanipulator (Eppendorf, cat. no. 920000011)
- BioPhotometer (Eppendorf, cat. no. 6132 000.008)
- Forma Series II Water-Jacketed CO_2 incubator (Thermo Scientific, cat. no. 3111)
- Piezo Micro Manipulator Controller (PRIME TECH, cat. no. CT-150)
- Millex Syringe-driven Filter Unit, 0.22 μm (Millipore, cat. no. SLGV004SL)
- Surgical suture needle with silk thread (GINHUAN Medical, cat. no. G5006g)
- Microscope slides (Vacht Brand, cat. no. 7015)

• Flattening table (Leica, cat. no. HI1220)
• UV-visible spectrophotometer (Thermo Scientific, NanoDrop 2000)
• Microcapillary tube (Sigma, cat. no. P1049)
• Sterilizer (Sigma, cat. no. Z378550)
• Cauterizer (Fine Science Tools, cat. no. 18010-00)
• Bulldog clamp (Serrafine, cat. no. D2085)
• Light Source Unit (Olympus, cat. no. LG-PS2)
• Glass needle (World Precision Instruments, cat. no. TW100F-4)
• PCR thermocycler (Applied Biosystems, cat. no. 4479071)
• Bio-imaging system (Eastwin, cat. no. BIS910)
• Centrifuge (Eppendorf, cat. no. 5415R)
• Centrifuge (Eppendorf, cat. no. 5424)
• Tubes, 0.2 ml (GeneEraBiotech, cat. no. PCR-20-C)
• Tubes, 1.5 ml (Quality Scientific Plastics, cat. no. 509-GRD-Q)
• Petri dishes, 35 × 10 mm (Corning, cat. no. 430165)
• Pipette tips, 1–200 µl (Quality Scientific Plastics, cat. no. 110-B-Q)
• Pipette tips, 0.1–10 µl (Quality Scientific Plastics, cat. no. 104- Q)
• Pipette tips, 100–1,250 µl (Quality Scientific Plastics, cat. no. 112XL-Q)

REAGENT SETUP

Construction of the Cas9-encoding plasmid and pGS3-T7-sgRNA plasmid for *in vitro* RNA synthesis Synthesize a DNA fragment harboring an SP6 promoter sequence followed by a Kozak sequence with an NcoI restriction site. Linearize plasmid PX260 (Addgene, plasmid #42229) using the NcoI restriction site, which is located immediately upstream of the initial codon of the NLS-hSpCas9-NLS expression cassette. Ligate the SP6 promoter with linearized PX260 plasmid, and transform it into competent DH5α bacterial cells. The plasmid containing the SP6 promoter and Kozak sequence is referred to as pSP6-Cas9. The resulting vector has a unique NotI restriction site for linearization for use as *in vitro* transcription template. pSP6-Cas9n is generated similarly by inserting the SP6-Kozak sequence into pSpCas9n(BB) (Addgene, plasmid #48873). pGS3-T7-sgRNA plasmid is generated by insertion of a synthesized DNA fragment, which contains a T7 promoter followed by two reverse-orientated BbsI restriction sites and the sgRNA scaffold, into the pGS3 vector. The sequences of the plasmids are supplied in **Supplementary Data 1** and **2**.

TAE electrophoresis buffer (50×) TAE electrophoresis buffer is 2 M Tris, 1 M glacial acetic acid and 1 M EDTA. Dissolve 242 g of Tris base, 57.1 ml of glacial acetic acid and 37.2 g of EDTA in 1 liter of distilled water to obtain a 50× stock solution. Store the 50× buffer at room temperature (25 °C) for at least 3 months. Dilute 50× TAE buffer in distilled water to 1× working solution for agarose gel preparation and for use as a gel electrophoresis buffer.

LB medium Add 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl to 1 liter of distilled water, and autoclave the mixture. For LB-ampicillin medium, add 1 ml of 100 mg ml^{-1} ampicillin stock solution to 1 liter of autoclaved LB medium, and store it at 4 °C for <1 month.

LB-ampicillin plates Dissolve 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl and 15 g of agar to 1 liter of distilled water. Autoclave and cool the medium to 50 °C. Then, add 500 µl of 100 mg ml^{-1} ampicillin stock solution and pour it into 10-cm dishes. Store the plates at 4 °C for up to 2 months.

TE microinjection buffer (10 mM Tris-HCl and 1 mM EDTA) To prepare 10 ml of TE microinjection buffer with water, add 100 µl of Trizma hydrochloride solution (pH 7.4, 1 M) and 20 µl of EDTA (pH 8.0, 0.5 M EDTA) to distilled water with a final volume of 10 ml. Filter the solution through a 0.22-µm Millex-GP Syringe Filter and store it in aliquots of 1 ml at 4 °C for up to 3 weeks.

Tris buffer (1 M, pH 8.0) Dissolve 121 g of Tris base in 1 liter of distilled water, mix it and adjust the pH to 8.0. Store the buffer at 25 °C for up to 6 months.

EDTA buffer (500 mM) Dissolve 186 g of EDTA in 1 liter of distilled water, mix it and adjust the pH to 8.0. Store the buffer at 25 °C for up to 6 months.

Tissue lysis buffer (0.1 M Tris, 0.05 M EDTA, 0.2 M NaCl and 0.2% (wt/vol) SDS) Add 10 ml of Tris (1 M, pH 8.0), 1 ml of EDTA (500 mM, pH 8.0), 1.17 g of NaCl and 2 ml of 10% (wt/vol) SDS to 100 ml of distilled water to obtain a 1× stock solution. Store the 1× buffer at room temperature for up to 6 months.

SDS buffer, 10% (wt/vol) Dissolve 10 g of SDS in 100 ml of distilled water. Store the buffer at 25 °C for up to 6 months.

Proteinase K stock solution Prepare a stock solution of 20 mg ml^{-1} in water, and store it in aliquots of 20 µl at −20 °C for up to 2 years.

Hyaluronidase stock solution (3 mg ml^{-1}) Prepare a stock solution by dissolving 30 mg of hyaluronidase in 10 ml of M2 medium. Filter-sterilize the solution and store it in aliquots of 200 µl at −20 °C for up to 6 months. Dilute the hyaluronidase stock solution with M2 medium at a 1:5 ratio when you are ready to perform embryo microinjection.

Pregnant mare serum gonadotropin (PMSG) stock solution Prepare a stock solution of 500 IU ml^{-1} in DPBS and store it in aliquots of 100 µl at −20 °C for up to 2 months. Dilute the PMSG stock solution with DPBS at a 1:10 ratio when you are ready to inject.

hCG stock solution Prepare a stock solution of 500 IU ml^{-1} in DPBS and store it in aliquots of 100 µl at −20 °C for up to 2 months. Dilute the hCG stock solution with DPBS at a 1:10 ratio before injection.

