Cloning-free CRISPR/Cas system facilitates functional cassette knock-in in mice

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Abstract

Although the CRISPR/Cas system has enabled one-step generation of knockout mice, low success rates of cassette knock-in limit its application range. Here we show that cloning-free, direct nuclear delivery of Cas9 protein complex with chemically synthesized dual RNAs enables highly efficient target digestion, leading to generation of knock-in mice carrying a functional cassette with up to 50% efficiency, compared with just 10% by a commonly used method consisting of Cas9 mRNA and single guide RNA. Our cloning-free CRISPR/Cas system facilitates rapid one-step generation of cassette knock-in mice, accelerating functional genomic research by providing various in vivo genetic tools.

Background

Although gene-targeted knockout and knock-in mice are invaluable tools for understanding the functions of genes in vivo, the production of such genetically modified mice has relied on gene targeting in embryonic stem cells, which is a complicated and time-consuming process [1]. The recent development of the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) system, a genome editing technology, has allowed for the direct manipulation of the genome in mouse zygotes in vivo (in vivo genome editing) with extremely high efficiency, enabling the highly convenient and ultra-rapid one-step generation of genetically modified mice without embryonic stem cells [2,3].

A flood of studies using CRISPR/Cas-mediated in vivo genome editing have reported the production of knockout mice [4-6] and knock-in mice carrying single nucleotide substitutions combined with oligo DNA donors [5,7,8]. In contrast, there has been only one report on the successful production of knock-in mice carrying reporter gene cassettes [9], essential tools for analyzing complex tissues such as brain in vivo [10], and the efficacy of the targeted insertion of the reporter gene was only about 10% [2,3,9,11]. The low success rates of gene cassette knock-in limit the applicability of CRISPR/Cas-mediated in vivo genome editing.

The CRISPR/Cas system was initially reported as an adaptive immune system in bacteria, consisting of three components including Cas9 nuclease and two small RNAs, CRISPR RNA (crRNA), which guides the Cas9 complex to the target sequence, and trans-activating crRNA (tracrRNA), which binds to crRNA and forms a ribonucleoprotein complex with Cas9 nuclease [12]. When it was harnessed as a genome editing tool [13,14], the dual-crRNA:tracrRNA was engineered as a chimeric single guide RNA (sgRNA) [13]. The CRISPR/Cas system consisting of two components - Cas9 nuclease and sgRNA - is the most common approach in the field of genome editing due to its enhanced convenience and robust targeting [15]. However, it is still unknown whether the commonly used sgRNA works more efficiently than the dual-crRNA:tracrRNA, especially for the production of knock-in mice carrying reporter gene cassettes.

Here, we show the highly efficient generation of knock-in mice carrying a functional gene cassette by a cloning-free CRISPR/Cas system using Cas9 protein combined with chemically synthesized dual-crRNA:tracrRNA.
Results
Generation of highly active guide sequence
In a previous study, we demonstrated that the insertion of a transgene downstream of the Actb polyadenylation signal allowed for sufficiently high levels of gene induction [16,17]. Thus, we chose the Actb locus as a model to be targeted for the generation of knock-in mice carrying a functional gene cassette. We first designed the guide sequence targeted to the locus 800 bp downstream of the mouse Actb polyA signal (Figure 1a) and inserted it into a bi-cistronic expression vector pX330 plasmid [18,19] containing sequences encoding Cas9 and sgRNA backbone sequences. Then, we determined its high activity of DNA digestion in vitro using a single-strand annealing (SSA) assay with episomal plasmid vectors containing a split luciferase gene and Actb target sequences in human HEK293T cell lines (Figure S1 in Additional file 1), and a Cel-I assay in mouse Neuro2A cell lines to target the endogenous mouse chromosome (Figure S2 in Additional file 1).

To further test the activity of Actb sgRNA in vivo, we injected Cas9 mRNA with Actb sgRNA - both in vitro transcribed with T7 RNA polymerase using PCR templates amplified from an Actb pX330 plasmid [11] - into one-cell stage mouse zygotes. We obtained 12 newborn mice and found that almost all the newborns...
were bi-allelically targeted mutants (Figure S3 and Table S1 in Additional file 1). Thus, the Actb sgRNA we designed is highly active both in vitro and in vivo for target digestion and subsequent induction of non-homologous end-joining (NHEJ).

