

One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering

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SUMMARY

Mice carrying mutations in multiple genes are traditionally generated by sequential recombination in embryonic stem cells and/or time-consuming intercrossing of mice with a single mutation. The CRISPR/Cas system has been adapted as an efficient gene-targeting technology with the potential for multiplexed genome editing. We demonstrate that CRISPR/Cas-mediated gene editing allows the simultaneous disruption of five genes (*Tet1*, *2*, *3*, *Sry*, *Uty* - 8 alleles) in mouse embryonic stem (ES) cells with high efficiency. Coinjection of Cas9 mRNA and single-guide RNAs (sgRNAs) targeting *Tet1* and *Tet2* into zygotes generated mice with biallelic mutations in both genes with an efficiency of 80%. Finally, we show that coinjection of Cas9 mRNA/sgRNAs with mutant oligos generated precise point mutations simultaneously in two target genes. Thus, the CRISPR/Cas system allows the one-step generation of animals carrying mutations in multiple genes, an approach that will greatly accelerate the *in vivo* study of functionally redundant genes and of epistatic gene interactions.

INTRODUCTION

Genetically modified mice represent a crucial tool for understanding gene function in development and disease. Mutant mice are conventionally generated by insertional mutagenesis (Copeland and Jenkins, 2010; Kool and Berns, 2009) or by gene-targeting methods (Capecchi, 2005). In conventional gene-targeting methods, mutations are introduced through homologous recombination in mouse embryonic stem (ES) cells. Targeted ES cells injected into wild-type (WT) blastocysts can

contribute to the germline of chimeric animals, generating mice containing the targeted gene modification (Capecchi, 2005). It is costly and time consuming to produce single-gene knockout mice and even more so to make double-mutant mice. Moreover, in most other mammalian species, no established ES cell lines are available that contribute efficiently to chimeric animals, which greatly limits the genetic studies in many species.

Alternative methods have been developed to accelerate the process of genome modification by directly injecting DNA or mRNA of site-specific nucleases into the one-cell embryo to generate DNA double-strand break (DSB) at a specified locus in various species (Bogdanove and Voytas, 2011; Carroll et al., 2008; Urnov et al., 2010). DSBs induced by these site-specific nucleases can then be repaired by error-prone nonhomologous end joining (NHEJ) resulting in mutant mice and rats carrying deletions or insertions at the cut site (Carbery et al., 2010; Geurts et al., 2009; Sung et al., 2013; Tesson et al., 2011). If a donor plasmid with homology to the ends flanking the DSB is coinjected, high-fidelity homologous recombination can produce animals with targeted integrations (Cui et al., 2011; Meyer et al., 2010). Because these methods require the complex designs of zinc finger nucleases (ZNFs) or Transcription activator-like effector nucleases (TALENs) for each target gene and because the efficiency of targeting may vary substantially, no multiplexed gene targeting in animals has been reported to date. To dissect the functions of gene family members with redundant functions or to analyze epistatic relationships in genetic pathways, mice with two or more mutated genes are required, prompting the development of efficient technology for the generation of animals carrying multiple mutated genes.

Recently, the type II bacterial CRISPR/Cas system has been demonstrated as an efficient gene-targeting technology with the potential for multiplexed genome editing. Bacteria and archaea have evolved an RNA-based adaptive immune system that uses CRISPR (clustered regularly interspaced short palindromic repeat) and Cas (CRISPR-associated) proteins to detect and destroy invading viruses and plasmids (Horvath and

Barrangou, 2010; Wiedenheft et al., 2012). Cas proteins, CRISPR RNAs (crRNAs), and *trans*-activating crRNA (tracrRNA) form ribonucleoprotein complexes, which target and degrade foreign nucleic acids, guided by crRNAs (Gasiunas et al., 2012; Jinek et al., 2012). It was shown that the Cas9 endonuclease from *Streptococcus pyogenes* type II CRISPR/Cas system can be programmed to produce sequence-specific DSB *in vitro* by providing a synthetic single-guide RNA (sgRNA) consisting of a fusion of crRNA and tracrRNA (Jinek et al., 2012). More intriguingly, Cas9 and sgRNA are the only components necessary and sufficient for induction of targeted DNA cleavage in cultured human cells (Cho et al., 2013; Cong et al., 2013; Mali et al., 2013) as well as in zebrafish (Chang et al., 2013; Hwang et al., 2013). A recent report also demonstrated disruption of a GFP transgene in mice using the CRISPR/Cas system (Shen et al., 2013). The ease of design, construction, and delivery of multiple sgRNAs suggest the possibility of multiplexed genome editing in mammals. Indeed, one study demonstrated that two loci separated by 119 bp could be cleaved simultaneously in cultured human cells at a low efficiency (Cong et al., 2013). The extent of achievable multiplexed genome editing has yet to be demonstrated in stem cells as well as in animals. Here, we use the CRISPR/Cas system to drive both NHEJ-based gene disruption and homology directed repair (HDR)-based precise gene editing to achieve highly efficient and simultaneous targeting of multiple genes in stem cells and mice.