Annealing the sgRNA oligos (sense and antisense strands) for Step 1B In a PCR tube, mix the following components: 1 µl of sgRNA sense (100 µM), 1 µl of sgRNA antisense (100 µM) and 1 µl of T4 ligation buffer (10×), and dilute to 10 µl with ddH_2O. Anneal the oligos in a thermocycler by using the following parameters: 95 °C for 5 min; ramp it down to 35 °C at 1 °C min^{1}. Annealed oligos should be stored at 4 °C for 2 weeks or at −20 °C for long-term storage.

PROCEDURE

Construction of sgRNA precursor

1| To generate the sgRNA expression construct, use either the PCR expression cassette method (option A) or the plasmid-based procedure (option B).

(A) PCR approach for T7-sgRNA-scaffold construction ● TIMING 1.5 h

(i) Set up the following reaction mixture to amplify the DNA fragment from PX330 for the sgRNA scaffold:

Component	Amount (µl)	Final
PX330, 20 ng $µl^{-1}$	1	20 ng
Scaffold-Fwd, 10 µM	1.5	0.3 µM
Scaffold-Rev, 10 µM	1.5	0.3 µM
KOD PCR buffer, 10×	5	1×
dNTP, 10 mM (2.5 mM each)	5	0.25 mM
KOD-plus-Neo polymerase	0.5	
$MgSO_4$, 25 mM	3	1.5 mM
ddH_2O	To 50	

(ii) Perform PCR using the following cycling conditions:

Cycle number	Denature	Anneal	Extend
1	95 °C, 2 min		
2–25	95 °C, 30 s	50 °C, 30 s	68 °C, 20 s
26			68 °C, 7 min

(iii) By using T7-target oligo and the amplified DNA fragment from Step 1A(ii) as template, set up the following reaction mixture to amplify the final DNA template for sgRNA synthesis; primer sequences can be found in **Supplementary Table 2**.

Component	Amount (μl)	Final
KOD PCR buffer, 10×	5	1×
dNTP, 10 mM (2.5 mM each)	5	0.25 mM
T7-target oligo, 10 μM	1.5	0.3 μM
sgRNA scaffold PCR product, 200 ng μl^{-1} (from Step 1A(ii))	0.5	100 ng
T7-Fwd primer, 10 μM	1.5	0.3 μM
Scaffold-Rev primer, 10 μM	1.5	0.3 μM
KOD-plus-Neo polymerase	0.5	
$MgSO_4$, 25 mM	3	1.5 mM
ddH_2O	30.5	
Total	50	

▲ **CRITICAL STEP** To minimize error in PCR products, it is important to use a high-fidelity polymerase. Other high-fidelity polymerases may be used, such as Pfu (Agilent) or Platinum Pfx polymerase (Invitrogen).

(iv) Perform PCR using the following cycling conditions:

Cycle number	Denature	Anneal	Extend
1	95 °C, 2 min		
2–25	95 °C, 30 s	50 °C, 30 s	68 °C, 20 s
26			68 °C, 7 min

? TROUBLESHOOTING

(v) Run 5 μl of the PCR product on a 2% (wt/vol) agarose gel in TAE buffer at 110 V for 25 min. Successful reactions should yield a single 130-bp-long product, and the template should be undetectable.

(vi) Purify the remaining PCR product by phenol:chloroform extraction and ethanol precipitation using standard methods.

▲ **CRITICAL STEP** Avoid transferring any of the organic phases during phenol:chloroform extraction. We usually use classical phenol:chloroform extraction to purify DNA, although DNA purification kits can be used to save time and labor.

(vii) After ethanol precipitation, resuspend the DNA pellet in 10 μl of nuclease-free water by pipetting up and down.

(viii) Determine the DNA concentration using a spectrophotometer, and store it at 4 °C.

■ **PAUSE POINT** DNA can be stored at 4 °C for at least 1 month or at –20 °C for long-term storage.

(B) Construction of an sgRNA expression vector ● TIMING 4 d

(i) Set up a digest of the vector PGS3-T7-sgRNA with BbsI endonuclease, as tabulated below, and incubate it at 37 °C for 5 h.

Component	Amount (μl)	Final
PGS3-T7-sgRNA vector, 500 ng μl^{-1}	2	1,000 ng
BbsI	1.5	
NEB buffer 2, 10×	5	1×
ddH_2O	To 50	

(ii) Run a 5-μl sample of the digest on a 1.5% (wt/vol) agarose gel in TAE buffer at 120 V for 30 min. Successful reactions should yield a single 3,000-bp-long product.

(iii) Purify the remainder of the digestion reaction using the QIAquick PCR purification kit according to the manufacturer's directions or phenol:chloroform extraction and ethanol precipitation.

(iv) Elute (or resuspend) the DNA in 30 μl of EB buffer (part of the Qiagen kit) or water.

(v) Dilute annealed sgRNA oligos (see Reagent Setup section) 1:200 in water. Set up a reaction to ligate them into PGS3-T7-sgRNA vector, as tabulated below, and incubate at 16 °C for 2 h. We recommend setting up a noninsertion PGS3-T7-sgRNA vector-only negative control for ligation.

Component	Amount (μl)	Final
Digested PGS3-T7-sgRNA (Step 1B(iv)), 50 ng μl^{-1}	1	50 ng
Annealed sgRNA oligo	1	50 ng
T4 ligase	1	
T4 ligase buffer, 10×	1	1×
ddH_2O	To 10	

▲ **CRITICAL STEP** Avoid freeze-thaw cycles of T4 ligase buffer. Prepare aliquots and store them at −20 °C.

■ **PAUSE POINT** Ligation reactions can be stored at 20 °C until transformation (Step 1B(v)).

(vi) Use 10 μl of ligation reaction for transformation of 100 μl of chemically competent DH5α bacterial cells, according to the manufacturer's instructions. Plate the transformed bacteria onto an LB plate containing 100 μg ml^{-1} ampicillin, and incubate the plate overnight at 37 °C.

(vii) On the next day, observe the plates for colony growth. Typically, there are no colonies on the negative control plates (ligation of BbsI-digested PGS3-T7-sgRNA alone without annealed sgRNA oligo insert), compared with hundreds of colonies on the cloning (sgRNA inserted into PGS3-T7-sgRNA) plates.

(viii) Pick five colonies from the experimental plates, and extract and purify the plasmid DNA using standard methods. Sequence the inserted fragment using the T7 promoter forward primer (T7-fwd; **Supplementary Table 2**).
? TROUBLESHOOTING

(ix) Set up a reaction to linearize sgRNA-containing plasmid PGS3-T7-sgRNA, as described below:

Component	Amount (μl)	Final
Plasmid (from Step 1B(viii)), 500 ng μl^{-1}	2	1,000 ng
DraI	2	
NEB buffer 4, 10×	5	1×
ddH_2O	To 50	

(x) Run 5 μl of the digest on a 2% (wt/vol) agarose gel in TAE buffer at 110 V for 25 min to verify successful digestion: successful reactions should yield a single 3,000-bp product.