**Generation of reporter knock-in mice by sgRNA combined with Cas9 mRNA**

As a model for knock-in mice carrying a functional gene cassette, we designed reporter mice highly expressing enhanced green fluorescent protein (EGFP) from the endogenous Actb locus only in a specific cell population intersectionally defined [20] by the expression of Cre recombinase and tetracycline transactivator (tTA). We constructed a 10.5 kb targeting vector for the mouse Actb locus containing a 2.5 kb TetO-FLEX-EGFP-polyA cassette (Tet operator (tetO) sequence concatamers fused to a minimal cytomegalovirus (CMV) promoter, beta-globin intron, inverted EGFP flanked by two pairs of loxP and lox2722 (FLEX switch) and polyA), and 2.0 kb left and right homology arms of the Actb locus (Actb-TetO-FLEX-EGFP-polyA; Figure 1a).

Next, we injected the circular Actb-TetO-FLEX-EGFP-polyA targeting vector together with Actb sgRNA and Cas9 mRNA, both in vitro transcribed as above, into one-cell-stage mouse zygotes (Figure 1b). We injected the mixture (5 ng/μl Cas9 mRNA, 2.5 ng/μl Actb sgRNA, and 10 ng/μl of the targeting vector) into pronuclei as previously described [9]. We obtained eight newborn mice and screened them by PCR with three different primer pairs (Figure 1a) and tail genomic DNA to detect homologous recombination (HR). We found one correctly targeted knock-in mouse carrying a TetO-FLEX-EGFP-polyA cassette at the Actb locus (Figure 1c), whereas the endogenous Actb loci were still targeted by NHEJ in all the newborn mice (Table 1; and Figure S4 in Additional file 1). The efficiency of the targeted insertion of the transgene by pronuclear injection was 12.5%, consistent with the previous report (Table 1) [9]. Next, we sequenced cloned PCR products of the left and right boundaries between Actb and the TetO-FLEX-EGFP-polyA cassette and found the precise knock-in of the cassette, as we designed (Figure 1d). These results confirm that the knock-in mouse carrying a functional gene cassette can be generated by CRISPR/Cas-mediated in vivo genome editing, although its efficiency is low even when using highly active sgRNA for NHEJ.

**Generation of reporter knock-in mice by sgRNA combined with Cas9 protein**

To digest target genomic DNA, Cas9 mRNA injected into pronuclei of mouse zygotes must be exported to the cytoplasm, translated into protein, and imported into the nucleus again. This long process [21] might delay target digestion, leading to a low rate of HR. Consistent with this idea, a recent in vitro cellular study reported that the target genomic DNA was almost immediately digested by the direct delivery of a Cas9 protein-sgRNA complex compared with the delivery of plasmid DNA expressing Cas9 and sgRNA [22]. Further, an in vivo study in mice and zebrafish revealed that the direct delivery of a Cas9 protein-sgRNA complex into embryos led to highly efficient generation of knockout animals [23]. Thus, we hypothesized that direct delivery of a Cas9 protein-RNA complex into the pronuclei of mouse zygotes and subsequent rapid digestion of target genomic DNA might improve the efficiency of knock-in mouse generation.