RESULTS

Simultaneous Targeting up to Five Genes in ES Cells

To test the possibility of targeting functionally redundant genes from the same gene family, we designed sgRNAs targeting the Ten-eleven translocation (Tet) family members, *Tet1*, *Tet2*, and *Tet3* (Figure 1A). Tet proteins (Tet1/2/3) convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) in various embryonic and adult tissues and mutant mice for each of these three genes have been produced by homologous recombination in ES cells (Dawlaty et al., 2011; Gu et al., 2011; Li et al., 2011; Moran-Crusio et al., 2011). To test whether the CRISPR/Cas system could produce targeted cleavage in the mouse genome, we transfected plasmids expressing both the mammalian-codon-optimized Cas9 and a sgRNA targeting each gene (Cong et al., 2013; Mali et al., 2013) into mouse ES cells and determined the targeted cleavage efficiency by the Surveyor assay (Guschin et al., 2010). All three Cas9-sgRNA transfections produced cleavage at target loci with high efficiency of 36% at *Tet1*, 48% at *Tet2*, and 36% at *Tet3* (Figure 1B). Because each target locus contains a restriction enzyme recognition site (Figure 1A), we PCR amplified an ~500 bp fragment around each target site and digested the PCR products with the respective enzyme. A correctly targeted allele will lose the restriction site, which can be detected by failure to cleave upon enzyme treatment. Using this restriction fragment length polymorphism (RFLP) assay, we screened 48 ES cell clones from each single-targeting experiment. Consistent with the Surveyor analysis, a high percentage of ES cell clones were targeted, with a high probability of having both alleles mutated (Figure S1A available online). The results summarized in Table 1 demonstrate that between 65% and

81% of the tested ES cell clones carried mutations in the *Tet* genes with up to 77% having mutations in both alleles.

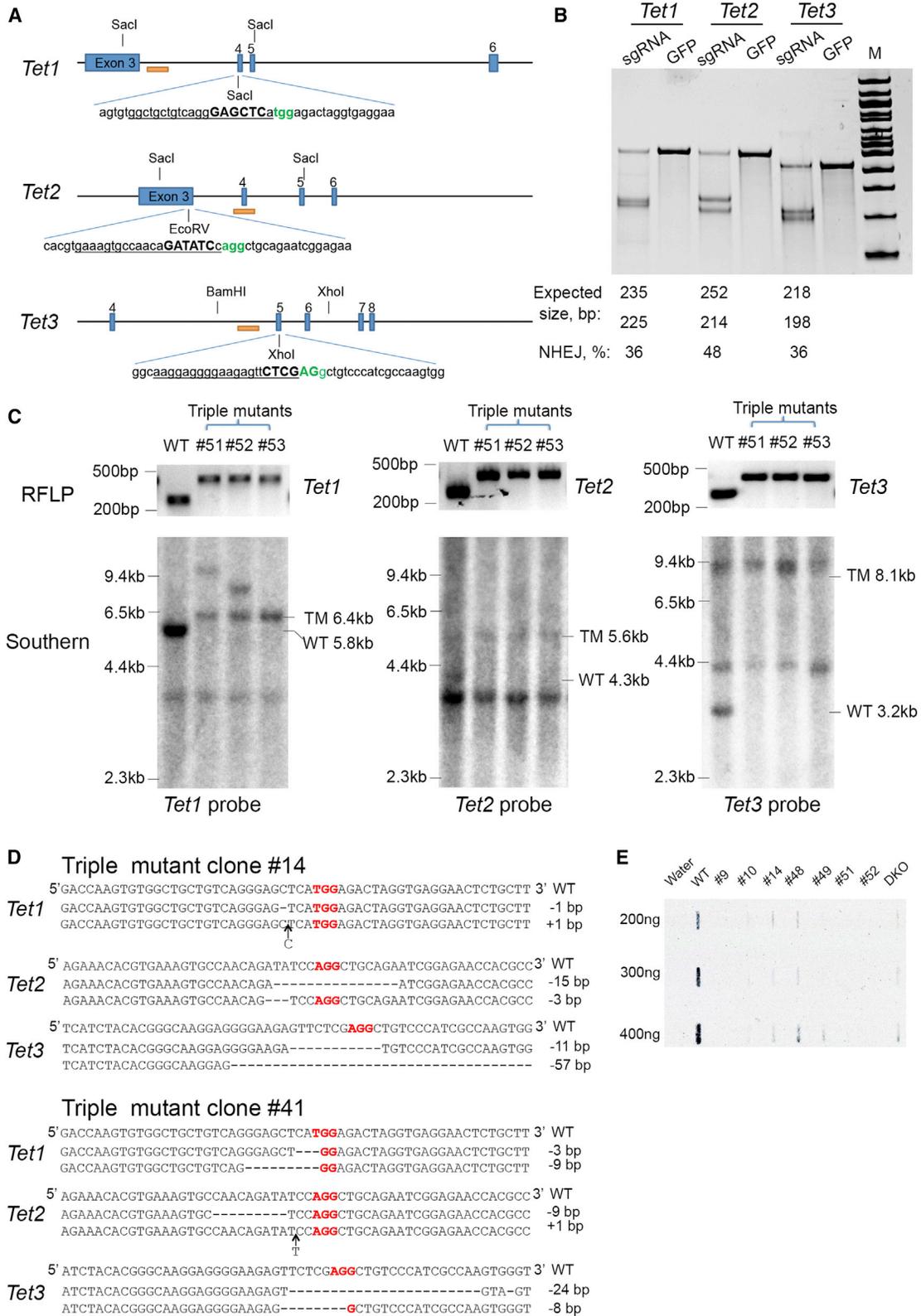
The high efficiency of single-gene modification prompted us to test the possibility of targeting all three genes simultaneously. For this we cotransfected ES cells with the constructs expressing Cas9 and three sgRNAs targeting *Tet1*, 2, and 3. Of 96 clones screened using the RFLP assay, 20 clones were identified as having mutations in all six alleles of the three genes (Figures 1C and S1B and Table 1). To exclude that a PCR bias could give false positive results, we performed Southern blot analysis and confirmed complete agreement with the RFLP results (Figure 1C). We subcloned and sequenced the PCR products of *Tet1*-, *Tet2*-, and *Tet3*-targeted regions to verify that all of eight tested clones carried biallelic mutations in all three genes with most clones displaying two mutant alleles for each gene with small insertions or deletions (indels) at the target site (Figure 1D). To test whether these mutant alleles would abolish the function of Tet proteins, we compared the 5hmC level of targeted clones to WT ES cells. Previously, we reported a depletion of 5hmC in *Tet1/Tet2* double-knockout ES cells derived using traditional gene-targeting methods (Dawlaty et al., 2013). As expected from loss of function alleles, we found a significant reduction of 5hmC levels in all clones carrying biallelic mutations in the three genes (Figure 1E).