(xi) Purify the DraI-digested sgRNA-containing plasmid using the QIAquick PCR Purification Kit according to the manufacturer's directions or by phenol:chloroform extraction and ethanol precipitation.

sgRNA production ● TIMING 3 h

2| Set up a 20-µl transcription reaction in a 1.5-ml tube by using the reagents from the *In vitro* Transcription T7 Kit, as tabulated below.

Component	Amount (µl)	Final
Transcription buffer, 10×	2	1×
Template DNA (from Step 1A or 1B), 200 ng µl^{-1}	5	1,000 ng
ATP, 50 mM	2	5 mM
GTP, 50 mM	2	5 mM
CTP, 50 mM	2	5 mM
UTP, 50 mM	2	5 mM
RNase inhibitor, 40 U	0.5	1 U
RNA polymerase, 50 U	2	5 U
RNase-free H_2O	To 20	

▲ **CRITICAL STEP** 10× transcription buffer for sgRNA production should be added last because it contains spermidine, which easily precipitates in the presence of DNA.

3| Pipette the mixture up and down to mix the reaction, and incubate it for 2 h at 42 °C.

4| Add 3 µl of RNase-free DNase I to the 20-µl transcription reaction, mix it well and incubate it for 30 min at 37 °C.

5| Add 77 µl of RNase-free water to the reaction tube to terminate the reaction.

6| Purify sgRNA by phenol:chloroform extraction and isopropanol precipitation using standard methods.

7| To confirm the quality of the sgRNAs, mix 2 µl of loading dye with 3 µl of sgRNA, and run the sgRNA on a 2% (wt/vol) agarose gel in TAE buffer with 0.4 µg ml^{-1} ethidium bromide at 100 V for 15 min. High-quality RNAs should give sharp bands without smears.

? TROUBLESHOOTING

■ **PAUSE POINT** Purified sgRNAs can be stored at –80 or –150 °C for up to 2–3 months.

Cas9 (or Cas9n) mRNA production ● TIMING 1 d

▲ **CRITICAL** Steps 8–18 can be carried out in parallel to Steps 2–7.

8| Linearize 5 µg of SP6-Cas9 plasmid or SP6-Cas9n by digesting it with 3 µl of NotI in a total reaction volume of 100 µl at 37 °C for 5 h.

9| Run 5 µl of the digested plasmid DNA and 1 µg of the DNA Ladder Mix on a 0.8% (wt/vol) agarose gel in 1× TAE buffer with 0.4 µg ml^{-1} ethidium bromide at 100 V for 30 min to check for complete digestion.

? TROUBLESHOOTING

10| Purify linearized SP6-Cas9 or SP6-Cas9n using the QIAquick PCR Purification Kit according to the manufacturer's directions or by phenol:chloroform extraction and ethanol precipitation. Determine the final concentration of the linearized SP6-Cas9 or SP6-Cas9n with a spectrometer, and store it at –20 °C.

▲ **CRITICAL STEP** The minimum DNA concentration of the linearized plasmid that is required for the *in vitro* transcription reaction is 160 ng µl^{-1}.

? TROUBLESHOOTING

■ **PAUSE POINT** Extracted DNA can be stored at –20 °C for at least 1 month before *in vitro* transcription.

11| Set up a 20-µl transcription reaction in a 1.5-ml tube using the mMessage mMachine SP6 kit according to the manufacturer's instructions. Mix the reaction by pipetting, and incubate it for 2 h at 37 °C.

Component	Amount (µl)	Final
Transcription buffer, 10×	2	1×
Linear plasmid DNA (Step 10), 200 ng µl^{-1}	5	1,000 ng
SP6 NTP/CAP, 2×	10	1×
SP6 enzyme mix	2	
RNA-free H_2O	To 20	

▲ **CRITICAL STEP** 10× reaction buffer should be added last because it contains spermidine, which precipitates easily in the presence of DNA.
▲ **CRITICAL STEP** The mMessage mMachine kit only produces capped transcripts. Although this works well in our hands, the transcripts can be further modified by adding poly(A) tails to increase the mRNA stability and translation efficiency.

12| Add 1 µl of TURBO DNase to each 20-µl transcription reaction, mix it well and incubate it for 15 min at 37 °C.

13| Add 30 µl of nuclease-free water and 30 µl of LiCl precipitation solution. Mix thoroughly and chill the mixture for 30 min at –20 °C.

14| Centrifuge the mixture at 4 °C for 10 min at 14,000*g* to pellet the mRNA. Carefully remove and discard the supernatant.

15| Wash the pellet once with 500 µl of 70% (vol/vol) ethanol, and recentrifuge it at 4 °C for 10 min at 14,000*g*.

16| Carefully remove and discard the ethanol, air-dry for 5 min and resuspend the mRNA in 20 µl of nuclease-free water by gently pipetting the mixture up and down.

17| Determine the RNA concentration, and store it frozen at –80 or –150 °C.
■ **PAUSE POINT** Purified Cas9 mRNAs can be stored at –80 or –150 °C for up to 2–3 months before use for embryo microinjections.

18| To check the quality of the mRNA, mix 2 µl of loading dye with 3 µl of mRNA, and run the samples on a 2% (wt/vol) agarose gel in TAE buffer with 0.4 µg ml^{-1} ethidium bromide at 100 V for 15–20 min. High-quality RNA should give sharp bands without smears.
? TROUBLESHOOTING

Preparation of injection mix ● TIMING 1 h
19| Thaw the Cas9 mRNA, target-specific sgRNA and donor template (ssODNs or plasmids for precise genome editing (see Reagents section)) on ice a few minutes before microinjection. For NHEJ-mediated targeting, prepare the injection mix described in option A. For HR-mediated precise genome editing, follow option B to prepare the injection mix.
▲ **CRITICAL STEP** To avoid any contamination of the microinjection samples with dust particles, which might clog the injection capillaries, use a 0.22-µm Millex-GP Syringe Filter to clean the solution before microinjection.
(A) Injection mix for NHEJ-mediated targeting
(i) Prepare a 40-µl single-use aliquot of 25 ng µl^{-1} Cas9 (or Cas9n) mRNA + 12.5 ng µl^{-1} sgRNA for each target.
(B) Injection mix for HR-mediated precise genome editing
(i) Prepare a 20-µl single-use aliquot of 10 ng µl^{-1} Cas9 (or Cas9n) mRNA + 5 ng µl^{-1} target-specific sgRNA.
(ii) Prepare a 20-µl single-use aliquot of donor templates (ssODNs or plasmids) at a concentration of 10 ng µl^{-1}.
(iii) Combine the two aliquots immediately before microinjection, and mix them well.