First, we injected a mixture consisting of 30 or 100 ng/μl Cas9 protein, 2.5 ng/μl Actb sgRNA, and 10 ng/μl targeting vector into the pronuclei of zygotes (Figure 2a). We obtained 39 newborn mice, although none carried the TetO-FLEX-EGFP-polyA cassette at the Actb locus (Figure S5 and Table S2 in Additional file 1). Next, we injected a mixture containing a higher dose of sgRNA (30 or 100 ng/μl Cas9 protein, 25 ng/μl Actb sgRNA, and 10 ng/μl targeting vector) into the pronuclei of zygotes. We obtained 19 newborn mice, but again none carried the TetO-FLEX-EGFP-polyA cassette at the Actb locus (Figure 2b,c; Table S2 in Additional file 1). We also found that the endogenous Actb locus was targeted by NHEJ in only one mouse (Figure S6 and Table S2 in Additional file 1). Next, we injected a mixture containing a higher dose of sgRNA (30 or 100 ng/μl Cas9 protein, 25 ng/μl Actb sgRNA, and 10 ng/μl targeting vector) into the pronuclei of zygotes. We obtained 58 newborn mice by direct pronuclear delivery of a Cas9 protein-sgRNA complex, we unexpectedly did not obtain any knock-in mice carrying a functional gene cassette (Table 1).

| Table 1 Generation of reporter knock-in mice by Cas9 mRNA or protein combined with sgRNA or crRNA/tracrRNA |
|-----------------------------------------------|-----------------|----------------|----------------|----------------|------------------|
| Cas9 | Guide RNA | Injected | Transferred (%) | Newborn (%) | Targeted (%) | Knock-in (%) |
|-------|-----------|----------|----------------|--------------|--------------|---------------|
| mRNA  | sgRNA    | 86       | 58 (67.4)      | 8 (13.8)     | 8 (100)      | 1 (12.5)      |
| Protein | sgRNA   | 135      | 112 (83.0)     | 19 (17.0)    | 5 (26.3)     | 0 (0)         |
| Protein | crRNA and tracrRNA | 107 | 65 (60.7) | 11 (16.9) | 9 (81.8) | 5 (45.5) |

Percentages were calculated using the number in each column as the numerator and the number in the column to its left as the denominator except for Knock-in. Percentages of Knock-in were calculated using the number in the Knock-in column as the numerator and the number in the Newborn column as the denominator. Note that the results of protein injections with high concentrations of guide RNAs are shown. All results are shown in Tables S2 and S3 in Additional file 1.
Cloning-free CRISPR/Cas system

To explore highly efficient knock-in systems based on the Cas9 protein, we tested the initial form of the CRISPR/Cas system consisting of three components including Cas9, crRNA and tracrRNA (dual RNAs). The short lengths of crRNA and tracrRNA are appropriate for their chemical synthesis, enabling a cloning-free CRISPR/Cas system when combined with Cas9 protein. Taking advantage of the cloning-free CRISPR/Cas system, we tried to simplify the evaluation process of CRISPR activity generally performed in cultured cells. We developed a cell-free in vitro digestion assay (IDA) system [13] using the target Actb PCR product, Cas9 protein, and chemically synthesized dual RNAs (Figure 3a). We chemically synthesized Actb crRNA containing identical 20 nucleotide guide sequences of Actb sgRNA and tracrRNA. We tested the target digestion activities of various concentrations of chemically synthesized dual RNAs combined with Cas9 protein by IDA. We found that chemically synthesized dual RNAs efficiently digested target DNA in a dose-dependent manner with Cas9 protein (Figure 3b). We then compared the target digestion activities of chemically synthesized dual RNAs with in vitro transcribed Actb sgRNA. We found that both the sgRNA and dual RNAs combined with Cas9 protein digested the target Actb PCR product with efficiencies of over 95% (Figure 3c). These results suggest that the digestion activity of CRISPR/Cas can be quickly and conveniently evaluated using IDA without cultured cells [13], and chemically synthesized RNAs can be used instead of in vitro transcribed sgRNA, enabling a cloning-free CRISPR/Cas system.