To further test the potential of multiplexed gene targeting by CRISPR/Cas system, we designed sgRNAs targeting two Y-linked genes, *Sry* and *Uty* (Figure S1C). Short PCR products encoding sgRNAs targeting all five genes (*Tet1*, *Tet2*, *Tet3*, *Sry*, and *Uty*) were pooled and cotransfected with a Cas9 expressing plasmid and the PGK puroR cassette into ES cells. Of 96 clones that were screened using the RFLP assay, 10% carried mutations in all eight alleles of the five genes (Figure S1D and Table S1), demonstrating the capacity of the CRISPR/Cas9 system for highly efficient multiplexed gene targeting.

One-Step Generation of Single-Gene Mutant Mice by Zygote Injection

We tested whether mutant mice could be generated *in vivo* by direct embryo manipulation. Capped polyadenylated Cas9 mRNA was produced by *in vitro* transcription and coinjected with sgRNAs. Initially, to determine the optimal concentration of Cas9 mRNA for targeting *in vivo*, we microinjected varying amounts of Cas9-encoding mRNA with *Tet1* targeting sgRNA at constant concentration (20 ng/ μ l) into pronuclear (PN) stage one-cell mouse embryos and assessed the frequency of altered alleles at the blastocyst stage using the RFLP assay. As expected, higher concentration of Cas9 mRNA led to more efficient gene disruption (Figure S2A). Nevertheless, even embryos injected with the highest amount of Cas9 mRNA (200 ng/ μ l) showed normal blastocyst development, suggesting low toxicity.

To investigate whether postnatal mice carrying targeted mutations could be generated, we coinjected sgRNAs targeting *Tet1* or *Tet2* with different concentrations of Cas9 mRNA. Blastocysts derived from the injected embryos were transplanted into foster mothers and newborn pups were obtained. As summarized in Table 2, about 10% of the transferred blastocysts developed to birth independent of the RNA concentrations used for injection suggesting low fetal toxicity of the Cas9



(legend on next page)

mRNA and sgRNA. RFLP, Southern blot, and sequencing analysis demonstrated that between 50 and 90% of the postnatal mice carried biallelic mutations in either target gene (Figures 2A, 2B, and 2C and Table 2).

Surprisingly, specific $\Delta 9$ *Tet1* and specific $\Delta 8$ and $\Delta 15$ *Tet2* mutant alleles were repeatedly recovered in independently derived mice. Preferential generation of these alleles is likely caused by a short sequence repeat flanking the DSB (see Figure S2B) consistent with previous reports demonstrating that perfect microhomology sequences flanking the cleavage sites can generate microhomology-mediated precise deletions by end repair mechanism (MMEJ) (McVey and Lee, 2008; Symington and Gautier, 2011) (Figure S2B). A similar observation was also made when TALEN mRNA was injected into one-cell rat embryos (Tesson et al., 2011).

We also derived blastocysts from zygotes injected with Cas9 mRNA and *Tet3* sgRNA. Genotyping of the blastocysts demonstrated that of eight embryos three were homozygous and three were heterozygous *Tet3* mutants (two failed to amplify) (Figure S2C). Some blastocysts were implanted into foster mothers and, upon C section, we readily identified multiple mice of smaller size (Figure S2D), many of which died soon after delivery. Genotyping shown in Figure S2E indicated that all pups with mutations in both *Tet3* alleles died neonatally. Only 2 out of 15 mice survived that were either *Tet3* heterozygous mutants or WT (Figure S2F). These results are consistent with the lethal neonatal phenotype of *Tet3* knockout mice generated using traditional methods (Gu et al., 2011), although we have not yet established which of the *Tet3* mutations produced loss of function rather than hypomorphic alleles.

