Easi-CRISPR for creating knock-in and conditional knockout mouse models using long ssDNA donors

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CRISPR/Cas9-based genome editing can easily generate knockout mouse models by disrupting the gene sequence, but its efficiency for creating models that require either insertion of exogenous DNA (knock-in) or replacement of genomic segments is very poor. The majority of mouse models used in research involve knock-in (reporters or recombinases) or gene replacement (e.g., conditional knockout alleles containing exons flanked by *LoxP* sites). A few methods for creating such models have been reported that use double-stranded DNA as donors, but their efficiency is typically 1–10% and therefore not suitable for routine use. We recently demonstrated that long single-stranded DNAs (ssDNAs) serve as very efficient donors, both for insertion and for gene replacement. We call this method efficient additions with ssDNA inserts–CRISPR (*Easi*-CRISPR) because it is a highly efficient technology (efficiency is typically 30–60% and reaches as high as 100% in some cases). The protocol takes ~2 months to generate the founder mice.

INTRODUCTION

The latest advances in genome-editing technologies, specifically those using the CRISPR/Cas9 system, have helped simplify the process of generating genetically engineered animal models. Genome editing is performed in cells or in zygotes through two molecular events. First, the Cas9 nuclease is taken to the target site through a guide RNA, where it creates a double-stranded DNA break (DSB). In the second step, the DSB is repaired by one of the two major DSB repair events, either through nonhomologous end joining (NHEJ), which usually leads to a change in the nucleotide sequence, or homology-directed repair (HDR), if an exogenous repair template is supplied that contains homology arms.

During the past 3–4 years, CRISPR/Cas9 technology has greatly impacted how mouse genome engineering is performed[1,2]. It is now routinely used to generate gene disruptions through short insertions or deletions (indels) via NHEJ, and also to insert short exogenous sequences provided as single-stranded oligodeoxynucleotides (ssODNs) via HDR. The ssODN repair templates used are typically 100 to 200 bases long, consisting of a few bases of altered sequence flanked by homology arms of 40–80 bases[3,4]. By contrast, double-stranded DNAs (dsDNAs) are used as repair templates for projects requiring insertion of longer sequences (such as reporter/recombinase knock-ins). As compared with the insertion efficiency of ssODN donors, however, that of dsDNA donors is poor[5], often requiring homology arms of at least 0.5–1 kb or longer[6,7]. Creating conditional knockout models requires even higher technical precision because these involve replacing a gene fragment (target exon(s)) with a *LoxP*-flanked exon (floxed) cassette gene replacement.

Development of *Easi*-CRISPR

We hypothesized that if longer ssDNAs could be used as donors, they, too, would be inserted at an efficiency similar to that of ssODNs, which is typically higher than that of the dsDNA donors. When we began to test our hypothesis, ssDNAs of >200 bases were not commercially synthesizable. To generate longer ssDNAs, we used classic molecular biology steps such as converting dsDNA to an RNA (using an *in vitro* transcription step) and reverting the RNA back to DNA (using a reverse transcription step) to obtain ssDNAs. We named this strategy (synthesizing ssDNAs from dsDNA templates) '*in vitro* transcription and reverse transcription' (*iv*TRT). Using long ssDNAs as donor templates, we developed highly efficient CRISPR–HDR protocols in three stages, as described below.

In the first stage, we demonstrated that longer sequences consisting of ~404 bases of new sequence with ~55-base homology arms on each side could be inserted at Cas9 cleavage sites[8]. These experiments demonstrated that artificial microRNA sequences can be inserted at introns of genes (host genes). Inserted cassettes are transcribed as part of the host gene transcription and eventually processed into mature microRNAs to knock down the protein expression of their target genes. We tested introns of the *eEF2* gene, which enabled ubiquitous expression of microRNA sequences. Insertion of artificial microRNA sequences into an intronic site at the *eEF2* locus resulted in knockdown of target genes (eGFP and *Otx2*), as analyzed at the embryonic stages. Targeted insertion was found in up to 83% of offspring.

Extending this work further, in the second stage, we demonstrated that sequences of up to ~1.5 kb can be inserted to express various types of proteins[9]. Our aim in those experiments was to further develop the method to achieve generation of germ-line-, transmittable knock-in models that can express protein-coding sequences such as recombinases, reporters, or transcriptional inducers. Indeed, the method worked at very high efficiency for precisely fusing expression cassettes to specific codons of genes. The method is shown to be robust (consistent performance at >6 loci) and is highly efficient (25–67%).

In the third stage of developing high-efficiency HDR protocols, we tested whether a gene segment can be excised by cleaving at two sites in the genome, and replacing the segment with a modified piece (such as a floxed-exon cassette). Even though the efficiency varied from locus to locus (8.5–100%), the method has worked for many loci.

TABLE 1 | Comparison of knock-in strategies.

Feature	*Easi*-CRISPR	Insertion via HR of dsDNA donor	MMEJ using dsDNA donor (PITCh)
Insert size	Up to ~1.5 kb (refs. 8,9,38)	Up to 11 kb (refs. 31,33,43–47)	Up to 4–5 kb (ref. 32)
Length of homology arms	55–105 bases	Typically, ~0.5–2 kb (up to ~7.5 kb)[31,33,43–47]	~40 bases
Difficulty of donor DNA construction	Easy	Difficult in most cases	Easy
Knock-in efficiency	8.5–100% (typically 30–60%)[8,9,38]	0–50% (typically ~10% or less)[31,33,43–47]	12.0% (without Exo1), 35.7% (with Exo1)[32]

HR, homologous recombination. MMEJ, microhomology-mediated end joining. PITCh, precise integration into the target chromosome.

The sgRNA and Cas9 mRNA were used as CRISPR reagents in the first stage of experiments[8], whereas separated crRNA + tracrRNA and Cas9 protein (ctRNP) were used in the second- and third-stage experiments[9].

Systematic testing of the above three ssDNA insertion approaches led to our establishing streamlined protocols for the generation of many types of commonly used mouse models, including knockdown, knock-in, and conditional knockout mouse models. These protocols have worked at high efficiency for >12 loci, and experiments have been performed in at least three different laboratories[9]. We named the method *Easi*-CRISPR, and here we provide a detailed protocol for it.

Applications of the *Easi*-CRISPR method

Many types of genetically engineered mouse models are used in biomedical research, and most of these rely on precise insertion of donor cassettes. Examples of a few commonly used models are (i) floxed models that contain two *LoxP* sites flanking a target exon to allow for conditional deletion of the gene segment[10]; (ii) Cre or CreER^{T2} driver lines that are used to delete a floxed gene segment in a given tissue and/or at a given time[11]; (iii) reporter strains used to monitor Cre specificity and sensitivity and/or to monitor gene expression[12]; and (iv) inducible transcriptional regulator strains (rtTA/tTA) that permit doxycycline-regulated expression of tetO-containing promoters[13]. The *Easi*-CRISPR approach can be used for the generation of all the above types of animal models. In addition, because the *Easi*-CRISPR strategy works very efficiently for gene replacements (demonstrated for up to ~1-kb ssDNAs thus far), the method can also be used for developing multiple point mutation knock-in animal models, and for swapping gene segments from other species in this size range. The method can also be applied to generate knockdown mice by introducing an artificial microRNA sequence at the intronic region of an endogenous gene, as described in Miura *et al.*[8].

The *Easi*-CRISPR method was developed using microinjection delivery in mice. The approach can also be adapted for use with electroporation or hydrodynamic gene delivery[14–16]. *Easi*-CRISPR can also be used to generate genome-edited animals in other species wherever zygote/embryo delivery of CRISPR components via microinjection or electroporation is possible (such as in fruit flies[17], zebrafish[18], rat[19], and rabbit[20], as well as livestock species such as cattle[21], sheep[22], goats[23], and pigs[24]).

Comparison of other methods of knock-in and conditional knockout model generation

Mouse genome-engineering experiments are commonly performed using one of two approaches: (i) direct injection of DNA to create transgenic models in which DNA is integrated at random genomic locations; and (ii) classic homologous recombination-mediated gene targeting in embryonic stem (ES) cells to create knock-in and (conditional) knockout models. The ES cell-based methods were the only choices before programmable nucleases were demonstrated for gene targeting. However, the ES cell-based methods are laborious, time-consuming, expensive, and, more importantly, may not lead to generation of a germ line–established mutant line. Soon after the CRISPR/Cas system was developed for genome editing in mammalian cells[25,26], it was demonstrated that mouse knock-in and conditional knockout alleles could be generated rapidly[6]. The research community anticipated that this technology would soon replace ES cell–based gene targeting[27], because the required components can be introduced directly into mouse embryos using the same microinjection techniques commonly used for random transgenesis. However, many laboratories have been unsuccessful in using CRISPR strategies for the generation of knock-in and conditional knockout alleles[28].

One of the main reasons for this lack of success is that HDR efficiency using dsDNA donors is generally poor. However, a few strategies for increasing the targeting efficiency of dsDNA donors have been reported. These include (i) inhibition of NHEJ or enhancement of HDR through chemical treatments[29,30]. Such methods have not become popular because they provide only a marginal gain in their efficiencies, and these strategies have been shown to be toxic to cells, disrupting fundamental DNA repair processes[31]. (ii) Certain strategies use circular donors with built-in synthetic guide sequences. The donors are linearized inside the cell/embryo through Cas9 cleavage (using synthetic guide recognition sites that are strategically placed flanking the insertion DNA cassettes) to enhance their insertion at the target site via microhomology-mediated end joining (MMEJ)—this targeting strategy was termed precise integration into the target chromosome (PITCh)[32]. The PITCh strategy offers a better solution for designing targeting constructs than does the classic dsDNA-based homologous recombination strategy because it does not require long homology arms. The insertion efficiency of PITCH–MMEJ was enhanced up to 35.7% when *Exo1* nuclease was included in the injection mix[33]. Comparison of different features of ssDNA-, dsDNA donor–, and MMEJ-based approaches are listed in **Table 1**.

In direct comparison to circular dsDNA donor-based strategies, the ssDNA donors used in *Easi*-CRISPR offer some advantages, such as (i) donor ssDNAs are simple to design; (ii) they do not require long homology arms (~50- to 100-base arms are long enough); and (iii) efficiency is very high (typically 30–60%; reaches up to 100% for some loci). The linear dsDNAs are the standard forms of DNA cassettes used in generating transgenic animals (in which the DNA is inserted at random locations). Although injection of dsDNAs would lead to many random transgene insertions, in our first-stage experiments developing *Easi*-CRISPR, we compared targeted insertion efficiency with linear dsDNA (PCR product) with that for ssDNA (synthesized using *iv*TRT) with the same sequence (and the same lengths of homology arms). We found that insertion efficiency was 2.5 times higher, and the viability of injected embryos was 2.4 times higher using an ssDNA donor than a dsDNA donor[8].

Conditional knockout models are the most commonly produced type of genetically engineered models. The genetic manipulation required to create such alleles using CRISPR/Cas strategies involves a much higher level of technical skill than that needed for knock-in designs because it requires two cleavages in the genome and precise replacement of the gene segment with a floxed DNA cassette between the cleavage sites. An approach in which two ssODN donors were used to insert *LoxP* sites, resulting in two cleavages in the genome, was initially feasible[6], but this approach has proven to be highly challenging because of many undesired outcomes. These include insertion of only one *LoxP*, deletion of the target exon between the genomic cleavages, and insertion of *LoxP* sites *in trans*[34]. The method has failed for many loci[28]. *Easi*-CRISPR is less likely to result in these unwanted outcomes, especially as compared with the two-ssODN oligo-based method. In addition, using circular dsDNAs (as standard knock-in donors) and inserting them through nicking (using Cas9 nickase) has been reported for the generation of conditional alleles[35], but this approach is also not routinely used because of low efficiency.

Limitations of *Easi*-CRISPR

The *Easi*-CRISPR strategy allows for generation of various kinds of mouse models at an efficiency much higher than that of any previously described method. Some of the limitations of *Easi*-CRISPR method are discussed below.