Highly efficient generation of reporter knock-in mice by dual-crRNA:tracrRNA combined with Cas9 protein

Next, we tested whether the knock-in efficiency was improved by the cloning-free CRISPR/Cas system. We first injected a mixture of 30 ng/μl Cas9 protein, 0.061 pmol/μl Actb crRNA, 0.061 pmol/μl tracrRNA, and 10 ng/μl targeting vector into the pronuclei of zygotes (Figure 4a). The molar concentrations of dual RNAs were equivalent.
to that of sgRNA in Figures 1 and 2. We obtained nine newborn mice, but none carried the TetO-FLEX-EGFP-polyA cassette at the Actb locus (Figure S8 and Table S3 in Additional file 1), consistent with the low digest activity at this concentration shown by IDA in vitro (Figure 3b), and the endogenous Actb loci were targeted by NHEJ in three out of eight mice (Figure S9 and Table S3 in Additional file 1). Next, we injected a mixture containing a higher dose of the dual RNAs (30 ng/μl Cas9 protein, 0.61 pmol/μl Actb crRNA, 0.61 pmol/μl tracrRNA, and 10 ng/μl targeting vector) into the pronuclei of zygotes. Surprisingly, we found 5 correctly targeted knock-in mice among 11 newborn mice (Figure 4b). The efficiency of the targeted insertion of the transgene by Cas9 protein injection combined with a higher dose of chemically synthesized crRNA and tracrRNA was 45.5% (Table 1). We further confirmed genotypes by Southern blotting with genomic DNA of knock-in newborns (Figure 4c). We also found the endogenous Actb loci were targeted by NHEJ in four out of six non-knock-in mice (Table 1; Figure S10 and Table S3 in Additional file 1). These results suggest that non-knock-in alleles were damaged in about half of the knock-in mice generated by Cas9 protein combined with dual RNAs.

We further investigated off-target cleavage in the knock-in mice, which is the most serious problem [24-27] associated with CRISPR/Cas-mediated genome editing. We chose 14 off-target candidate loci (OT1-14) containing up to 3 bp mismatches compared with the 20 bp guide sequence of Actb crRNA [18,25]. Among 13 off-target candidate loci (OT6 could not be amplified by PCR) in 6 knock-in mice, we did not find any sign of off-target digestion (Figure S12 and Table S4 in Additional file 1). These results suggest that in vivo genome editing by Cas9 protein combined with dual RNAs is highly specific for the on-target locus.

Finally, we crossed F0 knock-in mice with wild-type mice to investigate germline transmission of the knock-in alleles to the F1 generation. We tested four knock-in lines using PCR and Southern blotting and found that all showed successful germline transmission in F1 progeny with an average efficiency of 51.4% (ranging from 37.5% to 61.1% between lines) (Figures S13 and S14, and...
Table S5 in Additional file 1). These results suggest that the percentage of mosaicism in F0 knock-in mice was very low.

**Functionality of the reporter cassette inserted at the Actb locus**

Finally, we tested whether the TetO-FLEX-EGFP-polyA cassette inserted into the mouse Actb locus is effective. We transfected Cre-, tTA-, and DsRed-expressing plasmids into primary mouse fibroblasts derived from ear tips of three F0 knock-in and control littermates (Figure 5a). We found strong EGFP fluorescence only in fibroblasts derived from knock-in mice (Figure 5b). These results suggest that functional EGFP proteins are produced from the TetO-FLEX-EGFP-polyA cassette inserted into the endogenous Actb locus under the presence of Cre and tTA.