One-Step Generation of Double-Gene Mutant Mice by Zygote Injection

To test whether *Tet1/Tet2* double-mutant mice could be produced from single embryos, we coinjected *Tet1* and *Tet2* sgRNAs with 20 or 100 ng/ μ l Cas9 mRNA into zygotes. A total of 28 pups were born from 144 embryos transferred into foster mothers (21% live-birth rate) that had been injected at the zygote stage with high concentrations of RNA (Cas9 mRNA at 100 ng/ μ l, sgRNAs at 50 ng/ μ l), consistent with low or no toxicity of the Cas9 mRNA and sgRNAs (Table 3). RFLP, Southern blot analysis, and sequencing identified 22 mice carrying targeted mutations at all four alleles of the *Tet1* and *Tet2* genes (Figures 2D and 2E) with the remaining mice carrying mutations

Table 1. CRISPR/Cas-Mediated Gene Targeting in V6.5 ES Cells

| Gene | Mutant Alleles per Clone / Total Clones Tested | | | | | | |
|--------------------------|--|-------|------|------|-------|------|-------|
| | 6 | 5 | 4 | 3 | 2 | 1 | 0 |
| <i>Tet1</i> | N/A | | | | 27/48 | 4/48 | 17/48 |
| <i>Tet2</i> | | | | | 37/48 | 2/48 | 9/48 |
| <i>Tet3</i> | | | | | 32/48 | 3/48 | 13/48 |
| <i>Tet1+ Tet2 + Tet3</i> | 20/96 | 16/96 | 2/96 | 2/96 | 1/96 | 0/96 | 55/96 |

Plasmids encoding Cas9 and sgRNAs targeting *Tet1*, *Tet2*, and *Tet3* were transfected separately (single targeting) or in a pool (triple targeting) into ES cells. The number of total alleles mutated in each ES cell clone is listed from 0 to 2 for single-targeting experiment, and 0 to 6 for triple-targeting experiment. The number of clones containing each specific number of mutated alleles is shown in relation to the total number of clones screened in each experiment. See also Table S1.

in a subset of alleles (Table 3). Injection of zygotes with low concentration of RNA (Cas9 mRNA at 20 ng/ μ l, sgRNAs at 20 ng/ μ l) yielded 19 pups from 75 transferred embryos (25% live-birth rate), which is a higher survival rate than from embryos injected with 100 ng/ μ l of Cas9 RNA. Nevertheless, more than 50% of the pups were biallelic *Tet1/Tet2* double mutants (Table 3). These results demonstrate that postnatal mice carrying biallelic mutations in two different genes can be generated within one month with high efficiency (Figure 2F).

Although the high live-birth rate and normal development of mutant mice suggest low toxicity of CRISPR/Cas9 system, we sought to determine the off-target effects in vivo. Previous work in vitro, in bacteria, and in cultured human cells suggested that the protospacer-adjacent motif (PAM) sequence NGG and the 8 to 12 base “seed sequence” at the 3’ end of the sgRNA are most important for determining the DNA cleavage specificity (Cong et al., 2013; Jiang et al., 2013; Jinek et al., 2012). Based on this rule, only three and four potential off targets exist in mouse genome for *Tet1* and *Tet2* sgRNA, respectively (Table S2 and Experimental Procedures), with each of them perfectly matching the 12 bp seed sequence at the 3’ end and the NGG PAM sequence of the sgRNA (there is no potential off-target site for *Tet3* sgRNA using this prediction rule). From seven double-mutant mice produced from injection with high RNA concentration we PCR amplified 400 to 500 bp fragments from all seven potential off-target loci and found no cleavage in the Surveyor assay (Figure S3), suggesting a high specificity of CRISPR/Cas system.

Figure 1. Multiplexed Gene Targeting in mouse ES cells

(A) Schematic of the Cas9/sgRNA-targeting sites in *Tet1*, 2, and 3. The sgRNA-targeting sequence is underlined, and the protospacer-adjacent motif (PAM) sequence is labeled in green. The restriction sites at the target regions are bold and capitalized. Restriction enzymes used for RFLP and Southern blot analysis are shown, and the Southern blot probes are shown as orange boxes.

(B) Surveyor assay for Cas9-mediated cleavage at *Tet1*, 2, and 3 loci in ES cells.

(C) Genotyping of triple-targeted ES cells, clones 51, 52, and 53 are shown. Upper: RFLP analysis. *Tet1* PCR products were digested with *SacI*, *Tet2* PCR products were digested with *EcoRV*, and *Tet3* PCR products were digested with *XhoI*. Lower: Southern blot analysis. For the *Tet1* locus, *SacI* digested genomic DNA was hybridized with a 5’ probe. Expected fragment size: WT = 5.8 kb, TM (targeted mutation) = 6.4 kb. For the *Tet2* locus, *SacI* and *EcoRV* double-digested genomic DNA was hybridized with a 3’ probe. Expected fragment size: WT = 4.3 kb, TM = 5.6 kb. For the *Tet3* locus, *BamHI* and *XhoI* double-digested genomic DNA was hybridized with a 5’ probe. Expected fragment size: WT = 3.2 kb, TM = 8.1 kb.