The length of the ssDNA donor. One of the current technical limitations of *Easi*-CRISPR is the synthesis of longer ssDNA molecules. We have successfully made ssDNAs up to ~2 kb, which is sufficient for most of the commonly needed mouse models. Because of the enzymatic amplification steps involved in the *iv*TRT method, some sequences may be challenging to synthesize.

Possible sequence misincorporations arising during ssDNA preparation steps. Because of the enzymatic amplification steps, nucleotide misincorporations can occur during ssDNA synthesis. Some of the ssDNA preparations made at the first stage of developing this method had such mutations. We presume that a portion of these mutations may have resulted from the unintended use of standard-grade enzymes rather than high-fidelity enzymes. Some knock-in mouse models generated may contain such incorporations, and therefore, the full cassettes should be sequenced in both the founders and the F1 offspring (to exclude the possibility of undetected mutations transmitted because of mosaicism). Although most reverse transcriptase enzymes do not have proofreading capabilities, the development of enzymes with proofreading function will be helpful in preventing misincorporations[36]. The CRISPR-generated animal models, in general, can be mosaic, and therefore there is a certain level of uncertainty; the mutant allele is not guaranteed to be transmitted to the F1 offspring. As a general practice, we suggest breeding two or more founders, if more than one founder is available. Once germ-line transmission of the correctly inserted allele is confirmed from any of the founders for that locus, it can be decided, at that point, if it is necessary to continue breeding the rest of the founders.

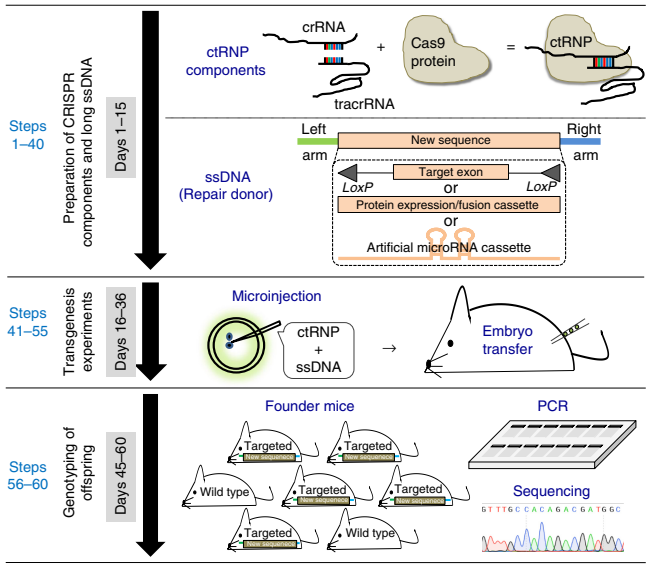

Figure 1 | Schematic of *Easi*-CRISPR. The procedure involves three broad stages: (i) assembly of CRISPR ribonucleoprotein components (crRNA + tracrRNA + Cas9 protein (ctRNP)) and generation of a long ssDNA donor (Steps 1–40); (ii) preparation of *Easi*-CRISPR components, their microinjection into mouse zygotes, and generation of founder offspring (Steps 41–55); and (iii) genotyping of offspring (Steps 56–60). All experimental procedures using animals should be carried out according to relevant institutional and governmental regulations for animal usage.

Possible random insertion of ssDNA donors. Even though ssDNAs are less likely to be randomly inserted into the genome, as compared with dsDNA, such a possibility cannot be completely excluded. Some founder mice, assessed as being negative for donor DNA insertion (using junction PCRs), turned out to contain donor DNA (when using internal donor-specific primers), suggestive of random insertion of the donor DNA in those samples.

Experimental design

The *Easi*-CRISPR method involves four major stages, as described in more detail below: (i) design of an *Easi*-CRISPR strategy; (ii) synthesis and purification of ssDNA and other CRISPR components for microinjection; (iii) preparation of *Easi*-CRISPR components and their microinjection into mouse zygotes; and (iv) genotyping of offspring. A diagram of the workflow is presented in **Figure 1**.

Design of an *Easi*-CRISPR strategy. Designing an *Easi*-CRISPR strategy involves two main parts: searching for CRISPR target

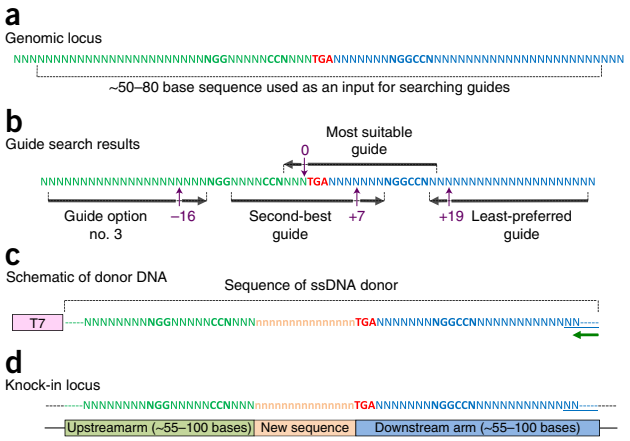

Figure 2 | Design principles of knock-in using *Easi*-CRISPR and the architecture of the ssDNA donor. (**a**) Genomic locus of a hypothetical gene's last exon, showing the stop codon in red (TGA). The green sequence upstream of and the blue sequence downstream of the stop codon will be included as parts of the upstream and downstream arms of the ssDNA donor, respectively. (**b**) Hypothetical guide search results showing four guide options along with their protospacer-adjacent motif (PAM) sequences (5′-NGG-3′). The guide that cleaves immediately upstream of the stop codon will be the most-preferred guide for use in the *Easi*-CRISPR procedure. If such a guide is not available for a given locus, a next-closest one should be chosen. In the example shown here, a guide that cuts seven bases downstream should be the second option. Guide option no. 3 and the 'least-preferred guide' cleave at −16 bases and +19 bases from the target site, respectively. The farther the guide from the target site, the poorer will be the correct insertion frequency because imprecise insertion rates become higher. If either of the latter two guides are chosen, the donor cassette should preferably contain mutation(s) in the guide recognition sites (or PAM) to prevent Cas9 from recleaving after the cassette is inserted. (**c**) Schematic of a donor DNA showing T7 promoter and the ssDNA region. The T7 promoter sequence is included in the dsDNA template (used for *iv*TRT), but it will not be included in the final ssDNA. The green arrow shows the primer for reverse transcription. (**d**) Knock-in locus showing correct fusion of the new sequence.

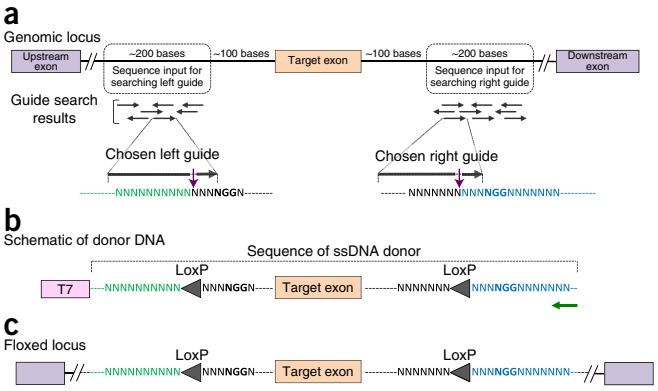

Figure 3 | Design principles of floxing using *Easi*-CRISPR and the architecture of the ssDNA donor. (**a**) Genomic locus of a hypothetical gene's target exon and its surrounding regions, used for guide search. Hypothetical guide search results showing multiple guide options for left and right guides. Two guides (one each for upstream and downstream ends) with high guide scores, and least (or no) off-target cleavage sites, are chosen. The typical distance between the two guide target sites is ~0.5–0.8 kb, and they should be placed sufficiently away from the target exon (at least ~100 bases), to prevent disruption of splice donor/acceptor regulatory elements in the floxed allele. PAM sequences are shown in bold. (**b**) Schematic of donor DNA, showing its components. Note that the T7 promoter will only be part of the dsDNA template and will not be present in the ssDNA synthesized from the template. The *LoxP* insertion sites are placed precisely at the Cas9 cleavage sites. The green arrow shows the primer for reverse transcription. (**c**) Floxed locus showing correct insertion of the new sequence.

sites and designing guide RNA sequences, and designing ssDNA donor cassettes for HDR.

Searching for CRISPR target sites and designing guide RNAs. CRISPR target search is an essential step in CRISPR genome engineering and has been described in numerous publications, including mouse genome–engineering protocols[37]. Briefly, the genomic sequence around the region of interest is retrieved from a genome browser such as http://asia.ensembl.org/index.html and the CRISPR target sites are found using the CHOPCHOP (http://chopchop.cbu.uib.no/index.php) and/or CRISPR Design (http://crispr.mit.edu/) sites. Select the target site as close as possible to the desired insertion site.

The *Easi*-CRISPR method was developed to be suitable for most commonly used mouse model designs, including insertion of protein-coding sequences to fuse with genes (also known as knock-in models), conditional knockout mouse models (also known as floxed models) and knockdown of genes through insertion of artificial microRNA sequences into intronic sites. In all three designs, long ssDNAs are used as donors, regardless of the differing location of guide sequences on genes and the types of insertion cassettes.

For knock-in models (**Fig. 2**), determine the target site for insertion of a desired DNA cassette to fuse with either the 5′ end or the 3′ end of the gene (start or stop codons, respectively). Identify guides that recognize sequences very close to the start or stop codons for precise fusion. For floxed/conditional knockout models (**Fig. 3**), determine the target exon, the deletion of which will lead to predicted loss of protein expression upon Cre-mediated recombination. Insert *LoxP* sites into the flanking introns of the target exon. As it is difficult to accurately predict the regulatory sequences necessary for splicing (e.g., splicing donor/acceptor sites, branch site, and polypyrimidine tract), we suggest placing *LoxP* sites at least ~100 bases away from the intron/exon boundaries. Care should be taken not to insert *LoxP*s at the evolutionary conserved regions, to avoid disruption of functional sequences such as enhancers. To knock down genes through insertion of artificial microRNA sequences into intronic sites of ubiquitously expressed genes and/or introns of genes with desired tissue-specific expression patterns (**Fig. 4**), it is necessary to determine the intron to target for insertion, avoid choosing evolutionary conserved regions that potentially contain sequences of some important (albeit unknown) function (choosing such regions may inadvertently cause disruption of the evolutionarily functional sequences), and, similar to floxed model design, choose regions sufficiently far away from exon boundaries to avoid disrupting splicing signals. Potential splicing events in a given sequence can be predicted using the GENESCAN tool (exon prediction algorithm; http://genes.mit.edu/GENSCAN.html). Both wild-type (untargeted) and theoretical sequence of the modified locus (after cassette insertion) should be analyzed using this tool.

The guide RNA for knock-in designs should be very close to the desired insertion site. The guide that cleaves at the exact insertion site would be the preferred guide. In the absence of a guide that cleaves exactly at the insertion site, the next closest one (preferably within a couple bases from the insertion site), should be chosen

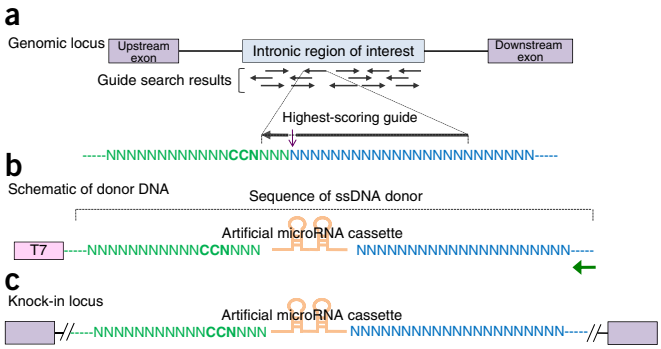

Figure 4 | Design principles of inserting a knockdown cassette using *Easi*-CRISPR and the architecture of the ssDNA donor. (**a**) Genomic locus of a hypothetical gene's target intron and its surrounding regions chosen for guide search. The guide search results show multiple guide options. A guide with a high score, and the least number of (or no) off-target cleavage sites, is chosen for the design. (**b**) Schematic of a donor DNA, showing T7 promoter and the ssDNA region. The T7 promoter sequence is included in the dsDNA template (used for *iv*TRT), but it will be excluded from the final ssDNA. The green arrow shows the primer for reverse transcription. (**c**) Knock-in locus showing correct insertion of the new sequence.