**Discussion**

In this study, we developed a cloning-free CRISPR/Cas-mediated genome editing system for highly efficient and convenient one-step generation of knock-in mice carrying a functional gene cassette. This system has several advantages. First, the CRISPR/Cas vector construction and in vitro RNA transcription can be omitted by using commercially available Cas9 protein and chemically synthesized crRNA and tracrRNA, leading to a cloning-free CRISPR/Cas system. Although chemical synthesis of sgRNA might also be possible and convenient, technical
limitations for the synthesis of long sgRNAs (more than 100 mer) must be considered. In contrast, shorter crRNAs and tracrRNAs can be chemically synthesized easily in a cost-effective manner. Furthermore, tracrRNAs can be commonly used independently of target sequences as well as Cas9 protein. The targeting vectors are already chemically synthesizable. Second, the efficiency of CRISPR/Cas-mediated digestion can be evaluated with a cell-free IDA system using target Actb PCR product, Cas9 protein, and chemically synthesized crRNA and tracrRNA, instead of cellular SSA or Cel-1 assays. Third, and most importantly, the direct delivery of Cas9 protein, chemically synthesized crRNA and tracrRNA, and targeting vector into the pronuclei of zygotes allowed for the highly efficient generation of knock-in mice carrying gene cassettes in the endogenous gene. Since we could not obtain any knock-in mice by the direct delivery of Cas9 protein, sgRNA, and targeting vector into the pronuclei of zygotes, the high knock-in efficiency of the cloning-free CRISPR/Cas system was mediated by use of crRNA and tracrRNA, not conventional sgRNA. Although both sgRNA and dual RNAs combined with Cas9 protein efficiently digested the target Actb PCR product in a biochemical IDA (Figure 3c), the enzymatic reaction conditions were completely different from those of zygote injection, leading to discrepancy in NHEJ and HR efficiencies in vivo. Consistent with this idea, although the sgRNA co-injected with Cas9 mRNA showed high NHEJ efficiency regardless of targeting vector (Table 1; Table S1 in Additional file 1), the sgRNA combined with Cas9 protein showed much less NHEJ efficiency (Table 1; Table S2 in Additional file 1), suggesting non-optimal assembly of Cas9 protein and sgRNA to form a highly active ribonucleoprotein complex. Taken together, the cloning-free CRISPR/Cas system facilitates functional cassette knock-in into the mouse chromosome.

In contrast to the highly efficient (almost 100%) generation of NHEJ-mediated knockout mice by the injection of Actb sgRNA and Cas9 mRNA, we demonstrated much lower efficiency of HR-mediated generation of knock-in mice by the injection of these RNAs combined with targeting vector. One possible explanation for this is the difference in the injection method. For knockout mice production, we injected Actb sgRNA and Cas9 mRNA into the cytoplasm, which presumably led to fast translation of Cas9 mRNA into protein, as described previously [5,6]. For knock-in mice production, because pronuclear injection [5,7-9] is a standard method for delivering RNA and targeting vector into one-cell-stage mouse zygotes, we injected the mixture of Actb sgRNA, Cas9 mRNA and targeting plasmid vector into the pronuclei, which might lead to delays in the translation of Cas9 mRNA into protein.

Rapid digestion of target genomic DNA within one-cell zygotes may also be critical for highly efficient induction of HR. In a previous cellular study, it was shown that the target genomic DNA was almost immediately digested by the direct delivery of the Cas9 protein-RNA complex [22]. Further, highly efficient generation of knockout mice by the direct delivery of the Cas9 protein-RNA complex was reported [23]. Thus, direct injection of the Cas9 protein-RNA complex into pronuclei may result in immediate digestion of the target endogenous locus of mouse zygotes at the early one-cell stage, leading to highly efficient knock-in of the functional cassette. Consistent with this view, knock-in alleles in four knock-in mice generated by injection of a Cas9 protein-dual RNAs complex were successfully transmitted to F1 progeny with about 50% efficiency.

It was also reported that a Cas9 protein-RNA complex was rapidly degraded in cultured cells, thus reducing undesired off-target effects [22,28], the most serious problem [24-27] associated with CRISPR/Cas-mediated genome editing. In contrast to high off-target effects in cultured cell lines [24-27], its frequency is thought to be relatively rare in embryonic stem cells and mouse embryos [9]. Our off-target analysis in several candidate loci in knock-in mice confirmed the high accuracy of the CRISPR/Cas
system with the Cas9 protein-dual RNAs complex in mouse embryos. Although non-biased genome-wide off-target analysis [29-31] or whole-genome sequencing [32-34] will be required, use of the Cas9 protein-dual RNAs complex is a promising approach for highly specific genome editing. Thus, our cloning-free CRISPR/Cas-mediated in vivo genome editing system provides highly efficient and highly accurate generation of knock-in mice carrying functional cassettes.