Preparation of one-cell-stage rat embryos ● TIMING 3 d
! CAUTION Researchers must hold a permit for handling animals. In addition, the experimental design and protocols must be approved by the institutional ethical review committee.
▲ **CRITICAL** Special training is required for inexperienced researchers to use the microinjection and surgical procedures. Although there are technical differences in the details of surgical procedures between mice and rats, the basic rules and steps are mostly the same. A comprehensive and detailed description of microinjection and surgical procedures for manipulating mouse embryos can be found in a manual by Nagy *et al.*[66]. A chapter in the book *The Laboratory Rat*, edited by Suckow *et al.*[63], is valuable for mastering the technique. In addition, helpful figures and procedures can be found in a protocol by Si-Hoe *et al.*[67].

20| *Day 1*. Treat 15–20 donor female rats (8-week-old, Sprague-Dawley strain or other specific strains suitable for subsequent studies) with 400 µl (40 IU) of a freshly diluted aliquot of PMSG stock solution through i.p. injection at 1 p.m.–2 p.m. House several hormone-treated female rats in each standard cage in a specific pathogen-free facility on a 12-h light/dark cycle with *ad libitum* access to food and water.

21| *Day 3*. Inject PMSG-treated female rats from day 1 with 300 µl (30 IU) of a freshly diluted aliquot of hCG stock solution at 2 p.m.–4 p.m. Mate hormone-treated female rats with male SD rats (10 weeks old) in single pairs overnight.
▲ **CRITICAL STEP** The number of embryos collected from each donor will vary depending on the genetic background, response to the hormone treatment, age and hormone dose. Outbred strains usually exhibit a consistently good response to superovulation and produce large numbers of oocytes. Generally, young sexually mature female rats (50–60 d old) respond to the superovulation treatment better than older or immature female rats of a given genetic background. It is better to test the optimal amount of PMSG and hCG in any facility just starting to manipulate rat embryos. Higher-concentration PMSG and hCG stock solutions are more stable for storage. The stocks should be stored at –20 °C in aliquots for up to 2 months. All hormone stocks are single use for optimal activity.

22| *Day 3*. Cage 40 female rats (10 weeks old) with vasectomized male rats (**Box 1**) in single pairs to get pseudopregnant female rats the next day (day 4).
▲ **CRITICAL STEP** Record the plug activity of each vasectomized male rat to eliminate poorly performing ones. If the number of vasectomized male rats is limited, the mating cage can be set up with two female rats and one male rat to increase the chance of getting more pseudopregnant female rats.

23| *Day 4*. In the morning, first check for the copulatory plug in the female rats mated with vasectomized male rats by spreading the vaginal opening with blunt forceps. Transfer plug-positive female rats to labeled cages in preparation for embryo transfer surgery in the afternoon.
▲ **CRITICAL STEP** The first step in the morning is to check the plugs before 9:30 a.m. for recipient female rats, as the plug will drop late in the morning. The unplugged female rats can be used 2 weeks later as recipients.

24| Prepare the transferring pipettes, as described in **Box 2**.

25| Kill the superovulated female rats (from Step 21) by CO_2 asphyxiation. Open the abdomen and dissect out and cut off the oviducts gently and place them into 2 ml of prewarmed (37 °C) M2 medium in a 35-mm dish.

26| Under a stereomicroscope and in a new 35-mm dish containing a mixture of 2 ml of prewarmed M2 medium with 40 µl of hyaluronidase stock solution, tear the ampulla with a pair of fine forceps and allow the embryos to fall out of the torn oviduct. If the embryos do not fall out easily, use a pair of forceps with bent tips to squeeze them out gently.

27| Incubate the fertilized oocytes in M2 medium with hyaluronidase until the cumulus cells fall off. Pipette the embryos and cumulus cell complex up and down a few times with a transfer pipette to accelerate the digestion process. Use a pipette tip with a wide opening to avoid crushing the embryos.
▲ **CRITICAL STEP** Do not let the embryos stand in M2 with hyaluronidase for more than a few minutes once the cumulus cells are detached, because this will potentially harm the embryos.
? TROUBLESHOOTING

28| After removal of the cumulus cells, reload fresh medium into the transfer pipette, pick up the embryos and transfer them into a dish containing 2 ml of fresh M2 medium. Wash the embryos by flushing them out several times to rinse off the hyaluronidase and cumulus cells.

29| Reload the pipette with prewarmed KSOM medium, and transfer the embryos into KSOM medium covered by mineral oil in a dish. Usually add six drops (50 µl per drop) of KSOM medium in a 35-mm dish. Rinse a group of 50 embryos in the central drop and then settle them in a fresh drop. Incubate the embryos at 37 °C with 5% CO_2 until microinjection. Typically, 200–300 fertilized oocytes are obtained from 15 superovulated female rats.

Microinjection of one-cell embryos ● TIMING 3 h
30| Prepare the holding pipette and the injection pipette as described in **Box 2**. Use silicified glass slides as the injection chamber by placing a drop of M2 medium in the center covered with mineral oil.

Box 2 | Preparation of glass pipettes ● TIMING 1 h

1. For microinjection and embryo handling, three kinds of glass pipettes are required: embryo transfer pipettes (option A); injection pipettes (option B); and holding pipettes (option C).

(A) Preparing pipettes for embryo transfer

(i) Hold the tip of a 50-µl microcapillary tube at both ends, and place the central region of the tube in the outer flame of the alcohol lamp and rotate it.

▲ CRITICAL STEP The fuel of the lamp should be 90% (vol/vol) ethanol to give a high enough temperature to melt the glass tube.

(ii) When the tube begins to melt, withdraw it from the flame and at the same time pull the two ends apart to draw out a thin region. Be sure to pull straight, or the tube will be bent. Quickly pulling the glass results in a small-diameter pipette with a long tip; a slower pull produces a wider but shorter-tip pipette.

(iii) Break the pipette with a diamond-point pencil at the site with an appropriate diameter. A small-diameter pipette is good for transferring embryos during microinjection. Shorter pipettes with wider diameters are useful for transferring embryos into the recipient rat.

(iv) Insert the pipette into the rubber nosepiece of the aspirator tube assemblies.

(B) Preparing pipettes for microinjection

Thin-wall (1 mm outer diameter, 0.75 mm inner diameter) single-barrel capillaries are used. Capillaries with or without inner filaments are suitable for making the holding pipette. For the injection pipette, only capillaries with inner filaments can be used. The pipette can be pulled on a mechanical pipette puller. The Sutter P-97 with 3-mm through filament is used as the example in this protocol.

(i) Insert the capillary in the puller and fix it firmly by twisting the rotary knob.

(ii) Run a ramp test for each batch of capillaries.

(iii) Initial settings are heat = ramp value-10; pull = 100; velocity = 150 and time = 100.

(iv) Use a microscope to evaluate whether the pipette is usable for injection. If not, adjust the settings.

(C) Preparing holding pipettes

(i) Prepare a pipette as described in Step 1B.

(ii) Fix the pulled pipette (drawn tip down) in the clamp of the microforge.

(iii) Under the ×10 objective of the microforge, move the capillary close to the glass ball on the filament and bring it into sharp focus. Adjust the glass ball to the same focus as the capillary wall.