(D) The sequence of six mutant alleles in triple-targeted ES cell clone 14 and 41. PAM sequence is labeled in red.

(E) Analysis of 5hmC levels in DNA isolated from triple-targeted ES cell clones by dot blot assay using anti-5hmC antibody. A previously characterized DKO clone derived using traditional method is used as a control. See also Figure S1.

Table 2. CRISPR/Cas-Mediated Single-Gene Targeting in BDF2 Mice

| Gene | Cas9/sg RNA (ng/ μ l) | Blastocysts/Injected Zygotes | Transferred Embryos (Recipients) | Newborns (Dead) | Mutant Alleles per Mouse/Total Mice Tested ^a | | |
|-------------|---------------------------|------------------------------|----------------------------------|-----------------|---|------|------|
| | | | | | 2 | 1 | 0 |
| <i>Tet1</i> | 200/20 | 38/50 | 19 (1) | 2 (0) | 2/2 | 0/2 | 0/2 |
| | 100/20 | 50/60 | 25 (1) | 3 (0) | 2/3 | 0/3 | 1/3 |
| | 50/20 | 40/50 | 40 (2) | 8 (3) | 4/7 | 2/7 | 1/7 |
| | 100/50 | 167/198 | 60 (3) | 12 (2) | 9/11 | 1/11 | 1/11 |
| <i>Tet2</i> | 100/50 | 176/203 | 108 (5) | 22 (3) | 19/20 | 0/20 | 1/20 |
| <i>Tet3</i> | 100/50 | 85/112 | 64 (4) | 15 (13) | 9/13 | 2/13 | 2/13 |

Cas9 mRNA and sgRNAs targeting *Tet1*, *Tet2*, or *Tet3* were injected into fertilized eggs. The blastocysts derived from injected embryos were transplanted into foster mothers and newborn pups were obtained and genotyped. The number of total alleles mutated in each mouse is listed from 0 to 2. The number of mice containing each specific number of mutated alleles is shown in relation to the total number of mice screened in each experiment. See also Table S2.

^aSome of the pups were cannibalized.

Multiplexed Precise HDR-Mediated Genome Editing In Vivo

The NHEJ-mediated gene mutations described above produced mutant alleles with different and unpredictable insertions and deletions of variable size. We explored the possibility of precise homology directed repair (HDR)-mediated genome editing by coinjecting Cas9 mRNA, sgRNAs, and single-stranded DNA oligos into one-cell embryos. For this we designed an oligo targeting *Tet1* so as to change two base pairs of a *SacI* restriction site and creating instead an *EcoRI* site and a second oligo targeting *Tet2* with two base pair changes that would convert an *EcoRV* site into an *EcoRI* site (Figure 3A). Blastocysts were derived from zygotes injected with Cas9 mRNA and sgRNAs and oligos targeting *Tet1* or *Tet2*, respectively. DNA was isolated, amplified, and digested with *EcoRI* to detect oligo-mediated HDR events. Six out of nine *Tet1*-targeted embryos and 9 out of 15 *Tet2*-targeted embryos incorporated an *EcoRI* site at the respective target locus, with several embryos having both alleles modified (Figure S4A). When Cas9 mRNA, sgRNAs, and single-stranded DNA oligos targeting both *Tet1* and *Tet2* were coinjected into zygotes, out of 14 embryos, four were identified that were targeted with the oligo at the *Tet1* locus, seven that were targeted with the oligo at the *Tet2* locus and one embryo (2) that had one allele of each gene correctly modified (Figure S4B). All four alleles of embryo 2 were sequenced, confirming that one allele of each gene contained the 2 bp changes directed by the oligo, whereas the other alleles were disrupted by NHEJ-mediated deletion and insertion (Figure S4C).

Blastocysts with double oligo injections were implanted into foster mothers and a total of 10 pups were born from 48 embryos transferred (21% live-birth rate). Upon RFLP analysis using *EcoRI*, we identified seven mice containing *EcoRI* sites at the *Tet1* locus and eight mice containing *EcoRI* sites at the *Tet2* locus, with six mice containing *EcoRI* sites at both *Tet1* and *Tet2* loci (Figure 3B). We also applied RFLP analysis using *SacI* and *EcoRV* to *Tet1* and *Tet2* loci, respectively, showing that all alleles not targeted by oligos contained disruptions, which is consistent with the high biallelic mutation rate by Cas9 mRNA and sgRNAs injection. These results were confirmed by sequencing demonstrating mutations in all four alleles of mouse 5 and 7 (Figure 3C). Our results demonstrate that mice

with HDR-mediated precise mutations in multiple genes can be generated in one step by CRISPR/Cas-mediated genome editing.