The insertion of several hundred nucleotides is often found at the targeted loci when using genome editing, although the origin of the inserted sequence is unknown [35]. One possible source of the insertion is the transcribed RNA. Recent studies suggest that DNA double-strand breaks (DSBs) are repaired by using the DNA that is reverse-transcribed from mRNA in mammalian cells [36], yeast [37], and fly [38]. We found trans-insertion of Trim33 mRNA into the Actb locus in our knock-in mouse, providing direct evidence of transcript RNA-templated DSB repair in mammalian organisms in vivo. Transcript RNA-templated repair of DNA DSBs is mutagenic and polymorphic in the human genome [36], suggesting that it may play an important role in human genetic diseases and evolution. Thus, our results shed light on transcript RNA-templated DNA DSB repair in mammalian organisms in vivo and stimulate functional research on this.

In addition to the generation of knock-in mice carrying complex gene cassettes, our method can be directly applied to the generation of knockout mice [23] and knock-in mice carrying single nucleotide substitutions with oligo DNA donors [5,7,8], as well as to other species [39-41] and cultured cells [22,28], and in vivo genome editing in adult animals [42]. Taken together, our streamlined cloning-free CRISPR/Cas-mediated in vivo genome editing system enables the highly efficient and extremely convenient one-step generation of knock-in mice carrying functional gene cassettes.

Conclusions
Cas9 protein and chemically synthesized crRNA and tracrRNA enabled cloning-free CRISPR/Cas system without CRISPR vector construction, cellular experiments for evaluation of the digestion activity of CRISPR/Cas, and in vitro RNA transcription. By the direct nuclear delivery of Cas9 protein complex combined with dual RNAs into one-cell mouse zygotes, knock-in mice carrying functional cassette were generated with extreme high efficiency, which could not be achieved by conventional mRNA pronuclear injection or Cas9 protein injection combined with commonly used sgRNA. Taken together, our streamlined cloning-free CRISPR/Cas-mediated in vivo genome editing system provides the highly efficient and extremely convenient one-step generation of knockout and knock-in animals, leading to acceleration of in vivo functional genomic research.

Materials and methods
Animal experiments
All research and animal care procedures were approved by the Tokyo Medical and Dental University Animal Care and Use Committee. Mice were housed in groups of three to five animals per cage and maintained on a regular 12 hours light/dark cycle (8:00 to 20:00 light period) at a constant 25°C. Food and water were available ad libitum.

CRISPR/Cas plasmid
A pair of oligo DNAs (Hokkaido Systems Science, Sapporo, Hokkaido, Japan) corresponding to Actb sgRNA was hybridized and ligated using Quick Ligase (New England Biolabs (NEB), Ipswich, MA, USA) into linearized pX330 plasmid (Addgene, 42230; Feng Zhang, MIT) digested with BbsI (NEB) as previously described [18,19]. Oligo DNAs and primers are listed in Table S6 in Additional file 1.

Targeting vector
The pActb-TetO-FLEX-EGFP-polyA targeting vector was constructed based on a pAAV-TetO-FLEX-HA-mKate2-TeNT-polyA plasmid (a gift from Akihiko Yamanaka, Nagoya University) with several modifications. First, HA-mKate2-TeNT was excised by digestion with XhoI (NEB) and HindIII (NEB), and replaced with PCR amplified inverted EGFP. Second, AAV2-ITR was excised by digestion with NarI (NEB) and HindIII (NEB), and replaced with a PCR-amplified 2.0 kb Actb fragment from C57BL/6 J mouse genomic DNA for left homology arm using a In-Fusion HD Cloning Kit (Takara, Otsu, Shiga, Japan). Finally, the PCR-amplified 2.0 kb Actb fragment for right homology arm was inserted into the plasmid digested with NotI (NEB) and MluI (NEB) by In-Fusion reaction.