(iv) Move the capillary to attach the glass ball at a point where the external diameter is 70–120 µm, as determined by using an eyepiece with a micrometer.

(v) Use the footpad to heat the filament until the pipette starts to melt.

(vi) Move the pipette in a straight line above the filament. Heat the filament while moving the tip of the pipette close to the filament until the glass melts.

(vii) When the inner-side diameter of the tip shrinks to ~15 µm, move the capillary away from the filament and turn it off. The glass capillary breaks with an even tip when heating is stopped. The opening of the tip should be straight and even. If the opening is too small, it will be unable to hold the embryos firmly. If the opening is too big, the fluid flow will not be easy to control.

(viii) Move the capillary beside the glass ball ~2–3 mm from the tip. Heat the filament and move the capillary close to it, until the pipette begins to soften and bend. Stop heating once it bends to a 15–20° angle.

31| Thaw an aliquot of the appropriate injection mix from Step 19 on ice. Fill the injection pipette by dipping the open end into the injection solution; the sample will enter the tip of the pipette by capillary force. Alternatively, load the injection pipette from the open end using a very long and thin microloader (Eppendorf). Place the pipette into the holder of the microinjector.

▲ CRITICAL STEP It is easy to generate air bubbles in the injection mix when using a microloader; we recommend loading the injection pipette through capillary force. The injection pipette must be made from a capillary with a filament; otherwise, the solution will not be delivered to the tip.

32| Transfer a group (usually 50) of embryos into the injection chamber of the microscope, and arrange them in a single vertical line under low power. Adjust both the holding pipette and the injection pipette to the same focal plane as the embryos in the center of the field. Then adjust the microscope to high power.

33| Break the tip of the injection pipette by gently tapping the injection capillary against the holding pipette. Move the holding pipette close to an embryo, and apply negative pressure using the injector to suck it in.

34| Focus the microscope to view the pronucleus. Adjust the pronuclear position to the middle of the embryo furthest from the holding pipette on the *x* axis with the help of the injection pipette. When the embryo is in a satisfactory position, firmly hold the embryo by applying extra negative pressure.

35| Refocus on the pronucleus and the tip of the injection pipette. For cytoplasmic injection (NHEJ injection mix), follow option A. For pronuclear injection (HR injection mix), follow option B.

(A) Cytoplasmic injection of injection mix for NHEJ-mediated targeting

(i) Push the pipette forward to penetrate the zona pellucida and then the membrane. As the injector gives continuous positive pressure to the pipette, the flow of the injection solution should be visible when the embryo membrane is penetrated. Rotate the injector counterclockwise to slow down the flow speed if a quick surge is observed in the cytoplasm.

(ii) Withdraw the injection pipette quickly but carefully.

(B) Pronuclear injection of injection mix for HR-mediated precise genome editing

(i) Push the tip of the pipette through the zona pellucida, the membrane and the pronuclear membrane; an immediate slow swelling of the pronucleus should be visible (**Supplementary Video 1**).

(ii) Quickly and carefully withdraw the pipette.

▲ **CRITICAL STEP** For pronuclear injection, the opening of the tip should be very small, and the pipette should be withdrawn immediately after pronuclear swelling is observed. Otherwise, too much solution will be injected too quickly, damaging the pronucleus and killing the embryo.

▲ **CRITICAL STEP** Withdrawing the pipette too slowly sometimes results in the tip attaching to the chromosomes, and the DNA is then pulled out of the embryo along with the pipette.

▲ **CRITICAL STEP** The first few embryos can be used for fine-tuning the pressure generated by the injector for optimal injection.

? TROUBLESHOOTING

36| Release the injected embryo to the left of the M2 drop (if the holding pipette is attached on the left side of the manipulator). Next, repeat Step 35 for the next embryo. If the oocyte is not fertilized or the embryo is dead, release to the right of the M2 drop.

37| Repeat Steps 35 and 36 until all embryos in a group have been injected. After one group of embryos has been injected, gather the embryos on the left (fertilized and viable) into a prewarmed KSOM drop in a 35-mm dish covered with mineral oil, and culture it in an incubator at 37 °C with 5% CO_2. M2 medium can also be used for short-term culture of rat embryos.

▲ **CRITICAL STEP** After injection of ~15 embryos, examine the injected embryos to estimate the survival rate. Survival rate should be over 60%. If too many embryos are lysed, twist the injector counterclockwise to decrease the pressure and the flow speed of injection.

38| After microinjection, transfer all of the embryos into a single 200-µl drop of M2 or KSOM medium in a 35-mm dish covered with oil. Discard lysed embryos that are swollen; lysis due to mechanical damage will occur within 1 h of injection. In general, over 60% of the embryos should survive the injection procedure.

▲ **CRITICAL STEP** Surviving embryos should be transferred immediately in groups of 15–20 into the oviducts of each plug-positive pseudopregnant female rat from Step 23.

Transfer of injected 1-d embryos to infundibulum of pseudopregnant female rats ● TIMING 1–2 h

39| Anesthetize a pseudopregnant female rat with 10% chloral hydrate by i.p. injection (3 ml kg^{-1}). Use an electric shaver to remove the fur from the posterior 1/3 of the back (optional). Wipe the skin with 70% (vol/vol) ethanol.

▲ **CRITICAL STEP** All surgical instruments should be sterilized either by autoclaving or by glass-bead sterilization.

40| Make a small skin incision parallel to the dorsal midline above the position of the left or right ovary. Make a very small incision penetrating to the body cavity, avoiding blood vessels. Through a process similar to blunt dissection, enlarge the incision by opening the blades of the surgical scissors underneath opposite sides of the incision, avoiding more bleeding.

41| Use blunt forceps to find the fat pad of the ovary, and pull it out. Using a hemostat or a Serrafine bulldog clamp it and fix the ovary and oviduct. Use fine forceps to find the oviduct infundibulum beneath the ovarian bursa. Usually, the oviduct opening is directly attached to the ovary between the oviduct-uterus junction and the ovary.

▲ **CRITICAL STEP** Make sure that only the fat pad is clamped. Do not clamp the ovary.

42| Transfer the embryos from the dish (Step 38) to the prewarmed M2 medium without mineral oil. Load the transfer pipette with M2 medium and then place two air bubbles in the pipette. Load 10–15 embryos close to each other in a minimal distance from the distal air bubble; take another air bubble at the tip of the pipette. The air bubbles are used for monitoring the position of the embryos during transfer.

43| Gently dissect the bursa using fine forceps by avoiding breaking the blood vessels. The rat ovarian bursa is highly vascularized, and during the surgical process some bleeding is unavoidable. In addition, the oviduct infundibulum is obscured in the bursa. Alternatively, find the oviduct ampulla and use ophthalmic scissors to make an incision in it.