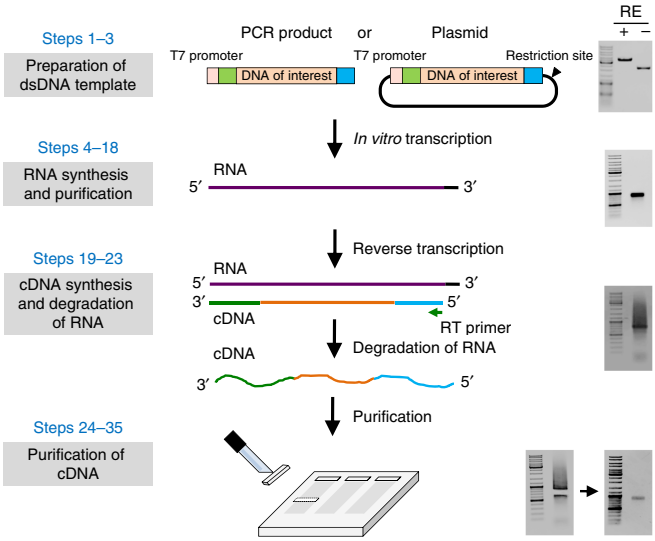

Figure 5 | Schematic of *iv*TRT and ssDNA preparation steps. A dsDNA template can be a PCR product or a plasmid with a suitable restriction enzyme (RE) site distal to the insertion cassette (Steps 1–3). The gel on the right shows a plasmid digested with an RE. RNA is synthesized using *in vitro* transcription (Steps 4–18). The gel on the right shows an RNA ~900 bases long. cDNA is synthesized by reverse transcription using a reverse primer (Steps 19–23). The gel on the right shows a sample ssDNA (cDNA). Note that the cDNA preparation typically runs like a smear with a prominent band within the smear. Purification of ssDNA (Steps 24–35). The image on the left shows the gel after excision of the prominent band (for purification) and the gel on the right shows the purified ssDNA. The GeneRuler DNA Ladder Mix was used in all the gels as a DNA size marker.

(see **Fig. 2** for examples of hypothetical guides and their locations with respect to the desired site of insertion and see **Supplementary Table 1** for information about the polarity of guides and ssDNA donors with respect to the genomic locus of previously published genes). Irrespective of the guide cleaving location, the inside termini of the homology arms of the ssDNA donor (that meet the new sequence) will remain the same (as though the cassette were still to be inserted at the desired location), whereas the lengths of homology arms are maintained as at least 55 bases long by extending their outside termini. Hypothetical scenarios for guide options for knock-in designs are shown in **Figure 2**. Examples of guide sequences and their cleavage sites—with respect to the desired insertion of the new cassette sequence—that were used in our previously published reports are shown in **Supplementary Table 1**. In case of floxing and knockdown designs, the guide locations can be somewhat flexible because the insertion sites are in the intronic regions in these designs. As *LoxP* sites/microRNA cassettes can be placed anywhere within a region that was originally narrowed for searching guides, any guide with a good score and with the lowest or no potential off-target cleavage can be chosen for the design. The floxing designs include the use of two guides (one in each of the introns flanking the target exon). Examples of guide options for floxing designs are shown in **Figure 3**. On the basis of our experience in engineering of several loci[9,38], we think that orientation of the two guides, in floxing designs, does not matter for the overall success in inserting a floxed cassette; the two guides can face each other or face opposite to each other, or they both can run in the same direction (**Supplementary Table 1**).

Designing ssDNA repair cassettes. The typical architecture of cassettes constitutes two homology arms (left and right) and a middle region (new sequence to be inserted). Homology arms are ~55–100 bases long. A T7 RNA polymerase promoter site is included, just upstream of one of the homology arms (referred as the upstream arm), and is needed for *in vitro* transcription. A unique restriction site is included after the distal arm (one that does not need a T7 promoter) for use in linearizing the plasmid (**Fig. 5**). The central region can be up to ~2 kb or more. The design principles and architecture of ssDNA donors and dsDNA templates for knock-in, floxing, and knockdown are shown in **Figures 2–4**, respectively. Note that the design principles for inserting artificial microRNA cassettes share some features between floxing and knock-in designs: one cleavage insertion, as in the case of knock-in, and insertion of cassettes at an intronic region, as in the case of floxing. The repair cassette should be carefully designed to not include the guide recognition sequence and thus avoid recleaving by the Cas9/guide RNA after the correct insertion. This can be achieved simply by inserting the new sequence at the exact cleavage site in the ssDNA design that will split the guide recognition site when the donor cassette is inserted into the genome. Although this can be an option in floxing and knockdown designs (as the insertion sites are flexible because they are in the intronic sites), this may not be an option in the case of knock-in designs (unless the chosen guide cleaves precisely at the insertion site). Even if the guide cleaves a little away from the cleavage site, the donor design should avoid recleavage of the modified allele. If necessary, silent mutations in the donor design can also be included to avoid recleavage. If suitable guides are not available for a locus for targeting via Cas9, targeting via other CRISPR enzymes such as Cpf1 can also be considered[16].

The T7 promoter sequence is inserted immediately upstream to the left or right homology arms. The arm to which the T7 promoter is added is referred as the upstream arm. Note that the final ssDNA donor strand synthesized will be the strand opposite to the one that contains the T7 promoter; if the T7 promoter is tethered to the sense strand, the final ssDNA donor synthesized will be the antisense strand and vice versa. In our experiments

thus far, even though we have added the T7 promoter to only the sense strands, we think that it can also be added to the antisense strand, which finally results in a sense ssDNA donor.

The upstream homology arm corresponds to the upstream sequence from the point in the genome at which the new sequence must be inserted (this is generally the sequence to the left of the cleavage point). We have not done a systematic study to identify optimal lengths for best performance; our initial studies contained arms from 55–105 bases long. It is preferable to choose a G nucleotide (ideally GG), at the 5′ end of the homology arm, to which the T7 promoter will be added upstream. It is known that the presence of one or two Gs immediately 3′ to the T7 promoter increases T7 RNA polymerase transcription efficiency. Note that if a suitable sequence that matches this criterion is not available in the left homology arm, the T7 promoter can be added to the right homology arm, in which case the right homology arm will be referred to as the upstream homology arm (with respect to donor DNA direction).

The downstream homology arm corresponds to the downstream sequence from the point in the genome at which the new sequence must be inserted (generally, the sequence on the right of the cleavage point). This arm is also typically ~55–105 bases long. We have kept the lengths of the (upstream and downstream) arms to somewhat similar lengths. A primer that binds at the 3′ end of downstream homology arm is used for reverse transcription. Therefore, the terminal region should possess an optimal sequence for primer binding to be suitable for reverse transcription (RT) reaction (e.g., optimal GC content and the sequence devoid of unusual repetitive nucleotides). A unique restriction site (that produces a 5′ overhang or a blunt end) should be added downstream of the right arm to linearize the plasmid before using it in *in vitro* transcription reaction (**Fig. 5**).

The middle region constitutes the new sequence to be inserted. The 3′ end of the upstream arm will continue to the 5′ end of the middle region (new sequence) and the 3′ end of the middle region will continue to the 5′ end of the downstream arm. For floxing designs (and for knockdown designs), once the theoretical sequences of the ssDNA cassette are designed, the cassettes can be built by custom synthesis from commercial vendors (such as Integrated DNA Technologies, GENEWIZ, GenScript, or Gene Art by Thermo Fisher Scientific). For knock-in designs consisting of expression cassettes such as recombinases or reporters, any known plasmids can be used as templates to amplify the cassette using primers that contain the homology arms (and the T7 promoter sequence).

Synthesis and purification of ssDNA and other CRISPR components for microinjection. The components of the *Easi*-CRISPR system are ssDNA donor and CRISPR reagents (crRNA + tracrRNA + Cas9 protein). The experimental methods for preparing these reagents are described below.

Synthesis of ssDNA using the ivTRT method. This method transcribes a dsDNA template into RNA (Steps 1–18). The RNA is then reverse-transcribed back to DNA (to generate ssDNA molecules), followed by RNase H degradation of RNA and purification of the ssDNA (Steps 19–35). The dsDNA templates can be either PCR products or plasmids that contain a T7 promoter and the insertion cassette (homology arms with the new sequence of interest in the middle). A schematic depicting the *iv*TRT and ssDNA preparation steps is shown in **Figure 5**. Another method of generating ssDNAs was reported recently that uses DNA-nicking endonucleases to make nicks on a plasmid dsDNA, followed by separation and purification of the desired ssDNA fragment from a denaturing agarose gel[39]. Very recently, Integrated DNA Technologies launched a custom ssDNA synthesis service offering DNA cassettes up to 2-kb long (Megamers). Megamers can be commercially procured, as an alternative to generating them via *iv*TRT.

Preparation of Easi-CRISPR microinjection mix. The ssDNA synthesized by *iv*TRT (or purchased as a Megamer's) is then used for microinjection along with the other CRISPR components. Our first set of *Easi*-CRISPR method development experiments used in-house-generated Cas9 mRNA and single-guide RNA (sgRNA), via *in vitro* transcription, as CRISPR components, but in our recent experiments, we observed that commercial synthetic RNAs (as separate crRNA and tracrRNA molecules) and the Cas9 protein have better rates of correct insertion. One reason for the high efficiency of Cas9 protein-containing complex could be that the pre-assembled RNP would be immediately available for cleavage upon injection, whereas there would be some delay in formation of Cas9 protein from the mRNA. Note that sgRNAs are also commercially available and they can be complexed with Cas9 protein *in vitro*. We proposed the terms ctRNP (crRNA + tracrRNA + Cas9 Protein) and sgRNP (sgRNA + Cas9 Protein) ribonucleoprotein complexes for these combinations of CRISPR reagents[9]. One major advantage of RNP compositions (in single, or two-part guide formats), is that all reagents can be commercially synthesized at much lower cost than by preparing sgRNAs and Cas9 mRNA through *in vitro* transcription methods (that were described in Harms *et al.*[37] and were quite prevalent before commercial synthesis was possible). To prepare *Easi*-CRISPR components for microinjection, ctRNP and ssDNA are mixed together; the process is described in detail in Steps 36–40.

Microinjection of *Easi*-CRISPR components into mouse zygotes. The microinjection step involves a series of transgenic technologies that are typically performed at specialized core facility labs. These steps have been published previously[37,40] and have now become standard methods. The *Easi*-CRISPR microinjection protocol also follows these standard steps (Steps 41–53). We used both pronuclear and cytoplasmic injection in the initial stage of development of this method, particularly when Cas9 mRNA was used in our first report[8]. We switched to performing only pronuclear injection after we switched to using ctRNP components. On the basis of the efficiencies we have observed while using pronuclear-only injection of ctRNP components, we suggest that pronuclear injection alone would be sufficient for creating knock-in, conditional knockout, and knockdown mouse models using the *Easi*-CRISPR method.

Genotyping of offspring. A general schematic of genotyping strategies is shown in **Figure 6**. At least two PCRs, one each for the 5′ and 3′ junctions, are essential for genotyping *Easi*-CRISPR-derived offspring (Steps 54–59). A third PCR, specific to internal sequences (in case of longer knock-in cassettes), to identify *cis*-inserted *LoxP* sites (in case of floxing) would be necessary.