Single-strand annealing assay
SSA assay using HEK293T cells was performed as described previously [43]. Briefly, Actb-pX330 or empty pX330 plasmids, firefly luciferase reporter vector containing the PCR-amplified Actb target sequence (Table S6 in Additional file 1), and renilla luciferase-expressing reference vector were co-transfected into HEK293T cells in a 96-well plate using Lipofectamine LTX (Life Technologies, Grand Island, NY, USA). At 24 hours post-transfection, luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer’s instructions.
Cel-I assay in Neuro2A cells

Cel-I assay using Mouse Neuro2A cells was performed as described previously [43]. Briefly, Actb-pX330 or empty pX330 plasmids was transfected into Neuro2A cells in a 6-well plate using Lipofectamine LTX (Life Technologies). After 72 hours post-transfection, genomic DNA was isolated using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). Then, Actb loci were PCR amplified from the purified genomic DNA with primers (Table S6 in Additional file 1). PCR products were denatured, digested at 42°C for 30 minutes with a Surveyor Mutation Detection Kit (Transgenomic, Omaha, NE, USA), and analyzed by electrophoresis in 3% agarose gel stained with ethidium bromide. Gel images were obtained with a ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA) and analyzed by Image Lab software (Bio-Rad).

In vitro RNA transcription

Cas9 mRNA and Actb-sgRNA were prepared as described previously [5]. Cas9 and Actb-sgRNA were PCR amplified from Actb-pX330 with T7 promoter-attached primers (Table S6 in Additional file 1). T7-Cas9 and T7-Actb-sgRNA PCR products were purified with a PCR Purification Kit (Qiagen, Valencia, CA, USA) and analyzed by sequencing. The quality of RNAs was analyzed using a NanoDrop 0.1 TE buffer to a working concentration of 30 or 100 ng/μl, or 0.061 or 0.61 pmol/μl, respectively, as previously described [5]. The mixture of Cas9 mRNAs and Actb-sgRNA was injected into the cytoplasm using a micromanipulator and microscope (Leica, Wetzlar, Germany) and injected into ICR female mice (CLEA Japan).

For knock-in mouse production by RNA injection, Cas9 mRNAs, Actb-sgRNA, and pActb-TetO-FLEX-EGFP-polyA were diluted and mixed in 0.1 TE buffer to a working concentration of 50 and 20 ng/μl, respectively. One-cell-stage zygotes were obtained by mating of BDF1 males and females (CLEA Japan, Meguro, Tokyo, Japan), and then frozen and stored until use. The mixture of Cas9 mRNAs and Actb-sgRNA was injected into the cytoplasm using a micromanipulator and microscope (Leica, Wetzlar, Germany) and injectors (Eppendorf, Hauppauge, NY, USA). After incubation at 37°C for 24 hours, two-cell-stage embryos were transferred into pseudopregnant ICR female mice (CLEA Japan).

In vitro digestion assay

Cas9 proteins (30 ng/μl) and chemically synthesized Actb-crRNA (8.7 ng/μl) and -tracrRNA (14.3 ng/μl), or in vitro transcribed Actb-sgRNA (25 ng/μl) were incubated with Actb target PCR products (30 ng/μl) in a Cas9 Nuclease Reaction Buffer (NEB) at 37°C for 60 minutes as previously described [13], then treated with RNase A (5 mg) and incubated at 37°C for 30 minutes to remove RNA [44]. Reactions were stopped with 6× DNA loading buffer containing 30% glycerol, 1.2% SDS and 250 mM EDTA, and analyzed by electrophoresis in 2% agarose gel as described above.

Injection

For knockout mouse production, Cas9 mRNAs and Actb-sgRNA were diluted and mixed in 0.1 TE buffer (10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0)) to a working concentration of 50 and 20 ng/μl, respectively. One-cell-stage zygotes were obtained by mating of BDF1 males and females (CLEA Japan, Meguro, Tokyo, Japan), and then frozen and stored until use. The mixture of Cas9 mRNAs and Actb-sgRNA was injected into the cytoplasm using a micromanipulator and microscope (Leica, Wetzlar, Germany) and injectors (Eppendorf, Hauppauge, NY, USA). After incubation at 37°C for 24 hours, two-cell-stage embryos were transferred into pseudopregnant ICR female mice (CLEA Japan).

For knock-in mouse production by RNA injection, Cas9 mRNAs, Actb-sgRNA, and pActb-TetO-FLEX-EGFP-polyA were diluted and mixed in 0.1 TE buffer to a working concentration of 5, 2.5, and 10 ng/μl, respectively, as previously described [5]. The mixture injection was injected into pronuclei of one-cell-stage zygotes.