44| Use fine forceps to help locate the oviduct infundibulum, and then insert the prepared transfer pipette into the oviduct opening (or the incision on the ampulla). Eject embryos from the transfer pipette gently until the air bubbles have entered the ampulla. Let the pipette stay in position for a few seconds and then withdraw the pipette slowly.
▲ **CRITICAL STEP** Make sure that all the bubbles are in the oviduct, but avoid ejecting too much liquid into the oviduct. Increased pressure in the oviduct will cause back-flow of the fluid out of the oviduct when withdrawing the pipette.

45| Unclamp the fat pad and place the reproductive organs back into the body cavity. Sew the body wall musculature together with 1–2 stitches, and close the skin with wound clips.

46| Transfer another group of embryos into the oviduct on the contralateral side of the same recipient female rat.

47| Transfer the anesthetized rat to a warm plate for recovery. When the rats are fully recovered, return them to breeding cages. Usually, the pups are born 3 weeks after oviduct transfer.
▲ **CRITICAL STEP** If the number of pseudopregnant female rats is insufficient to transfer all embryos, culture the embryos in KSOM medium and transfer them the next day through the infundibulum. The healthy embryos will be in the two-cell stage; discard any single-cell embryos.

Isolation of genomic DNA and genotyping of the founder rat ● **TIMING 3 d**

48| Take the tail tip from each pup (10–15 d old) and place it in a separate 1.5-ml tube. Add 500 µl of lysis buffer and 1 µl of proteinase K stock solution.

49| Place the tubes in a water bath at 55 °C overnight or until the tails are completely lysed.
? TROUBLESHOOTING

50| Purify genomic DNA from the digested tail tips by using phenol:chloroform extraction and ethanol precipitation, by resuspending the DNA pellet in 30–50 µl of nuclease-free water.
? TROUBLESHOOTING
■ **PAUSE POINT** Genomic DNA can be stored at 4 °C for at least 1 month or at –20 °C for 6 months.

51| Amplify the targeted genomic region with locus-specific PCR primers. Set up a 50-µl PCR containing the following components:

Components	Volume (µl)	Final
Genomic DNA from Step 50, 100 ng μl^{-1}	1	100 ng
Q5 reaction buffer, 5×	10	1×
Forward primer, 10 µM	2.5	0.5 µM
Reverse primer, 10 µM	2.5	0.5 µM
dNTPs, 10mM (2.5 mM each)	5	1 mM
Q5 high-fidelity DNA polymerase	0.5	
Nuclease-free water	To 50	

▲ **CRITICAL STEP** To avoid introducing mutations during amplification, use a high-fidelity polymerase.

52| Perform PCR using the following cycling conditions:

Cycle number	Denature	Anneal	Extend
1	98 °C, 30 s		
2–29	98 °C, 10 s	T_m, 30 s	72 °C, 30 s per kb
30			72 °C, 2 min

53| Run 5 µl of each PCR product and 5 µl of the DNA Ladder Mix on a 1.5% (wt/vol) agarose gel in 1× TAE buffer with 0.4 µg ml^{-1} ethidium bromide at 100 V for 30 min. Check for the presence of a single band of the expected size.
? TROUBLESHOOTING

54| Heat 20 µl of each PCR product at 98 °C for 5 min, and ramp it down to 35 °C at 1 °C min^{-1}.

55| Combine 17.5 µl of PCR product (from Step 54), 2 µl of 10× NEB buffer 2 and 0.5 µl of T7EI in a 20-µl reaction volume for 1 h at 37 °C.

56| Run 10 µl of each endonuclease-digested PCR product (Step 55) and 1 µg of the DNA Ladder Mix on a 1.5% (wt/vol) agarose gel in 1× TAE buffer with 0.4 µg ml^{-1} ethidium bromide at 100 V for 30 min. Wild-type control will show only one band of the full-length PCR product, and heterozygous mutant genotypes will show one or two additional bands due to endonuclease cleavage within the Cas9 target region.
? TROUBLESHOOTING

Subcloning PCR products to characterize the mutation ● TIMING 1–2 d
57| Purify the remaining 25 µl of PCR product from Step 52 using the QIAquick Nucleotide Removal Kit according to the manufacturer's instructions, and determine the DNA concentration. Characterize the mutation either by subjecting the PCR products to direct sequencing using forward or reverse PCR primer from Step 51 (check chromatograms for a doublet at the desired mutation site, indicating heterozygous deletions or a knock-in in one allele) or by subcloning the PCR products into a blunt vector and then sequencing them with universal sequencing primers M13R or M13F, as described in Steps 58–60 below.

58| Prepare the ligation mixture containing the following components, and incubate it at 25 °C for 10 min.

Components	Volume (µl)	Final
Purified PCR product from Step 57, 20 ng μl^{-1}	1	20 ng
pEASY-Blunt cloning vector	1	
ddH_2O	To 5	

59| Use 5 µl of ligation mixture to transform 50 µl of chemically competent DH5α bacterial cells, according to the manufacturer's instructions. Plate transformations onto an LB plate containing 100 µg ml^{-1} ampicillin, and incubate it overnight at 37 °C.

60| The next day, pick 5–10 colonies and extract the plasmid for sequencing. Check the sequence of the inserted alleles by using the M13F or M13R sequencing primer, by comparing the sequence with a wild-type sequence to identify the type of mutation.
▲ CRITICAL STEP Rats identified as founders are possibly mosaic owing to Cas9-sgRNA activity in the 2- to 4-cell embryo stage. It is important to produce and analyze the F_1 generation for the establishment of rat strains containing specific mutations.
? TROUBLESHOOTING

Identification of germline transmission offspring ● TIMING 4 weeks
61| Mate founder mutants to wild-type rats at the age of 8 weeks to obtain heterozygous F_1 progeny.

62| Check the F_1 pups for the inheritance of the mutant allele by using the genotyping procedure established for the analysis of founder mutants (Steps 48–60).
▲ CRITICAL STEP The genotype of the F_1 generation should be determined by DNA sequencing, as the founder could be a chimera containing multiple mutations.

63| Intercross confirmed F_1 heterozygous mutants to generate homozygous mutant F_2 rats.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1A(iv)	No amplification of sgRNA	Incorrect template or primer concentration	Titrate oligo concentration to 0.3 μM for a 50-μl reaction. Titrate primer concentration to a final concentration of 0.1–0.5 μM
1B(viii)	Colonies growing on negative control plate	Incomplete digestion of PGS3-T7-sgRNA plasmid	Increase the amount of restriction enzymes or increase the reaction time of phosphatase treatment of the plasmid digestions
7,18	Smeared band in *in vitro* transcription samples	Improper transfection conditions	Repeat *in vitro* transcription reaction with RNase-free reagent and material
9	Two or three bands of digested DNA	Enzyme failure; incomplete digestion of cloning plasmid	Change to a new aliquot of enzyme; extend time of digestion
10,50	Low concentration of DNA	DNA pellet aspirated with ethanol	Repeat enzyme digestion (Step 8) carefully; repeat purifying genomic DNA from digested tail tips (Step 50)
27	Few embryos were obtained	Rats not responding well to the hormone treatment	Increase the hormone amount; use 7- to 8-week-old females
35B(ii)	Pronuclear swelling is not visible	Clogged injection pipette; failure to penetrate the membrane of the pronucleus	Change to a new pipette; refocus on the pronucleus and the pipette tip
49	No digestion of tail tip	Proteinase K failure; insufficient proteinase K	Change a new aliquot of proteinase K; double the proteinase K concentration
53	Unspecific PCR products; no PCR products	Improper primer pairs; improper T_m; high GC content in target genomic DNA	Redesign primer pairs; check for optimal T_m in a gradient PCR; add 5% DMSO to reaction sample
56	Smeared bands after T7E1 digestion	Long digestion time	Digest within 1 h
60	Low mutation rate	Inaccessibility of the genomic target locus; degeneration of RNA for injection	Double the Cas9 mRNA and sgRNA concentrations; change to a second sgRNA; check the RNA quality