Genotyping of floxed alleles. Primers that can amplify each of the two separate *LoxP* sites are necessary for detecting *LoxP* insertions. When genotyping floxed alleles, we always use primer sets that

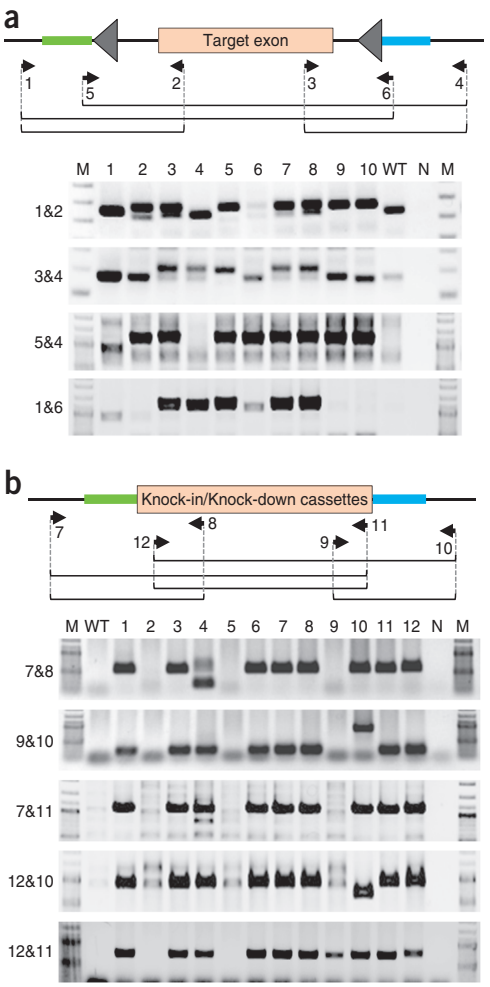

Figure 6 | Genotyping schematics. (**a**) Genotyping of floxed alleles. Primer sets 1&2 and 3&4 amplify single *LoxP* insertions at the two separate sites but cannot indicate whether they are inserted *in cis* or *in trans*. Correct insertion genotype (*in cis*) can be determined by PCR using the primer sets 5&4 and 1&6, and can be confirmed by sequencing the PCR products. Note that the 3′ ends of primers 5&6 bind to the first 15 bases of *LoxP* sites (primer 5: 5′-NNNNNNNNNNNNNNNNNNNataacttcgtatagc-3′; primer 6: 5′-NNNNNNNNNNNNNNNNNNNataacttcgtataat-3′). (**b**) Genotyping knock-in (and knockdown) alleles. Three PCRs are performed, one each for 5′ and 3′ junctional regions (primer sets 7&8 and 9&10), and the third for insert-specific regions (primer set 12&11). PCR with outer primer sets (7&11 and 12&10) amplifies longer PCR fragments, including the full knock-in cassette. If amplification of longer sequences is not successful (e.g., if primers 7&11 and 12&10, which amplify nearly the full length of the cassette, do not work well), alternative primers within the insertion cassette should be tried. The amplified fragments in both **a** and **b** should be sequenced to ensure sequence fidelity. The examples of PCR of primer pairs 1&2 and 3&4 (for *Pitx1* floxing), and 7&8 and 9&10 (*MMP9*-T2AmCitrine knock-in) were previously reported in Quadros *et al.*[9]. Image adapted from ref. 9; license at https://creativecommons.org/licenses/by/4.0/, All experimental procedures using mice were carried out according to Tokai University institutional regulations for animal usage (permit no. 165009).

amplify a single *LoxP* insertion (for both *LoxP*s; **Fig. 6a**). As the *LoxP*-inserted allele can be seen as a slightly larger band, as compared with that of the wild type, PCR products from a wild-type control should be included in agarose gel electrophoresis. When both *LoxP*s are positive, the pups can be candidates for floxed mice. However, it is often difficult to judge whether these two *LoxP*s are inserted *in cis*. Complete insertion of a floxed cassette (*in cis*) can be determined by PCR using primer sets (one outer primer and one other primer that binds to a distantly located *LoxP* site; **Fig. 6a**) and subsequent sequencing of the amplified fragments to determine whether the fragment contains an internal *LoxP* site. Optimization of PCR conditions may be necessary, when *LoxP* sequence-containing primers are used for PCR, because *LoxP* sites contain palindromic sequences and thus they may not yield optimal PCR reactions for certain loci. We used the following PCR conditions when using *LoxP*-containing primers in 10 µl of 1× PrimeSTAR Buffer (Mg^{2+} plus) containing genomic DNA (1 µl), dNTP (0.2 mM each), primers, and 0.25 U of PrimeSTAR HS DNA polymerase: denaturation (94 °C for 3 min), 35 cycles of 98 °C for 10 s, 62 °C for 5 s, 72 °C for 2 min, and extension (72 °C for 10 min).

Even when the correctly inserted floxed cassette is identified, it is desirable to check for the presence of a floxed allele and its nucleotide sequence in the next generation. The recombination capability of the floxed allele is confirmed by breeding the mouse line with a Cre driver line. A quick analysis for recombination of the floxed allele can also be performed using an *in vitro* Cre reaction, in which the target region is PCR-amplified and incubated with a Cre protein, and the recombination products are analyzed on an agarose gel[6].

Genotyping of knock-in and knockdown alleles. We recommend that PCR be performed for both the 5′ and 3′ junctional regions, and for insert-specific regions (using internal primer sets) (**Fig. 6b**). Even though all three sets of PCR reactions can show expected amplicons, all junctions and the insertion cassette must be fully sequenced to ensure sequence fidelity, to rule out any indels near the junctions, and to check for nucleotide misincorporations in the insert regions that may arise from the enzymatic synthesis steps in preparing the ssDNA donor. PCR with an outer primer set (that binds outside the homology arms) amplifies longer PCR fragments, including the entire knock-in cassette, as well as the shorter wild-type sequence (including alleles that lack the insertion cassette but that may contain indels). In this PCR, amplification of only the wild-type (or indel) band does not always guarantee that it can serve as a negative sample, because a smaller amplicon (wild type) is preferentially amplified rather than the longer band (the correctly targeted allele). The insertion allele (larger band) may be amplified efficiently if the insertion event is biallelic (with no wild-type or indel alleles)[9]. Regardless of the PCR amplification results, sequencing of the entire knock-in cassette is mandatory to confirm the correctness of the targeted allele. Sequencing should also be performed in F1 animals to rule out any hidden mosaic alleles that may contain mutations from the founders and be transmitted to the offspring.

MATERIALS

REAGENTS

CRISPR reagents

- crRNA (minimum modified) and tracrRNA (cat. no. 1072534) from Integrated DNA Technologies (IDT). The crRNA part is custom-synthesized for each specific guide RNA, whereas tracrRNA is universal.
- Nuclease-Free Duplex Buffer (IDT, cat. no. 11-05-01-12)
- Alt-R S.p. Cas9 Nuclease 3NLS (IDT, cat. no. 1074181)

Reagents for ssDNA synthesis

▲ **CRITICAL** The standard kits and reagents that have been tried in our laboratories are listed below. Comparable kits and reagents from other vendors may also be used in place of these.

- NcoI (New England BioLabs, cat. no. R0193S) or another suitable restriction enzyme
- Standard desalt ultramers (long primers) to use as primers for PCR of insert or custom gene-synthesized plasmid (from Integrated DNA Technologies or Gene Art from Thermo Fisher Scientific)
- KOD plus neo buffer (Toyobo, cat. no. KOD-401) or any suitable high-fidelity PCR mix
- dNTP mix (New England BioLabs, cat. no. N0447L)
- SeaKem ME agarose (Lonza, cat. no. 50011)
- SeaPlaque GTG agarose (Lonza, cat. no. 50110)
- Wizard SV Gel PCR Clean-up System (Promega, cat. no. A9282) or NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, cat. no. 740609)
- MEGAclear Kit (includes binding, washing, and elution solutions; Ambion-Life Technologies, cat. no. AM1908)
- T7 RiboMax Express Large Scale RNA Production System (Promega, cat. no. P1320)
- Ethanol, 200 proof, ACS grade (Deacon Laboratories) or Ethanol (99.5) (Wako, cat. no. 057-00456)
- M-MuLV Reverse Transcriptase (New England BioLabs, cat. no. M0253S) or SuperScript III Reverse Transcriptase (Life Technologies, cat. no. 18080051) or SuperScript IV Reverse Transcriptase (Life Technologies, cat. no. 18090010)
- RNaseH (New England BioLabs, cat. no. M0297L)
- S1 nuclease (TaKaRa, cat. no. 2410A)
- 3 mol/l Sodium acetate buffer solution (pH 5.2) (Nakalai Tesque, cat. no. 06893-24)
- Phenol, saturated with TE buffer (Nakalai Tesque, cat. no. 26829-54)
  ! CAUTION Phenol is toxic and causes burns. The container should be opened in a fume hood while wearing proper protective equipment.
- Modified TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0; Affymetrix, cat. no. 75793)
- Ethidium bromide (10 ml; Sigma, cat. no. E1510) **! CAUTION** Ethidium bromide is carcinogenic and mutagenic. Wear personal protective equipment.
- Embryo Max Microinjection Buffer (EMD Millipore, cat. no. MR-095-10F), or a microinjection buffer containing 10 mM Tris-Cl, pH 7.5, 0.1 mM EDTA
- Millex-GX 0.22-μm filter unit (EMD Millipore, cat. no. SLGV004SL)

Reagents for mouse transgenesis experiments

- Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG) (National Hormone and Peptide Program (Harbor–UCLA Medical Center); http://www.humc.edu/hormones/material.html)
- EmbryoMax M2 media for embryo handling (Millipore, cat. no. MR-015-D)
- Hyaluronidase (Millipore, cat. no. MR-051-F)
- KSOM + AA for embryo incubation (Millipore, cat. no. MR-106-D)
  ▲ CRITICAL Media should be stored at −20 °C; after opening the container, store at 4 °C and use the remainder before expiration date.
- Light mineral oil (Millipore, cat. no. ES-005-C)

Mice

! CAUTION Experimental procedures involving animals should be carried out according to relevant institutional and governmental regulations.

- Donor females (three-week-old C57BL6 females from Charles River Laboratories or Jackson Laboratories)
- Stud males (C57BL6 males from Charles River Laboratories or Jackson Laboratories)
- Pseudopregnant recipients (CD-1(ICR) female mice at 5–6 weeks of age from Charles River Laboratories)
- Vasectomized males (5- to 6-week-old CD-1 mice from Charles River Laboratories). Perform vasectomies as described previously[41].