DISCUSSION

The genetic manipulation of mice is a crucial approach for the study of development and disease. However, the generation of mice with specific mutations is labor intensive and involves gene targeting by homologous recombination in ES cells, the production of chimeric mice, and, after germline transmission of the targeted ES cells, the interbreeding of heterozygous mice to produce the homozygous experimental animals, a process that may take 6 to 12 months or longer (Capecchi, 2005). To produce mice carrying mutations in several genes requires time-consuming intercrossing of single-mutant mice. Similarly, the generation of ES cells carrying homozygous mutations in several genes is usually achieved by sequential targeting, a process that is labor intensive, necessitating multiple consecutive cloning steps to target the genes and to delete the selectable markers.

As summarized in Figure 4, we have established three different approaches for the generation of mice carrying multiple genetic alterations. We demonstrate that CRISPR/Cas-mediated genome editing in ES cells can generate the simultaneous mutations of several genes with high efficiency, a single-step approach allowing the production of cells with mutations in five different genes (Figure 4A). We chose the three *Tet* genes as targets because the respective mutant phenotypes have been well defined previously (Dawlaty et al., 2011, 2013; Gu et al., 2011). Cells mutant for *Tet1*, 2 and 3 were depleted of 5hmC as would be expected for loss of function mutations of the genes (Dawlaty et al., 2013). However, we have not as yet established, which of the Cas9-mediated gene mutations produced loss of function rather than hypomorphic alleles.

We also show that mouse embryos can be directly modified by injection of Cas9 mRNA and sgRNA into the fertilized egg resulting in the efficient production of mice carrying biallelic mutations in a given gene. More significantly, coinjection of Cas9 with *Tet1* and *Tet2* sgRNAs into zygotes produced mice that carried mutations in both genes (Figure 4B, upper). We

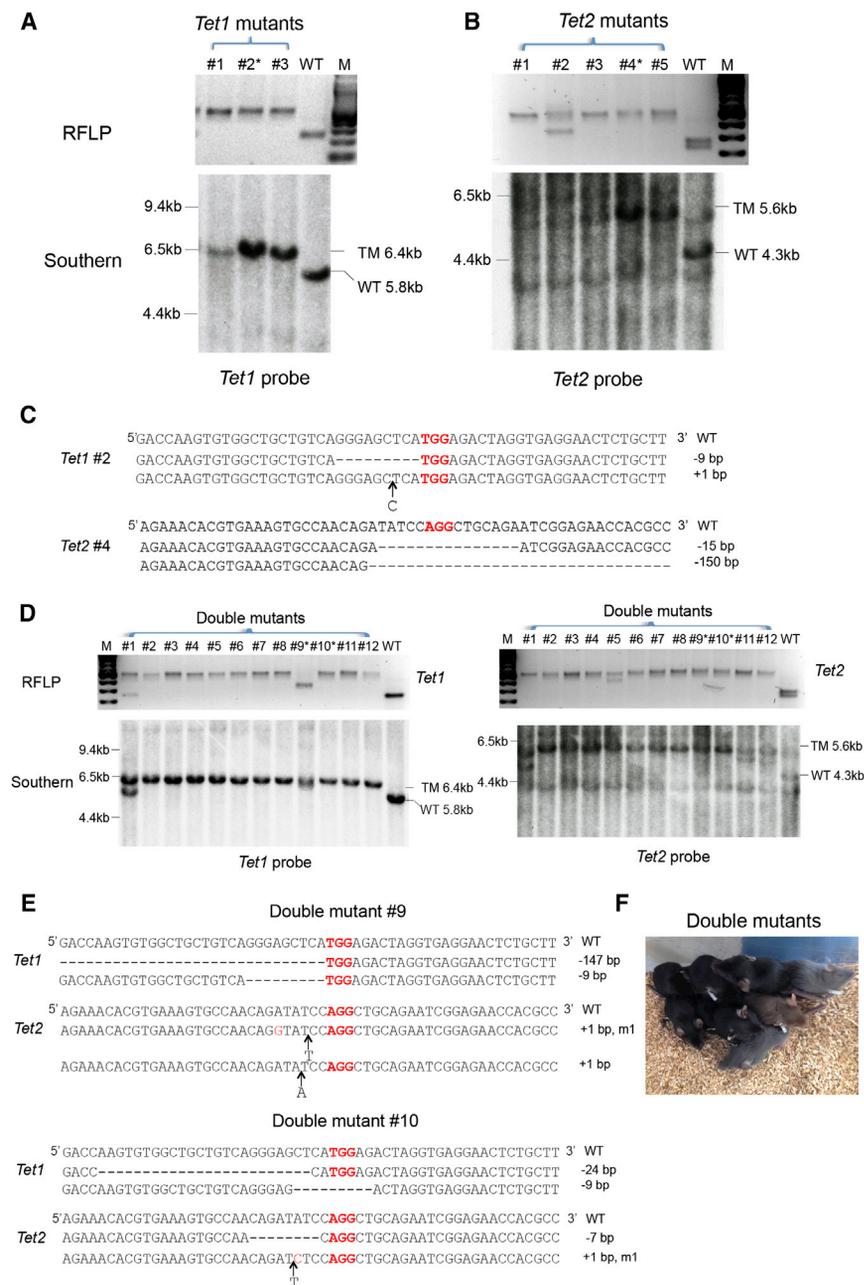


Figure 2. Single- and Double-Gene Targeting In Vivo by Injection into Fertilized Eggs