● TIMING

Step 1A, PCR approach for T7-sgRNA-scaffold construction: 1.5 h
Step 1B, construction of an sgRNA expression vector: 4 d
Steps 2–7, sgRNA production: 3 h
Steps 8–18, Cas9 (or Cas9n) mRNA production: 1 d
Step 19, preparation of injection mix: 1 h
Steps 20–29, preparation of one-cell-stage rat embryos: 3 d
Steps 30–38, microinjection of one-cell embryos: 3 h
Steps 39–47, transfer of injected 1-d embryos to the infundibulum of pseudopregnant female rats: 1–2 h
Steps 48–56, isolation of genomic DNA and genotyping of founder rat: 3 d
Steps 57–60, subcloning PCR products to characterize the mutation: 1–2 d
Steps 61–63, identification of germline transmission offspring: 4 weeks
Box 1, generation of vasectomized sterile male rats: 3 h
Box 2, glass pipette preparation: 1 h

ANTICIPATED RESULTS

We and others have demonstrated that the CRISPR/Cas system is a highly efficient genetic tool for gene editing in rats and can be used to target multiple genes in one embryo through a single microinjection[15]. Usually, 10–20% of the injected embryos will develop to term, and ~70% of the live pups will contain mutations at the target loci. By using two sgRNAs independently targeting the *Il2rg* and *Fah* genomic loci, we were able to demonstrate simultaneous knockout of these two genes in the rat. We found that 72% of the F_0-generation rats were mutant at the *Fah* locus, and 39% were mutant at the *Il2rg* locus; 5 of the 18 (28%) pups contained mutations in both loci (**Supplementary Fig. 3**). With co-injection of donor template with Cas9-sgRNA mix into the rat pronuclei, founders with precise genome edits can be produced. We also demonstrated precise gene editing with ssODN-mediated HR at the *Tgr5* genomic locus by Cas9-D10A-induced DNA nicking (**Supplementary Fig. 4**). 5 of 12 rats produced after microinjection contained a knock-in of the exogenous DNA sequence. None contained indels generated by NHEJ. Two founders contained the exact knock-in of both EcoRI and LoxP sites at the desired location. The remaining three founders contained a partial knock-in of the template ssODNs. The imprecise repair is probably due to the short homology arms, as we used only 30-bp homology arms at each side.

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

ACKNOWLEDGMENTS We thank F. Zhang of the Broad Institute of MIT and Harvard for kindly providing the Cas9 expression vector. We thank S. Siwko for comments and advice. This work was partially supported by grants from the State Key Development Programs of China (2010CB945403 to D.L. and 2012CB910400 to Mi.L.), grants from the National Natural Science Foundation of China (no. 31371455, 31171318 and 81330049) and a grant from the Science and Technology Commission of Shanghai Municipality (12XD1406100) and 14140900300.

AUTHOR CONTRIBUTIONS Y.S., Y.G., L. Wang, Z.Q., Me.L., Y.C., L. Wu and Y.L. performed the experiments; Y.S., Y.G., X.M., Mi.L. and D.L. analyzed the data; and Y.S., Y.G., Mi.L. and D.L. wrote the manuscript.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

1. Jacob, H.J., Lazar, J., Dwinell, M.R., Moreno, C. & Geurts, A.M. Gene targeting in the rat: advances and opportunities. *Trends Genet.* **26**, 510–518 (2010).
2. Thomas, K.R. & Capecchi, M.R. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51**, 503–512 (1987).
3. Koller, B.H., Marrack, P., Kappler, J.W. & Smithies, O. Normal development of mice deficient in beta 2M, MHC class I proteins, and CD8+ T cells. *Science* **248**, 1227–1230 (1990).
4. Schwartzberg, P.L., Goff, S.P. & Robertson, E.J. Germ-line transmission of a c-abl mutation produced by targeted gene disruption in ES cells. *Science* **246**, 799–803 (1989).
5. Mullins, L.J., Brooker, G. & Mullins, J.J. Transgenesis in the rat. *Methods Mol. Biol.* **180**, 255–270 (2002).
6. Lois, C., Hong, E.J., Pease, S., Brown, E.J. & Baltimore, D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* **295**, 868–872 (2002).
7. Zan, Y. *et al.* Production of knockout rats using ENU mutagenesis and a yeast-based screening assay. *Nat. Biotechnol.* **21**, 645–651 (2003).
8. Kitada, K. *et al.* Transposon-tagged mutagenesis in the rat. *Nat. Methods* **4**, 131–133 (2007).
9. Katter, K. *et al.* Transposon-mediated transgenesis, transgenic rescue, and tissue-specific gene expression in rodents and rabbits. *FASEB J.* **27**, 930–941 (2013).
10. Tong, C., Li, P., Wu, N.L., Yan, Y. & Ying, Q.L. Production of p53 gene knockout rats by homologous recombination in embryonic stem cells. *Nature* **467**, 211–213 (2010).
11. Geurts, A.M. *et al.* Knockout rats via embryo microinjection of zinc-finger nucleases. *Science* **325**, 433 (2009).
12. Cui, X. *et al.* Targeted integration in rat and mouse embryos with zinc-finger nucleases. *Nat. Biotechnol.* **29**, 64–67 (2011).
13. Tesson, L. *et al.* Knockout rats generated by embryo microinjection of TALENs. *Nat. Biotechnol.* **29**, 695–696 (2011).
14. Menoret, S. *et al.* Generation of Rag1-knockout immunodeficient rats and mice using engineered meganucleases. *FASEB J.* **27**, 703–711 (2013).
15. Li, D. *et al.* Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. *Nat. Biotechnol.* **31**, 681–683 (2013).
16. 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).
17. Hu, X. *et al.* Heritable gene-targeting with gRNA/Cas9 in rats. *Cell Res.* **23**, 1322–1325 (2013).
18. Tong, C., Huang, G., Ashton, C., Li, P. & Ying, Q.L. Generating gene knockout rats by homologous recombination in embryonic stem cells. *Nat. Protoc.* **6**, 827–844 (2011).
19. Men, H. & Bryda, E.C. Derivation of a germline competent transgenic Fischer 344 embryonic stem cell line. *PloS ONE* **8**, e56518 (2013).
20. Men, H., Bauer, B.A. & Bryda, E.C. Germline transmission of a novel rat embryonic stem cell line derived from transgenic rats. *Stem Cells Dev.* **21**, 2606–2612 (2012).
21. Hirabayashi, M. *et al.* Establishment of rat embryonic stem cell lines that can participate in germline chimerae at high efficiency. *Mol. Reprod. Dev.* **77**, 94 (2010).
22. Hirabayashi, M. *et al.* Effect of leukemia inhibitory factor and forskolin on establishment of rat embryonic stem cell lines. *J. Reprod. Dev.* **60**, 78–82 (2013).
23. Hirabayashi, M. *et al.* Rat transgenesis via embryonic stem cells electroporated with the Kusabira-orange gene. *Mol. Reprod. Dev.* **77**, 474 (2010).
24. Bibikova, M., Beumer, K., Trautman, J.K. & Carroll, D. Enhancing gene targeting with designed zinc finger nucleases. *Science* **300**, 764 (2003).
25. Miller, J.C. *et al.* An improved zinc-finger nuclease architecture for highly specific genome editing. *Nat. Biotechnol.* **25**, 778–785 (2007).
26. Kim, Y.G., Cha, J. & Chandrasegaran, S. Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc. Natl. Acad. Sci. USA* **93**, 1156–1160 (1996).
27. Mashimo, T. *et al.* Generation of knockout rats with X-linked severe combined immunodeficiency (X-SCID) using zinc-finger nucleases. *PloS ONE* **5**, e8870 (2010).
28. Porteus, M.H. & Baltimore, D. Chimeric nucleases stimulate gene targeting in human cells. *Science* **300**, 763 (2003).
29. Qiu, Z. *et al.* High-efficiency and heritable gene targeting in mouse by transcription activator-like effector nucleases. *Nucleic Acids Res.* **41**, e120 (2013).
30. Wefers, B. *et al.* Generation of targeted mouse mutants by embryo microinjection of TALEN mRNA. *Nat. Protoc.* **8**, 2355–2379 (2013).
31. Boch, J. *et al.* Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* **326**, 1509–1512 (2009).
32. Moscou, M.J. & Bogdanove, A.J. A simple cipher governs DNA recognition by TAL effectors. *Science* **326**, 1501 (2009).
33. Sung, Y.H. *et al.* Knockout mice created by TALEN-mediated gene targeting. *Nat. Biotechnol.* **31**, 23–24 (2013).
34. Cong, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823 (2013).