Cas9 proteins

The recombinant Cas9 proteins were obtained from NEB and PNA Bio (Thousand Oaks, CA, USA).

Chemical synthesis of crRNA and tracrRNA

Actb-crRNA (5′-caauagagccauagugGUUUAGAC UAUGCUUUUAAG-3′) and -tracrRNA (5′- AACAG CAAUGCAAGUAAAAAGCUAGCUUUAUCACUUGAAAAAGUGGCACCCGAGUGCGUUCU-3′) were designed with some modification of previously reported methods [12,19], and chemically synthesized and purified by polyacrylamide gel electrophoresis (Fasmac, Atsugi, Kanagawa, Japan).

PCR screening

Genomic DNA was prepared from F0 and F1 newborn tails by proteinase K treatment and a subsequent standard phenol extraction method as described previously [45,46]. Knock-in mice were screened by PCR with ExTaQ (Takara) and three different pairs of primers (Table S6 in Additional file 1) and analyzed by electrophoresis in 1 or 2% agarose gel as described above. PCR products were further cloned with TOPO TA Cloning Kit (Life Technologies) and analyzed by sequencing.

Southern blotting

Southern probe (0.7 kb) was PCR amplified (Primers: Table S6 in Additional file 1) from BDF1 genomic DNA and cloned with a TOPO TA Cloning Kit, and DIG-labeled with a DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche, Penzberg, Upper Bavaria, Germany). The genomic DNA was digested with HindIII and separated on 0.7% agarose gel, transferred to nylon.
membranes, positively charged (Roche), hybridized with DIG-labeled probe, and detected with CDP-Star (Roche) as previously described [47].

Off-target effects
The potential off-target candidate loci containing up to 3 bp mismatches compared with the 20 bp guide sequence of Actb sgRNA were predicted by the CRISPR design tool [18,25,48]. The off-target candidate loci were amplified by PCR using primers listed in Table S6 in Additional file 1 and analyzed by direct sequencing as previously described [47].

Primary fibroblast cultures
The ear tips derived from 2-week-old mice were diced into small pieces, incubated at 37°C for 30 minutes with 4 mg/ml collagenase L (Nitta Gelatin, Naniwa, Osaka, Japan) and 4 mg/ml dispase (Life Technologies), and then cultured with 10% fetal bovine serum/Dulbecco’s modified eagle medium at 37°C and 10% CO₂ for several days. pCAG-Cre, pCMV-tTA (Takara), and pCMV-DsRed (Takara) were co-transfected into primary fibroblast cells in a six-well plate using Lipofectamine LTX Plus reagent (Life Technologies). Images were acquired on a FV500 confocal microscope and Fluoview software (Olympus, Shinjuku, Tokyo, Japan).

Statistical analyses
All data are presented as the mean ± standard error of the mean. Statistical methods were described in the figure legends for each data set. Briefly, Student’s t-tests were used to compare differences between any two groups. One-way ANOVA with post hoc Tukey-Kramer tests were used to compare differences between three groups. Statistical significance was set at P < 0.05.

Additional file
The following additional data are available with the online version of this paper.

Additional file 1: Cellular assays, generation of knockout and knock-in mice, sequencing of all the newborns and non-knock-in alleles, germline transmission, off-target analysis, and tables of these results and a list of the oligo DNAs and primers used in this study.

Abbreviations
bp: base pair; Cas: CRISPR-associated protein; CMV: cytomegalovirus; CRISPR: clustered regularly interspaced short palindromic repeat; crRNA: CRISPR-RNA; CSB: double-strand break; EGFP: enhanced green fluorescent protein; FLEX: FLEX switch flanked by two pairs of loxP and loxO2722; HR: homologous recombination; IDA: in vitro digestion assay; iTR: inverted terminal repeat; NEB: New England BioLabs; NHEJ: non-homologous end-joining; PCR: polymerase chain reaction; sgRNA: single guide RNA; SSA: single-strand annealing; tET: Tet operator; tracrRNA: trans-activating crRNA; tTA: tetracycline transactivator.
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