Reagents for mouse genotyping

- Cell lysis solution (Qiagen, cat. no. 158908)
- Protein precipitate solution (Qiagen, cat. no. 158912)
- DNA hydration solution (Qiagen, cat. no. 158914)
- TaKaRa Taq (dNTP is included in the kit; TaKaRa, cat. no. R001) or PrimeSTAR HS DNA polymerase (buffer and dNTP are included in the kit; TaKaRa, cat. no. R010)
- 2× GC buffer (TaKaRa, cat. no. 9154)
- Allele-In-One Mouse Tail Direct Lysis Buffer (Kurabo, cat. no. ABP-PP-MT01500)
- Agarose (Phenix Research Products, cat. no. RBA 500)
- 50× TAE buffer (Thermo Fisher Scientific, cat. no. BP1332-20)
- Nuclease-free water (many common vendors)
- TA Cloning Kit, with pCR2.1 vector (Life Technologies, cat. no. K202020)
- LB agar ampicillin-100, plates (Sigma, cat. no. L5667-10EA)
- Mix & Go Competent Cells, strain Zymo 5α (Zymo Research, cat. no. T3007), used for DNA cloning
- QIAprep Spin Miniprep Kit (Qiagen, cat. no. 27106)
- Proteinase K solution (10 ml; 5Prime, cat. no. 2500150)
- GeneRuler DNA Ladder Mix (Thermo Fisher Scientific, cat. no. SM0331)
- Gel-loading dye, purple (6×), no SDS (New England BioLabs, cat. no. B7025S)
- Kimwipes for general cleaning (Kimtech Science, cat. no. KCK-280-4.4 × 8.4)

EQUIPMENT

- PCR tubes (Thermo Fisher Scientific, cat. no. AB-1114) or 8-strip of 0.2-ml thin-wall PCR tubes and caps (Thermo Fisher Scientific, cat. no. 435440-Q)
- 1.5-ml tube (BM Bio, cat. no. NT-175)
- Parafilm M (Sigma, cat. no. P7793-1EA)
- RNase-free pipette tips (aerosol-resistant tips (ARTs))
- IVF dish (Falcon, cat. no. 353653)
- Tissue culture dish, 35 × 10 mm (Falcon, cat. no. 353001)
- Tissue culture dish, 60 × 15 mm (Falcon, cat. no. 353002)
- MicroFil, 28-gauge/97-mm long (World Precision Instruments, item no. MF28G); used for administration of hormones
- Flexipet oocyte/embryo pipettes (Cook Medical, cat. no. K-FPIP-1130-10BS-5)
- Holding pipettes (Humagen, cat. no. MPH-SM-20)
- NORM-JECT tuberculin syringes (Henke-Sass, Wolf, cat. no. 4010-200VO), used for administration of hormones
- Thermocycler (Bio-Rad, model no. T100 or equivalent)
- UV spectrophotometer (Thermo Scientific, model no. NanoDrop 1000)
- Gel documentation System (Bio-Rad, model Gel Doc XR+)
- LED light (BioSpeed ethidium bromide-VIEWER)
- Tabletop microcentrifuge (Eppendorf, model no. 5417C or equivalent)
- Tabletop microcentrifuge, refrigerated (Eppendorf, model no. 5417R or equivalent)
- Micropipettes (Eppendorf Research Plus)
- Gel electrophoresis system (Bio-Rad, model no. Power Pac 300)
- Shaking heat block (Eppendorf, Thermomixer Comfort model)
- Heat block (dry thermo unit; TAITEC)
- Water baths (Fisher Scientific)
- Vortex Genie 2 (Scientific Industries)
- Microwave and weighing balances
- Glass pipette puller (Sutter Instrument, cat. no. P97), outfitted with a 2.5 × 2.5-mm box filament (Sutter Instrument, cat. no. FB255B)
- Glass capillaries, with filament, 1.0-mm, 4-inch (World Precision Instruments, item no. TW100F-4)
- Microinjection needles (injection capillaries are made fresh the morning of injection using the Sutter Instruments model no. P97 pipette puller outfitted with 2.5 × 2.5-mm box filament (model no. FB255B))
- Inverted microscope system (Nikon, model no. Eclipse TE2000-E with DIC (or Leica, model DM IRB) equipped with a Narishige model no. IM 300 microinjector and Nikon model no. NT-88-V3 micromanipulators (or Leica manipulators))
- Dissecting scope system (Leica, model no. MZ 9.5 with PLAN 0.5× condenser lens (model no. 10 446 157), base with tilt head (Leica, model no. 10 445 367), heating glass (Live Cell Instrument, cat. no. HG-T-Z002) and temperature controller (Live Cell Instrument, cat. no. CU-301)
- Tri-gas incubator equipped with Coda inline filters (Heraeus, model no. Heracell 150)
- Nunc Lab-Tek Chamber Slide System (Lab-Tek, cat. no. 177372)
- Forceps, no. 5 (INOX, cat. no. 5)
- Large slide warmer (Spectrum Scientifics, cat. no. 3875)
- Mouth-pipetting apparatus
- Microinjection scope (Leica, model no. DM IRB) with the following specifications: eyepiece: HC PLAN 10×/22 with tilt (model no. 11 507 804); condenser lens (model no. 0.30 S70); objectives: C PLAN 4×/0.10 (model no. 11 506 074), N PLAN L20×/0.40 CORR (model no. 11 506 057), N PLAN L40×/0.55 CORR (model no. 11 506 059)

REAGENT SETUP

Oligonucleotides and ultramers Resuspend oligonucleotides or ultramers to a final concentration of 100 μM in nuclease-free water and store at −80 °C for up to several years for future use.

1× TAE electrophoresis buffer Dilute 50× TAE buffer in dH_2O to a 1× working solution. The 1× solution can be stored at room temperature (25 °C) for up to 3 months.

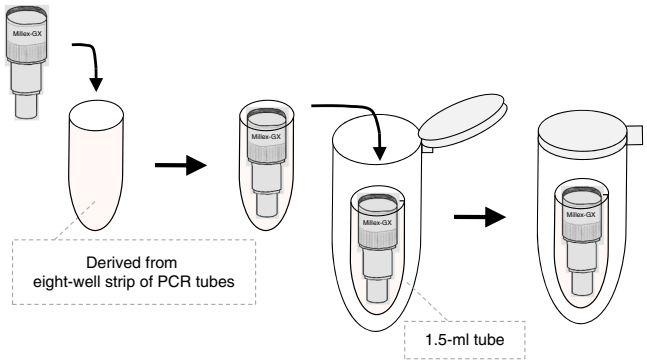

Figure 7 | Assembly of Millex-GX 0.22-µM filter unit. Insert a Millex-Gx 0.22-µM filter unit into a PCR tube (cut from an eight-well strip), then insert the PCR tube containing the filter into a 1.5-ml microcentrifuge tube.

Ethanol For 100 ml of ethanol (70% (vol/vol)) solution, combine 70 ml of 99.5% (vol/vol) ethanol with 30 ml of nuclease-free water. It can be stored in a tightly sealed tube at room temperature for at least 1 year.

Cas9 protein Dilute Cas9 protein in microinjection buffer to a working stock (e.g., 3.1 µM (500 ng/µl)), as described in Step 37. This can be stored at −20 °C for up to 18 months.

crRNA and tracrRNA Resuspend crRNA and tracrRNA to a final concentration of 100 µM (~1.2 µg/µl for crRNA and 2.2 µg/µl for tracrRNA) in microinjection buffer or Nuclease-Free Duplex Buffer and store aliquots at −80 °C for up to 18 months for future use. Dilute each RNA to a concentration of 6.1 µM with microinjection buffer before annealing (Step 36).

The ctRNP complex preparation The CRISPR system comprises three components: crRNA, tracrRNA, and Cas9 protein. The crRNA is unique to each project, whereas the other two components are universal. The crRNA is 36 bases long and tracrRNA is 67 bases long. The crRNA can be custom synthesized (e.g., using the Alt-R system from IDT); the crRNA and tracrRNA can also be chemically modified, which is known to increase their stability[42]. The two RNAs (crRNA and tracrRNA) are annealed as described in Step 36 and mixed with Cas9 protein to generate ctRNP complexes (Step 38)

▲ **CRITICAL** The size of crRNA and tracrRNA may vary from vendor to vendor.

Preparation of Millex-GX (0.22-µm filter unit) Cut one PCR tube from an 8-strip of 0.2-ml thin-wall PCR tubes and place the Millex-GX filter unit (0.22-µm filter unit) into the 0.2-ml PCR tube (**Fig. 7**). Insert this column (consisting of the filter unit in the PCR tube) into a 1.5-ml microcentrifuge tube.

Preparation of dishes and reagents for embryo collection and microinjection

Oviduct collection dish: Add 2 ml of M2 medium to each 35-mm tissue culture dish (one dish per female for up to ten females).

Hyaluronidase dish: Add 1.5 ml of hyaluronidase medium to each 35-mm tissue culture dish (one dish per female for up to ten females).

Wash dish: Add 1.5 ml of M2 medium to each 35-mm tissue culture dish.

KSOM rinse dish: Add 1.5 ml of (pre-equilibrated) KSOM medium to each 35-mm tissue culture dish.

Incubation dish: Add 1 ml of KSOM medium (pre-equilibrated in a 5% CO_2 incubator) to each IVF/organ culture dish.

Embryo transfer dish: Add 1.5 ml of M2 medium to each 35-mm tissue culture dish.

PROCEDURE

Preparation of dsDNA template ● TIMING 5 h

1| There are two options for the generation of the dsDNA template: use a linearized plasmid DNA (option A) or use of a PCR product (option B).

(A) Use of a linearized plasmid as a template

(i) Digest 2.5–4 µg of plasmid with a suitable restriction enzyme (e.g., NcoI) in a 100-µl reaction volume at 37 °C for 3 h.

(B) Use of PCR product as a template

(i) Generate ~50–100 µl of PCR product using any high-fidelity *Taq* DNA polymerase (e.g., KOD plus neo), by setting up and running the following PCR. The approximate yield of DNA from the PCR reaction should be ~5–15 µg.

Reagent	Volume per reaction	Final concentration
10× buffer	5 µl	1×
2 mM dNTP	5 µl	200 µM
25 mM $MgSO_4$	3 µl	1.5 mM
Primers	1 µl sense primer	0.4 µM
(20 µM stock)	1 µl antisense primer	0.4 µM
Enzyme (KOD plus neo) 1 U/µl	1 µl	1 U/50 µl
Water	33 µl	
Template DNA	1 µl	<1 ng
Total	50 µl	

Cycle number	Denature	Anneal/Extend
1	95 °C for 1 min	
2–31	95 °C for 15 s	68 °C for 3 min
32		68 °C for 7 min
Hold at 4 °C		

▲ **CRITICAL STEP** Use high-fidelity *Taq* polymerase (e.g., KOD plus neo) for the generation of a template using PCR.
▲ **CRITICAL STEP** Primers used for amplifying PCR products can be standard desalt-grade and custom-synthesized by any commercial vendor.

2| Run the sample on an agarose gel to check for complete digestion of the plasmid or for efficient amplification of the PCR product. Load ~1–2 µl of the RE-digested sample from Step 1A, or 1/10 volume of the PCR product from Step 1B, after addition of 2 µl of 6× gel loading buffer, onto a 1% (wt/vol) agarose gel (SeaKem ME) in 1× TAE. Run at 135 V for 30 min.

3| Purify the dsDNA template prepared in Step 1 using option A, if linearized plasmid was used as a template, or option B, if PCR product was used as a template.

(A) Purification of dsDNA if linearized plasmid was used as a template

(i) Add 100 µl of modified TE to the sample tube (total = 200 µl).
(ii) Add 200 µl of TE-saturated phenol and centrifuge (21,000*g*, 20 °C, 6 min).
! CAUTION Phenol is toxic and causes burns. The container should be opened in a fume hood while wearing proper protective equipment.
(iii) Transfer the supernatant to a new tube, add 20 µl of 3 M sodium acetate (pH 5.2) and 500 µl of 99.5% (vol/vol) ethanol, vortex, and centrifuge at 21,000*g* for 10 min at room temperature.
(iv) Decant the supernatant, add 130 µl of 70% (vol/vol) ethanol, and centrifuge at 21,000*g* for 2 min at 4 °C.
(v) Completely remove the supernatant and dry the pellet. Dissolve the pellet in 10 µl of modified TE buffer and use ~1 or 2 µl to estimate the DNA concentration using a NanoDrop spectrophotometer.

(B) Purification of dsDNA if PCR product was used as a template

(i) Gel-purify the PCR product using the Wizard SV Gel PCR Clean-Up System and following the manufacturer's instructions; perform two elutions in 20 µl of elution buffer. Estimate the concentration using a NanoDrop spectrophotometer.
! CAUTION Phenol is toxic and causes burns. The container should be opened in a fume hood while wearing proper protective equipment.

RNA synthesis using T7 RiboMax Express ● TIMING 1–4 h

4| Synthesize RNA using the T7 RiboMAX Express Large Scale RNA Production System according to the manufacturer's instructions. Add the following reagents to a PCR tube or microcentrifuge tube, mix by pipetting and centrifuge at 21,000*g* for 30 s at room temperature. Use ~1 µg of template DNA from Step 3 for *in vitro* transcription.
! CAUTION Avoid repeated freeze–thaw of buffers. Thaw all the reagents on ice and be sure to use RNase-free tips and gloves.

Component	Amount per reaction (µl)
RiboMAX Express T7 2× buffer	10 µl
Template DNA from Step 3	× µl[a]
Nuclease-free water	Up to 20 µl
Enzyme mix, T7 Express	2 µl
Total	20 µl

[a]1 µg of template from Step 3A or 3B is used (adjust the volume according to the DNA concentration).