(A) Genotyping of *Tet1* single-targeted mice. (B) Upper: genotyping of *Tet2* single-targeted mice. RFLP analysis; lower: Southern blot analysis. (C) The sequence of both alleles of targeted gene in *Tet1* biallelic mutant mouse 2 and *Tet2* biallelic mutant mouse 4. (D) Genotyping of *Tet1/Tet2* double-mutant mice. Analysis of mice 1 to 12 is shown. Upper: RFLP analysis; lower: southern blot analysis. The *Tet1* locus is displayed on the left and the *Tet2* locus on the right. (E) The sequence of four mutant alleles from double-mutant mouse 9 and 10. PAM sequences are labeled in red. (F) Three-week-old double-mutant mice. All RFLP and Southern digestions and probes are the same as those used in Figure 1. See also Figures S2 and S3.

lines, and mouse by ZNF or TALEN along with DNA oligo has been demonstrated previously (Chen et al., 2011; Soldner et al., 2011; Wefers et al., 2013). We demonstrate that CRISPR/Cas-mediated targeting is useful to generate mutant alleles with predetermined alterations, and coinjection of single-stranded oligos can introduce designed point mutations into two target genes in one step, allowing for multiplexed gene editing in a strictly controlled manner (Figure 4B, lower). It will be of great interest to assess whether this targeting system allows for the production of conditional alleles, or precise insertion of larger DNA fragments such as GFP markers so as to generate conditional knockout and reporter mice for specific genes.

There are several potential limitations of the CRISPR/Cas technology. First, the requirement for a NGG PAM sequence of *S. pyogenes* Cas9 limits the target space in the mouse genome. It has been shown that the *Streptococcus*

thermophilus LMD-9 Cas9 using different PAM sequence can also induce targeted DNA cleavage in mammalian cells (Cong et al., 2013). Therefore, exploiting different Cas9 proteins may enable to target most of the mouse genome. Second, although the sgRNAs used here showed high targeting efficiency, much work is needed to elucidate the rules for designing sgRNAs with consistent high targeting efficiency, which is essential for multiplexed genome engineering. Third, although our off-target analysis for the seven most likely off targets of *Tet1* and *Tet2* sgRNAs failed to detect mutations in these loci, it is possible that other mutations were induced following as yet unidentified rules. A more thorough sequencing analysis for a large number

found that up to 95% of newborn mice were biallelic mutant in the targeted gene when single sgRNA was injected and when coinjected with two different sgRNAs, up to 80% carried biallelic mutations in both targeted genes. Thus, mice carrying multiple mutations can be generated within 4 weeks, which is a much shorter time frame than can be achieved by conventional consecutive targeting of genes in ES cells and avoids time-consuming intercrossing of single-mutant mice.

The introduction of DSBs by CRISPR/Cas generates mutant alleles with varying deletions or insertions in contrast to designed precise mutations created by homologous recombination. The introduction of point mutations into human ES cells, cancer cell

lines, and mouse by ZNF or TALEN along with DNA oligo has been demonstrated previously (Chen et al., 2011; Soldner et al., 2011; Wefers et al., 2013). We demonstrate that CRISPR/Cas-mediated targeting is useful to generate mutant alleles with predetermined alterations, and coinjection of single-stranded oligos can introduce designed point mutations into two target genes in one step, allowing for multiplexed gene editing in a strictly controlled manner (Figure 4B, lower). It will be of great interest to assess whether this targeting system allows for the production of conditional alleles, or precise insertion of larger DNA fragments such as GFP markers so as to generate conditional knockout and reporter mice for specific genes.

There are several potential limitations of the CRISPR/Cas technology. First, the requirement for a NGG PAM sequence of *S. pyogenes* Cas9 limits the target space in the mouse genome. It has been shown that the *Streptococcus*

Table 3. CRISPR/Cas-Mediated Double-Gene Targeting in BDF2 Mice

| Gene | Cas9/sgRNA (ng/ μ l) | Blastocyst/Injected Zygotes | Transferred Embryos (Recipients) | Newborns (Dead) | Mutant Alleles per Mouse/Total Mice Tested ^a | | | | |
|--------------------|-----------------------------|--------------------------------|-------------------------------------|--------------------|---|------|------|------|------|
| | | | | | 4 | 3 | 2 | 1 | 0 |
| <i>Tet1 + Tet2</i> | 100 / 50 | 194/229 | 144(7) | 31(8) | 22/28 | 4/28 | 1/28 | 1/28 | 0/28 |
| | 20 / 20 | 92/109 | 75(5) | 19(3) | 11/19 | 1/19 | 2/19 | 3/19 | 2/19 |

Cas9 mRNA and sgRNAs targeting *Tet1* and *Tet2* were coinjected into fertilized eggs. The blastocysts derived from the injected embryos were transplanted into foster mothers and newborn pups were obtained and genotyped. The number of total alleles mutated in each mouse is listed from 0 to 4 for *Tet1* and *Tet2*. The number of mice containing each specific number of mutated alleles is shown in relation to the number of total mice screened in each experiment.