35. Mali, P. *et al.* RNA-guided human genome engineering via Cas9. *Science* **339**, 823–826 (2013).
36. 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).
37. Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821 (2012).
38. Hwang, W.Y. *et al.* Heritable and precise zebrafish genome editing using a CRISPR-Cas system. *PloS ONE* **8**, e68708 (2013).
39. Cho, S.W., Kim, S., Kim, J.M. & Kim, J.S. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* **31**, 230–232 (2013).
40. Shen, B. *et al.* Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. *Cell Res.* **23**, 720–723 (2013).
41. Garneau, J.E. *et al.* The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* **468**, 67–71 (2010).
42. Marraffini, L.A. & Sontheimer, E.J. Self versus non-self discrimination during CRISPR RNA-directed immunity. *Nature* **463**, 568–571 (2010).
43. Makarova, K.S. *et al.* Evolution and classification of the CRISPR-Cas systems. *Nat. Rev. Microbiol.* **9**, 467–477 (2011).
44. Mali, P., Esvelt, K.M. & Church, G.M. Cas9 as a versatile tool for engineering biology. *Nat. Methods* **10**, 957–963 (2013).
45. Sung, P. & Klein, H. Mechanism of homologous recombination: mediators and helicases take on regulatory functions. *Nat. Rev. Mol. Cell Biol.* **7**, 739–750 (2006).
46. Lieber, M.R. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu. Rev. Biochem.* **79**, 181–211 (2010).
47. Chen, F. *et al.* High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases. *Nat. Methods* **8**, 753–755 (2011).
48. Richardson, C., Moynahan, M.E. & Jasin, M. Double-strand break repair by interchromosomal recombination: suppression of chromosomal translocations. *Genes Dev.* **12**, 3831–3842 (1998).
49. Kim, E. *et al.* Precision genome engineering with programmable DNA-nicking enzymes. *Genome Res.* **22**, 1327–1333 (2012).
50. Mashiko, D. *et al.* Generation of mutant mice by pronuclear injection of circular plasmid expressing Cas9 and single guided RNA. *Sci. Rep.* **3**, 3355 (2013).
51. Pattanayak, V. *et al.* High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat. Biotechnol.* **31**, 839–843 (2013).
52. Hsu, P.D. *et al.* DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol.* **31**, 827–832 (2013).
53. 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).
54. Fu, Y., Sander, J.D., Reyon, D., Cascio, V.M. & Joung, J.K. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat. Biotechnol.* **32**, 279–284 (2014).
55. Ran, F.A. *et al.* Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* **154**, 1380–1389 (2013).
56. Ran, F.A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281–2308 (2013).
57. Xiao, A. *et al.* CasOT: a genome-wide Cas9/gRNA off-target searching tool. *Bioinformatics* **30**, 1180–1182 (2014).
58. Hwang, W.Y. *et al.* Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotechnol.* **31**, 227–229 (2013).
59. Deng, C. & Capecchi, M.R. Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. *Mol. Cell. Biol.* **12**, 3365–3371 (1992).
60. Brown, A.J. *et al.* Whole-rat conditional gene knockout via genome editing. *Nat. Methods* **10**, 638–640 (2013).
61. Ma, Y. *et al.* Generating rats with conditional alleles using CRISPR/Cas9. *Cell Res.* **24**, 122–125 (2014).
62. Bryksin, A.V. & Matsumura, I. Overlap extension PCR cloning: a simple and reliable way to create recombinant plasmids. *BioTechniques* **48**, 463–465 (2010).
63. Mark, A. & Suckow, S.H.W.a.C.L.F. *The Laboratory Rat* 2nd edn. (Academic Press, 2006).
64. Brinster, R.L., Chen, H.Y., Trumbauer, M.E., Yagle, M.K. & Palmiter, R.D. Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc. Natl. Acad. Sci. USA* **82**, 4438–4442 (1985).
65. Mangerich, A. *et al.* A caveat in mouse genetic engineering: ectopic gene targeting in ES cells by bidirectional extension of the homology arms of a gene replacement vector carrying human PARP-1. *Trans. Res.* **18**, 261–279 (2009).
66. Nagy, A., Gertsenstein, M., Vintersten, K. & Behringer, R. *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory Press, 2003).
67. Si-Hoe, S.L., Wells, S. & Murphy, D. Production of transgenic rodents by the microinjection of cloned DNA into fertilized one-cell eggs. *Mol. Biotechnol.* **17**, 151–182 (2001).