5| Incubate the reaction at 37 °C in a thermocycler for 30 min to 3 h.

6| Add 1 µl of RQ1 RNase-Free DNase, mix well, and incubate at 37 °C in a thermocycler or in a water bath, for 15 min to eliminate the DNA template.
! CAUTION Wrap the microcentrifuge tube caps with Parafilm film to prevent contamination if a water bath is used for the incubation.

Purification of RNA using a MEGAclear Kit ● TIMING 1 h
7| To purify RNA using a MEGAclear Kit according to the manufacturer's instructions, first preheat a dry heat block to 65–70 °C and prewarm the elution solution.

8| Add 80 µl of elution solution to the sample from Step 6 (total volume = 101 µl) and mix by gentle pipetting.

9| Add 350 µl of binding solution and mix by gentle pipetting.

10| Add 250 µl of >99.5% (vol/vol) ethanol and mix by gentle pipetting.

11| Transfer the sample to the column and centrifuge (21,000*g*, 1 min, room temperature).

12| Discard the flow-through and re-insert the column into the microcentrifuge tube.

13| Add 500 µl of washing solution to the column and centrifuge (21,000*g*, 1 min, room temperature).

14| Discard the flow-through and repeat washing (as in Step 13).

15| Discard the flow-through and centrifuge (21,000*g*, 30 s, room temperature) to completely remove traces of ethanol.

16| Insert the column into a newly labeled 1.5-ml microcentrifuge tube and add 25–50 µl of prewarmed elution solution from Step 7 directly to the column bed, incubate for 10 min, and centrifuge at 21,000*g* for 1 min at room temperature. A second elution can be performed to recover more RNA.

17| Check the quality and concentration of RNA by analyzing 1 µl of sample in a NanoDrop spectrophotometer.

18| Confirm the quality of the RNA by agarose gel electrophoresis (**Fig. 5**).
▲ CRITICAL STEP Prepare aliquots of ~3–5 µg of RNA/tube to avoid repeated freezing and thawing of RNA.
? TROUBLESHOOTING
■ PAUSE POINT Store the samples at 80 °C until use.

Synthesis of cDNA from RNA ● TIMING 1.5 h
19| To synthesize cDNA according to the manufacturer's instructions following the Superscript III Reverse Transcriptase protocol, add the following reagents to a PCR tube or microcentrifuge tube (Tube A), mix by pipetting and centrifuge at 21,000*g* for 30 s at room temperature. Use ~3–5 µg of purified RNA from Step 18 as a template for cDNA synthesis. Mix the reaction by pipetting and centrifugation at 21,000*g* for 30 s at room temperature.

Component	Amount per reaction (µl)	Final (in 30 µl)
RNA template from Step 18	× µl	~5 µg
RT primer (100 µM)	1.5 µl	5 µM
dNTP mix, NEB (10 mM)	3.0 µl	1 mM
Nuclease-free water	Up to 30 µl	
Total	30 µl[a]	

[a]Reactions can be scaled up to obtain a higher quantity of cDNA. This reaction is 3× the scale (3 × 10 µl) of the manufacturer's protocol.

20| Incubate the tube at 65 °C for 5 min.

21| Immediately place the tube (Tube A) on ice, wait for at least 1 min, and then proceed to cDNA synthesis. Mix the following reagents in a new tube (Tube B).

Component	Amount per reaction (µl)	Final
10× RT buffer	6 µl	
0.1 M DTT	3 µl	0.01 M
25 mM $MgCl_2$	12 µl	10 mM
RNase out	3 µl	
Super Script III Reverse Transcriptase	3 µl	
Nuclease-free water	3 µl	
Total	30 µl	

! CAUTION DTT is harmful when inhaled, and it may cause irritation to skin and eyes. Wear gloves and proper equipment when working with DTT.

22| Combine the contents of the two tubes (Tubes A and B). Mix well and incubate the reaction at 50 °C for 50 min in a water bath or thermocycler. Stop the reaction by heat-inactivating at 85 °C for 5 min. Cool the reaction to room temperature.
! CAUTION Wrap the microcentrifuge tube caps with Parafilm to prevent contamination if a water bath is used for incubation.

23| Add 3 µl of RNase H to the tube, mix by pipetting, and incubate the tube at 37 °C for 20 min in a water bath.
! CAUTION Wrap the microcentrifuge tube caps with Parafilm to prevent any contamination from the water bath.
? TROUBLESHOOTING
■ PAUSE POINT The samples can be stored at –20 °C for up to 2 years until use.

Ethanol precipitation of cDNA and gel purification ● TIMING 1.5 h
24| Add 40 µl of modified TE, 10 µl of 3 M sodium acetate (pH 5.2), and 250 µl of 99.5% (vol/vol) ethanol to 63 µl of sample from Step 23. Vortex and centrifuge at 21,000*g* at 4 °C for 10 min. Discard the supernatant, add ~130 µl of 70% (vol/vol) ethanol, and centrifuge (21,000*g*, 4 °C, 2 min).

25| Completely remove the supernatant using a micropipette and air dry the pellet. Dissolve the pellet in 8 µl of modified TE.

26| Add 2 µl of 6× gel loading buffer, load the sample into 1% low-melting gel (SeaPlaque GTG agarose), and perform electrophoresis (135 V, 30 min).

27| Stain the gel with ethidium bromide, excise the gel piece containing the major band under LED light (BioSpeed ethidium bromide-VIEWER), and transfer it to a new 1.5-ml microcentrifuge tube. A sample electrophoresis image of a cDNA preparation and excision of a prominent band for gel purification is shown in **Figure 5**.

! CAUTION Ethidium bromide is carcinogenic and mutagenic. Use care and wear personal protective equipment while handling it. Also wear personal protective equipment to avoid exposure to the UV light, if a UV light source is used for viewing the gels. Long-wavelength UV can be used to prevent damage to the DNA sample.

28| Extract DNA from the gel slice using a NucleoSpin Gel and PCR Clean-up (TaKaRa) kit (option A) or by phenol extraction and ethanol precipitation (option B).

(A) DNA extraction using a NucleoSpin Gel and PCR Clean-up kit (column purification) ● TIMING 20 min
(i) Use a NucleoSpin Gel and PCR Clean-up kit for purification of cDNA according to the manufacturer's instructions: add 200 µl of Buffer NT1 to each 100 mg of agarose gel (containing cDNA) and mix well. Determine the weight of the gel piece before adding Buffer NT1.
(ii) Incubate the tube for 10 min at 50 °C to completely dissolve the gel piece (briefly vortex the sample every 2–3 min).
(iii) Transfer the sample (up to 700 µl) to a column and centrifuge for 30 s at 11,000*g* at room temperature.
(iv) Discard the flow-through and add 700 µl of Buffer NT3 to the column and centrifuge for 30 s at 11,000*g* at room temperature.
(v) Repeat Step 28A(iv) washing with Buffer NT3.
(vi) Centrifuge for 1 min at 11,000*g* at room temperature to completely remove Buffer NT3.

(vii) Place the column into a new microcentrifuge tube, add 15–30 µl of Buffer NE, and incubate at room temperature for 1 min.
(viii) Centrifuge for 1 min at 11,000*g* (first elution) at room temperature.
(ix) Place the column into the tube and add 15–30 µl of Buffer NE and incubate at room temperature for 1 min.
(x) Centrifuge for 1 min at 11,000*g* (second elution at room temperature) (total elution volume will be 30–60 µl).
▲ **CRITICAL STEP** Multiple elutions yield higher cDNA recovery; for example, three elutions of 20 µl each can also be performed.

(B) DNA extraction by phenol extraction and ethanol precipitation ● TIMING 50 min
(i) Add 200 µl of modified TE to the tube containing the gel piece and place it at –80 °C for >20 min (can be left overnight).
(ii) Thaw the sample at room temperature and transfer the solution to a new tube.
(iii) Add 200 µl of TE-saturated phenol and centrifuge (21,000*g*, 20 °C, 6 min).
! **CAUTION** Phenol is toxic and causes burns. The container should be opened in a fume hood while wearing proper protective equipment.
(iv) Transfer the supernatant to a new tube, then add 20 µl of 3 M sodium acetate (pH 5.2) and 500 µl of 99.5% (vol/vol) ethanol. Vortex and centrifuge at 21,000*g*, 10 min, at room temperature.
(v) Decant the supernatant, add 130 µl of 70% (vol/vol) ethanol, and centrifuge at 21,000*g* for 2 min at 4 °C.
(vi) Completely remove the supernatant and dry the pellet. Dissolve the pellet in 11 µl of injection buffer.
■ **PAUSE POINT** Store the samples at –20 °C (short term (up to 3 months)) or –80 °C (long term (up to 2 years)).

Filter purification of cDNA solution ● TIMING 1.5 h
29| Precool the centrifuge to 4 °C.

30| Add injection buffer to the Millex-GX (0.22-µm filter unit: see Reagent Setup for assembly of filter) and centrifuge the tube (13,000*g*, 4 °C, 1 min) (**Fig. 7**).

31| Take the filter unit only from the PCR tube and place it into a new PCR tube, and then re-insert into the 1.5-ml microcentrifuge tube.

32| Add 11 µl of sample to the filter and centrifuge (13,000*g*, 4 °C, 2 min).

33| Take the filter-containing PCR tube from the 1.5-ml microcentrifuge tube and transfer the filtered sample (in the bottom of the PCR tube) solution to a new 1.5-ml tube.

34| Transfer 1 µl of sample to another new tube and dilute with 4 µl of injection buffer (total 5 µl). Use 1 µl for concentration determination using a NanoDrop spectrophotometer, and the remaining 4 µl for agarose gel electrophoresis (**Fig. 5**).
?TROUBLESHOOTING

35| Store the remaining sample (10 µl) at –20 or –80 °C for up to 2 years for future use.

Preparation of guide RNA by annealing crRNA and tracrRNA ● TIMING 30 min
36| Anneal crRNA and tracrRNAs by mixing equimolar ratios. Mix 10 µl of 6.1 µMcrRNA (~72 ng/µl) and 10 µl of 6.1 µM tracrRNA (~135 ng/µl) and anneal in a thermocycler (94 °C for 2 min and then place at room temperature for ~10 min). The crRNA/tracrRNA complex (3.1 µM each) can be stored at –80 °C until use (for up to 1 year).
▲ **CRITICAL STEP** Annealing of crRNA and tracrRNA is an important step to obtain active guide RNA and for the formation of ctRNP complex.

Preparation of ctRNP + ssDNA injection mix ● TIMING 1 h
▲ **CRITICAL** The injection mix should be prepared immediately before zygote injection. Steps 37–40 are performed concurrently with zygote preparation on the day of microinjection (Steps 44–47).

37| To prepare the ctRNP injection mix (10- to 20-µl volume), dilute the Cas9 protein in microinjection buffer to a concentration of 500 ng/µl (equivalent to 3.1 µM).

38| Mix the components on ice as shown below (10-µl scale).

▲ **CRITICAL STEP** Microinjection reagent mix should be prepared just before performing the injections.

Component	Amount per reaction (μl)	Final concentration[a]
crRNA/tracrRNA complex (3.1 μM (104 ng/μl)) from Step 36	2 μl	0.61 μM (20.7 ng/μl)
ssDNA (30–100 ng/μl) from Step 35	× μl	5–10 ng/μl
Cas9 protein (3.1 μM (500 ng/μl)) from Step 37	1 μl	0.3 μM (50 ng/μl)
Microinjection buffer	Up to 10 μl	
Total	10 μl	

[a]We typically use 0.3 μM (10.3 ng/μl) of guide RNA and 0.3 μM (50 ng/μl) of Cas9 protein for mouse zygote microinjections. When two guides are used (e.g., floxing), use 0.15 μM of each guide RNA. Many other concentrations, even though not equimolar, have also worked successfully (e.g., 20 ng/μl guide 1 + 50 ng/μl Cas9 + 10 ng/μl ssDNA (20:50:10); 10 ng/μl guide 1 + 10 ng/μl guide 2 + 50 ng/μl Cas9 + 10 ng/μl ssDNA (2 × 10:50:10); 10 ng/μl guide 1 + 10 ng/μl Cas9 + 10 ng/μl ssDNA (10:10:10); 10 ng/μl guide 1 + 10 ng/μl guide 2 + 10 ng/μl Cas9 +10 ng/μl ssDNA (2 × 10+10+10); 5 ng/μl guide 1 + 20 ng/μl Cas9 + 10 ng/μl ssDNA (5:20:10); 5 ng/μl guide 1 + 5 ng/μl guide 2 + 10 ng/μl Cas9 + 10 ng/μl ssDNA; + 10 ng/μl ssDNA (2 × 5:10:10)).

39| Incubate the injection mixture at 37 °C for 10 min just before injection and keep it at room temperature until use for injection. The injection mix can remain at room temperature until the injection process is completed, which usually takes ~1–2 h.

40| Load the injection mix into needles, following the microinjection procedures described in ref. 37. They are now ready for use to inject zygotes in Step 48.
▲ **CRITICAL STEP** Preparation of zygotes in Steps 41–47 should be performed concurrently with Steps 37–40, so that the injection mix is prepared and loaded into microinjection needles immediately before microinjection.

Mouse transgenesis ● TIMING 5–6 h
▲ **CRITICAL** These experiments follow well-established standard protocols of mouse transgenesis, typically performed at specialized core facility laboratories. Such protocols have been described in detail elsewhere[37,40]. As mentioned above, we switched to performing only pronuclear injection after we switched to ctRNP components. On the basis of the efficiencies we have observed using the pronuclear-only injection of ctRNP components, we suggest that pronuclear injection only would be sufficient for the *Easi*-CRISPR method.
! CAUTION Experimental procedures involving animals should be carried out according to relevant institutional and governmental regulations.

41| House mice in individually ventilated cages (IVCs) on a 12/12 light cycle (light on at 07:00, light off at 19:00).

42| Intraperitoneally inject each female donor mouse with 5.0 IU of PMSG around noon on day 1.

43| Approximately 48 h after the PMSG injection, intraperitoneally inject each female mouse with 5.0 IU of hCG on day 3 and breed with stud males overnight.

44| Check for plugged donor females the next morning on day 4 and euthanize plugged females.

45| Dissect oviducts and place the samples at 37 °C on a heated slide warmer.
▲ **CRITICAL STEP** Dissection of oviducts should be done as quickly as possible, ~10 min after euthanasia.

46| Quickly dissect cumulus–oocyte complexes by using no. 5 forceps to disrupt the ampulla and then pick oocytes from the dish by mouth pipetting.
▲ **CRITICAL STEP** Transfer the oocytes to the wash dish and wash a few times by mouth pipetting to remove cumulus complexes and hyaluronidase.

47| Record the number of zygotes and unfertilized zygotes, transfer healthy embryos to the KSOM dish, and culture at 5% CO_2 in an incubator at 37 °C until used for injection (up to ~1–2 h).
! CAUTION Zygotes should not be allowed to pass the one-cell stage because they will no longer be suitable for injection.

48| Prepare a slide using a Lab-Tek chamber by placing two side-by-side 150-μl drops of M2 medium. Flatten the drops into disks with a pipette tip to minimize their height. Overlay the drops with ~1 ml of mineral oil. Assemble the microinjection setup, including the micromanipulator system, holding pipettes, and microinjection needles containing the injection mix

prepared in Step 40. Place the slide on the microscope stage, mounted with a slide warmer adjusted to 37 °C, and transfer ~20–30 zygotes from Step 47 at a time to one of the drops of M2 medium for each injection session.
▲ CRITICAL STEP Maintain the temperature at 37 °C on the slide warmer. Two drops of M2 medium are placed on the slide so that the second drop can be used if the first drop is accidentally blown during mouth pipetting.

49| Perform pronuclear injection of zygotes from Step 47, maintaining positive pressure on the injection needle at all times, by inserting the needle into the zona pellucida and bringing the needle to the closest pronucleus.
▲ CRITICAL STEP Inject ~20–30 zygotes per batch. All zygotes, in each batch, must be injected within 10 min; thus, the number of zygotes taken per batch depends on the efficiency of the injector. A beginner may start with as few as 4–6 per batch; an experienced technician can inject as many as 50 zygotes in 10 min.

50| Penetrate the membrane of the pronucleus. A slight swelling of the pronucleus may be noticed once the membrane is penetrated. Otherwise, press the injection foot pedal to observe a slight swelling of the pronucleus.

51| Carefully withdraw the needle from the zygote.

52| After injecting all the zygotes, use the mouth-pipetting apparatus to collect and transfer zygotes to the embryo transfer dish.
▲ CRITICAL STEP Discard lysed zygotes.
? TROUBLESHOOTING

53| Incubate surviving zygotes at 37 °C in KSOM until embryo transfer (usually within the next 1–2 h).

54| *Transfer of injected embryos into pseudopregnant mice.* Obtain pseudopregnant mice by mating 8- to 12-week-old CD-1 females to vasectomized CD-1 males. On the morning of microinjection day, use plugged females for oviduct transfers.
▲ CRITICAL STEP Typically, 10–20 CD-1 females are bred in each session to obtain an average of four to eight plugged females.

55| Transfer viable embryos from Step 53 to the oviducts of pseudopregnant foster mothers from Step 54, following established surgical procedures as previously described[41]. Transfer ~15–25 injected zygotes per female. The optimal number of embryos transferred is 18 in total per female (nine per side). After the embryo transfer surgery, the animals are monitored until they recover from anesthesia, and are housed and cared for until they deliver offspring.
▲ CRITICAL STEP If an insufficient number of pseudopregnant females are available on the day of microinjection, the microinjected embryos can be cultured overnight and two-cell-stage embryos can be transferred the next day to newly generated pseudopregnant females.
? TROUBLESHOOTING

Genotyping of offspring to identify transgenic founders ● TIMING 1–3 d
▲ CRITICAL Samples from the offspring (tail or ear piece) are collected at a suitable age, following the institutional animal care and use committee–approved protocols, and genotyping of each offspring is done using multiple sets of PCRs.

56| *Mouse tail or ear DNA extraction.* Use option A, the longer protocol, to produce cleaner DNA, or option B, the rapid protocol, to produce crude DNA for quick genotyping. DNA preparations from both methods work well for genotyping PCRs.
! CAUTION Experimental procedures involving animals should be carried out according to relevant institutional and governmental regulations.

(A) Longer protocol ● TIMING 3 h

(i) Collect ~2- to 3-mm tail pieces from each offspring (generated from Step 55) in 1.5-ml microcentrifuge tubes. Collect a sample from a wild-type mouse as control. Add 300 µl of cell lysis solution containing 3 µl of proteinase K and incubate at 65 °C overnight in a heat block.
▲ CRITICAL STEP To save time and prevent reagent loss, make a master mix for the lysis solution and proteinase K. This can then be distributed in 300-µl aliquots in separate microcentrifuge tubes.

(ii) The next day, cool the samples to room temperature. Add 100 µl of protein precipitation solution and vortex thoroughly for ~20 s.

(iii) Place the tubes on ice for 10 min, and then centrifuge at 21,000*g* for 5 min at room temperature.

(iv) Transfer the supernatants to newly labeled microcentrifuge tubes containing 800 µl of 100% (vol/vol) ethanol. Mix by inverting the tubes eight to ten times.
▲ CRITICAL STEP Use ethanol-resistant markers for labeling the tubes because spills of ethanol solutions on the tubes can wash away the labels.
(v) Centrifuge at 21,000*g* for 5 min at room temperature.
(vi) Slowly discard the supernatant, add 700 µl of 70% (vol/vol) ethanol, and mix by inverting 8–10 times.
(vii) Centrifuge at 21,000*g* for 5 min at room temperature, and then slowly discard the supernatant.
(viii) Centrifuge at 21,000*g* for 4 min at room temperature.
(ix) Aspirate the remaining 70% (vol/vol) ethanol using a 200-µl pipette tip and air dry the DNA pellet for ~5 min (do not exceed 5 min).
! CAUTION It is important to change tips between samples to avoid cross-contamination.
▲ CRITICAL STEP The DNA pellet is very loose at this step; the aspiration step should be performed with care to avoid losing the pellet.
(x) Add 100 µl of DNA hydration solution to the pellet and flick the side of the tube to mix. Incubate the tubes at 65 °C in a dry bath for 15–30 min to solubilize the DNA.
■ PAUSE POINT Genomic DNA samples can be stored at 4 °C for ~2 months.

(B) Rapid protocol ● TIMING 4 h

(i) Collect ~2-mm ear pieces from each offspring (generated from Step 55) in 1.5-ml microcentrifuge tubes. Collect a sample from a wild-type mouse as control. Add 40 µl of Allele-In-One Mouse Tail Direct Lysis Buffer and incubate at 55 °C with shaking, using a shaking heat block, for >3 h, or overnight.
(ii) Incubate the tubes at 85 °C in a heat block for 45 min to inactivate the protease in the solution.
■ PAUSE POINT Genomic DNA samples can be stored at –20 °C for years.
(iii) Centrifuge at 21,000*g* for 2 min at room temperature (as the samples are crude) and use for PCR reactions.

57| Perform genotyping PCRs using the appropriate combinations of primer sets on all samples, including a DNA sample from wild-type mouse as a control. Prepare PCR reactions as follows, distribute the master mix, and add ~1 µl of mouse tail DNA (from Step 56), in a total volume of 10 µl per sample.
▲ CRITICAL STEP Genotyping of each offspring is done using multiple sets of PCR (see Experimental design section for further details): For knock-in alleles, both junction PCRs are performed, including amplification of all of the internal sequences, either as small overlapping segments or as one full-length PCR product. The genotyping of conditional alleles is performed by at least three independent PCR reactions, one short fragment for each *LoxP i*nsertion, and a full-length PCR to include both *LoxPs* in one amplicon. The genotyping of knockdown alleles is performed in a way similar to that for the knock-in alleles, which includes two-junction PCRs along with either a full-length or multiple small-amplicon PCRs to cover the full insertion cassette. Schematics, and examples of PCRs, are shown in **Figure 6**.

To amplify short PCR fragments (**Fig. 6a**,**b** 1&2, 3&4, 7&8, 9&10, 12&11):

Reagent	Volume per reaction	Volume for ten reactions	Final concentration
2× GC buffer	5 µl	50 µl	1×
2.5 mM dNTPs	0.8 µl	8 µl	200 µM
primers	0.25 µl sense primer	2.5 µl sense primer	0.5 µM
(20 µM stock)	0.25 µl antisense primer	2.5 µl antisense primer	0.5 µM
Enzyme (TaKaRa Taq: 5 U/µl)	0.025 µl	0.25 µl	0.125 U/10 µl
Water	2.675 µl	26.75 µl	
Genomic DNA	1 µl		–~100 ng
Total	10 µl		

The following PCR mixtures are suggested for the long PCR reactions shown in (**Fig. 6a**,**b** 5&4, 1&6, 7&11, 12&10):

Reagent	Volume per reaction	Volume for ten reactions	Final concentration
5× PrimeSTAR buffer	2 µl	20 µl	1×
2.5 mM dNTPs	0.8 µl	8 µl	200 µM
primers	0.12 µl sense primer	1.2 µl sense primer	0.24 µM
(20 µM stock)	0.12 µl antisense primer	1.2 µl antisense primer	0.24 µM
Enzyme (PrimeSTAR HS DNA Polymerase: 2.5 U/µl)	0.1 µl	1 µl	0.25 U/10 µl
Water	5.86 µl	58.6 µl	
Genomic DNA	1 µl		~100 ng
Total	10 µl		

58| Run the PCR reactions in a thermocycler using the following standard PCR conditions:

The following cycling conditions are suggested for PCR amplification with TaKaRa Taq polymerase. These conditions were used for the generation of short PCR fragments (**Fig. 6a**,**b** 1&2, 3&4, 7&8, 9&10, 12&11):

Cycle number	Denature	Anneal	Extend
1	95 °C for 5 min		
2–36	95 °C for 45 s	58 °C for 30 s	72 °C for 1 min
37			72 °C for 5 min
Hold at 4 °C			

The following cycling conditions are suggested for PCR amplification with PrimeSTAR HS DNA polymerase. These conditions were used for the generation of the long PCR fragments shown in **Fig. 6a**,**b** 5&4, 1&6, 7&11, 12&10):

Cycle number	Denature	Anneal	Extend
1	94 °C for 3 min		
2–36	98 °C for 10 s	62 °C for 5 s	72 °C for 1 min/kb
37			72 °C for 10 min
Hold at 4 °C			

59| After adding gel loading dye to the samples, run PCR products on a 1% (wt/vol) agarose gel electrophoresis system, along with control PCRs and DNA markers (GeneRuler DNA Ladder Mix) at 135 V for 30–60 min. Stain the gel with ethidium bromide. Analyze gels using a gel documentation system.

! CAUTION Ethidium bromide is carcinogenic and mutagenic. Wear personal protective equipment.

? TROUBLESHOOTING

60| Sequence PCR products directly, or clone PCR products with a TA Cloning Kit by following standard DNA-cloning protocols[3] and sequence them to confirm the genotypes.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
18	Poor RNA yield	Possible batch-to-batch variation among RNA synthesis kits	Some kits, such as the T7 RiboMAX Express and NEB HiScribe kits, have yielded more consistent results than others
			Incubating a reaction for longer than 3 h improves the RNA yield
			Reaction can be scaled up to obtain more RNA
		T7 promoter-driven transcription is not adequate	Including two Gs (GG) in the T7 promoter sequence enhances transcription
	Synthesis of shorter and stronger RNA bands in addition to an expected-size band	Presence of trace amounts of primer dimers (that contain T7 promoter), in the dsDNA template	Gel purification of the dsDNA template will reduce this problem
		Presence of cryptic terminator sequences for RNA polymerase	Synthesizing the opposite strand can eliminate problems arising from the unknown terminators
23	RNA degradation	Contamination of RNase	Ensure that the reagents and equipment are RNase-free
34	Poor cDNA synthesis yield	Degraded RNA template (RNA can become degraded very quickly)	Make aliquots of RNA and store them at –80 °C for long-term use
		Less template was used to start the reaction	Reaction can be scaled up to increase the yield (**Supplementary Fig. 3**)
	ssDNA appears like a smear in the gel	Smear-like appearance of ssDNA preparations is normal, especially when larger a quantity of preparation is loaded into the gel	Prominent band(s) can be gel-extracted (**Supplementary Fig. 4**)
		There will be detectable prominent band(s) present in the smear, corresponding to the expected size of the ssDNA of interest	
		If there are prominent shorter-sized bands, they could be arising from partial-length strands (from premature reverse transcription termination)	Synthesizing the opposite strand can eliminate the problems arising from the unknown terminators
52	Embryo lysis during microinjection	Clogging of injection needles	Centrifugation (e.g., 21,000*g*, 2 min, room temperature) and filtration of microinjection mix can prevent clogging of needles
55	Loss of embryos and low birth rate	Some component in the injection mix may be toxic	Rule out toxicity of each component by injecting the mix without one component in a few zygotes (~20) and assess the toxicity by culturing them up to blastocyst stage
			Prepare a fresh batch of ssDNA, and purchase a new batch of crRNA/tracrRNA if the toxicity persists
		Some unknown animal husbandry factor/s affecting birth rates	Examine whether any changes in general animal husbandry practices need to be made

(continued)

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
59	Insertion frequency of ssDNA is low	Inefficient annealing of crRNA and tracrRNA, and ctRNP complexes are not formed efficiently	Anneal in the thermocycler and incubate at room temperature with Cas9 protein for 15 min to form RNP complex
		Degraded ssDNA donor	Check the intactness of ssDNA in agarose gel electrophoresis and prepare a new batch if necessary
		Errors in guide RNA and ssDNA donor designs, or poor cleaving efficiency of the chosen guide(s)	Revisit the designs to check for flaws Change the guides if your design allows
60	Mutations in the insertion cassette	Misincorporations arising from PCR and/or reverse transcription reactions	Use high-fidelity enzymes for ssDNA preparation steps
		Inaccurate repair	Additional founder pups may be created and sequence may be verified

● TIMING

A typical *Easi*-CRISPR mouse genome-engineering project can be completed in ~2 months from the time the CRISPR strategy is designed to identification of the genome-edited G0 founder mice. The general time frame required for different stages of *Easi*-CRISPR is outlined in **Figure 1**. Even though designing the overall strategy (first stage) can take only ~2–3 h for an experienced person, this is the most critical part of the *Easi*-CRISPR protocol. Even slight errors in the donor design can result in a mouse model that may not be exactly as intended. We recommend that two or three independent persons verify the targeting strategy, particularly the donor design. One of these people should preferably be an expert with previous experience in designing genetically engineered models. The next critical step is the preparation of ssDNA donors, which requires the most hands-on time of the *Easi*-CRISPR protocol.

Steps 1–3, preparation of dsDNA template: 5 h
Steps 4–6, RNA synthesis using T7 RiboMax Express: 1–4 h
Steps 7–18, purification of RNA using a MEGAclear Kit: 1 h
Steps 19–23, synthesis of cDNA from RNA: 1.5 h
Steps 24–28, ethanol precipitation of cDNA and gel purification: 1.5 h
Step 28A, using a NucleoSpin Gel and PCR Clean-up kit (column purification): 20 min
Step 28B, phenol extraction and ethanol precipitation: 50 min
Steps 29–35, filter purification of cDNA solution: 1.5 h
Step 36, preparation of guide RNA by annealing crRNA and tracrRNA: 30 min
Steps 37–40, preparation of ctRNP + ssDNA injection mix: 1 h
Steps 41–55, mouse transgenesis: 5–6 h
Steps 56–60, genotyping of offspring to identify transgenic founders: 1–3 d

ANTICIPATED RESULTS

Easi-CRISPR has the potential to generate various kinds of mouse models, including conditional knockout mice (floxed), knock-in mice (to fuse protein expression cassettes with genes), and knockdown mice (artificial microRNA knock-in mice). This method invariably generates the intended mouse model by injecting ~50–100 zygotes. This method has been successful for >14 loci thus far, and the experiments were performed in at least three independent laboratories. The anticipated results for each of the major experimental stages are described below.

Designing guide RNAs

In some cases, we have chosen guides up to ~15 bases away from the insertion site, but have noticed imprecise insertions in some founders (deletion of the 15 nucleotides)[38]. If the chosen guide is farther from the desired insertion site, more founders may need to be generated to obtain correctly inserted founders.

Preparation of ssDNA

This involves three stages: preparation of dsDNA templates, preparation of RNA from dsDNA, and preparation of ssDNA from RNA. The anticipated results in each of these steps are detailed below.

Preparation of dsDNA templates

This stage (either using a plasmid source or a PCR product) invariably yields more than several micrograms of DNA.

Preparation of RNA from dsDNA.

Typical yields range between 5 and 140 µg of RNA but are dependent on the kits used. Even though ~5 µg of RNA is sufficient for the next step, in our experience, a total yield of about ~30 µg indicates an optimal quantity for an *in vitro* transcription reaction of 20-µl volume. A sample gel image depicting variable results is shown in **Supplementary Figure 1**.

Preparation of ssDNA from RNA.

Typical yields, after the final step of gel purification, range from 0.2 to 2 µg of cDNA, depending on kits and scale of synthesis used. A sample gel showing comparable performance of three different reverse transcription reactions is shown in **Supplementary Figure 2**. The effects of the total amount of input RNA and incubation periods on the total yield of cDNA are shown in **Supplementary Figure 3**. These data indicate that ~5 µg of input RNA and an at least 10 min (per the manufacturer's recommendations) or longer incubation is necessary to obtain optimal ssDNA yields. Note that gel extraction of cDNA is a critical step to remove partially synthesized ssDNAs, and that the presence of incompletely synthesized ssDNA molecules can hamper insertion of full-length molecules. In this step, we load higher amounts of cDNA preparations to reduce the gel volume. When higher amounts are loaded, we often see a smear with one prominent band and another less prominent band migrating just above the prominent band. This is shown in **Supplementary Figure 4**, in which the prominent band and the slower-migrating band are marked as 1 and 2, respectively. Purification of the two bands and running them at lower concentrations reveal that both bands migrate at the same rate (**Supplementary Fig. 4**). On the basis of this experiment, we presume that a differential migration of molecules can occur when loaded at a higher concentration; one reason could be that some molecules may migrate at different rates due to their secondary structures. Despite the fact that the majority of ssDNA is lost during purification steps, the amount obtained at the end would be sufficient for at least two to three sessions of microinjections, given that 50–200 ng of ssDNA is used for one injection session. Finally, the ssDNA preparation can be tested by subjecting an aliquot to S1 nuclease digestion, which digests it completely if the preparation contains only ssDNA (**Supplementary Fig. 5**).

Microinjection and transgenesis

Similar to transgenic microinjection experiments, overall birth rates vary from 10 to 30% among *Easi*-CRISPR experiments. However, insertion frequencies among live-born offspring are quite high in *Easi*-CRISPR, with the majority of projects completed by injecting ~50, or even fewer, zygotes.

Genotyping

In general, genotyping of CRISPR-generated animal models is very challenging, given the potential for mosaicism and for many possible genome-editing outcomes, including NHEJ-indels and large deletions or insertions. Identification of desired insertion alleles among such mixtures is certainly a difficult task. To ensure identification of correctly targeted alleles, we perform at least three independent sets of PCR (**Fig. 6**), followed by full sequencing of the targeted alleles. We have noticed higher rates of imprecise or partial insertions in some loci more than others[8], which could be attributed to the mechanism of ssDNA donor-mediated DNA repair and is not yet fully understood. Furthermore, some loci may be challenging for PCR genotyping and may require testing of many primers, until some are found that work. It should be noted that, even though a desired allele is detected by PCR(s), the CRISPR-generated alleles may not be transmitted to the next generation. In addition, the germ line–transmitted alleles must be reconfirmed by all sets of genotyping primers and sequencing of the PCR products to ensure that the correct allele is transmitted and established in the next generation. Genotyping by southern blotting can additionally ensure the correctness of the targeted allele. Inclusion of suitable restriction enzyme sites within the knock-in cassette can be useful for southern blot-based genotyping.

Overall efficiency of *Easi*-CRISPR protocol

Experiments to target long ssDNA cassettes at 14 independent loci have been reported[9,38]. The results from these studies indicate that *Easi*-CRISPR is a robust and highly efficient method for creating knock-in and conditional knockout animal models. Among the 97 live-born animals generated by injecting 680 zygotes, 38 contained the correctly inserted cassettes. The overall targeting efficiency, for any given locus, ranged from 8.5% to 100% of live-born animals, most commonly showing a frequency of 30–60%[9,38]. The cleaving efficiency of the guides could be one of the major factors affecting insertion frequencies, and thus targeting efficiencies for some loci can be more efficient than others.

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

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AUTHOR CONTRIBUTIONS M.O. conceived the idea of using long ssDNAs as donors in genome editing, which was further tested and improved upon by the other three authors. All authors contributed equally in writing the manuscript.

COMPETING FINANCIAL INTERESTS The authors declare competing financial interests: details are available in the online version of the paper.

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EDITORIAL SUMMARY

AOP: This protocol describes *Easi*-CRISPR, a method for creating knock-in, conditional knockout, and knockdown mouse models by CRISPR/Cas9-based genome engineering using long single-stranded DNA donor templates.

COMPETING FINANCIAL INTERESTS

C.B.G., M.O., and H.M. have filed a patent application relating to the work described in this paper with international application number PCT/US2016/035660, filed June 3, 2016 (DNA editing using single-stranded DNA).