^aSome of the pups were cannibalized.

of sgRNAs will provide more information about the potential off-target cleavage of the CRISPR/Cas system and lead to a better prediction of potential off-target sites. Last, oligo-mediated repair allows for precise genome editing, but the other allele is often mutated through NHEJ (Figures 3B, 3C, and S4C). We have shown that using lower Cas9 mRNA concentration generates more mice with heterozygous mutations. Therefore, it may be possible to optimize the system for more efficient generation of mice with only one oligo-modified allele. In addition, employment of Cas9 nickase will likely avoid this complication because it mainly induces DNA single-strand break, which is typically repaired through HDR (Cong et al., 2013; Mali et al., 2013).

It is likely that a much larger number of genomic loci than targeted in the present work can be modified simultaneously when pooled sgRNAs are introduced. The methods presented here open up the possibility of systematic genome engineering in mice, facilitating the investigation of entire signaling pathways, of synthetic lethal phenotypes or of genes that have redundant functions. A particularly interesting application is the possibility to produce mice carrying multiple alterations in candidate loci that have been identified in GWAS studies to play a role in the genesis of multigenic diseases. In summary, CRISPR/Cas-mediated genome editing makes possible the generation of ES cells and mice carrying multiple genetic alterations and will facilitate the genetic dissection of development and complex diseases.

EXPERIMENTAL PROCEDURES

Procedures for Generating sgRNAs Expressing Vector

Bicistronic expression vector expressing Cas9 and sgRNA (Cong et al., 2013) were digested with BbsI and treated with Antarctic Phosphatase, and the linearized vector was gel purified. A pair of oligos for each targeting site (Table S3) was annealed, phosphorylated, and ligated to linearized vector.

Cell Culture and Transfection

V6.5 ES cells (on a 129/Sv x C57BL/6 F1 hybrid background) were cultured on gelatin-coated plates with standard ES cell culture conditions. Cells were transfected with a plasmid expressing mammalian-codon-optimized Cas9 and sgRNA (single targeting), three plasmids expressing Cas9 and sgRNAs targeting *Tet1*, *Tet2*, and *Tet3* (triple targeting), or five PCR products each coding for sgRNA targeting *Tet1*, *Tet2*, *Tet3*, *Sry*, and *Uty*, along with a plasmid expressing PGK-puroR using FuGENE HD reagent (Promega) according to the manufacturer's instructions. Twelve hours after transfection, ES cells were replated at a low density on DR4 MEF feeder layers. Puromycin (2 μ g/ml) was added 1 day after replating and taken off after 48 hr. After recovering for 4 to 6 days, individual colonies were picked and genotyped by RFLP and Southern

blot analysis, and the leftover ES cells on plate were collected for Suveryor assay.

Suveryor Assay and RFLP Analysis for Genome Modification

Suveryor assay was performed as described by (Guschin et al., 2010). Genomic DNA from treated and control ES cells or targeted and control mice was extracted. Mouse genomic DNA samples were prepared from tail biopsies. PCR was performed using *Tet1*-, *Tet2*-, and *Tet3*-specific primers (Table S3) under the following conditions: 95°C for 5 min; 35 \times (95°C for 30 s, 60°C for 30 s, 68°C for 40 s); 68°C for 2 min; hold at 4°C. PCR products were then denatured, annealed, and treated with Suveryor nuclease (Transgenomic). DNA concentration of each band was measured on an ethidium bromide-stained 10% acrylamide Criterion TBE gel (BioRad) and quantified using ImageJ software. The same PCR products for Suveryor assay were used for RFLP analysis. Ten microliters of *Tet1*, *Tet2*, or *Tet3* PCR product was digested with ScaI, EcoRV, or XhoI, respectively. Digested DNA was separated on an ethidium bromide-stained agarose gel (2%). For sequencing, PCR products were cloned using the Original TA Cloning Kit (Invitrogen), and mutations were identified by Sanger sequencing.

Dot Blot

DNA was extracted from ES cells following standard procedures. DNA was transferred to nylon membrane using BioRad slot blot vacuum manifold apparatus. Anti-5hmC (Active Motif 1:10,000) was used to detect 5hmC following manufacturer's protocol.

Production of Cas9 mRNA and sgRNA

T7 promoter was added to Cas9 coding region by PCR amplification using primer Cas9 F and R (Table S3). T7-Cas9 PCR product was gel purified and used as the template for in vitro transcription (IVT) using mMACHINE mMACHINE T7 ULTRA kit (Life Technologies). T7 promoter was added to sgRNAs template by PCR amplification using primer *Tet1* F and R, *Tet2* F and R, and *Tet3* F and R (Table S3). The T7-sgRNA PCR product was gel purified and used as the template for IVT using MEGAshortscript T7 kit (Life Technologies). Both the Cas9 mRNA and the sgRNAs were purified using MEGAclear kit (Life Technologies) and eluted in RNase-free water.

One-Cell Embryo Injection

All animal procedures were performed according to NIH guidelines and approved by the Committee on Animal Care at MIT. B6D2F1 (C57BL/6 X DBA2) female mice and ICR mouse strains were used as embryo donors and foster mothers, respectively. Superovulated female B6D2F1 mice (7–8 weeks old) were mated to B6D2F1 stud males, and fertilized embryos were collected from oviducts. Cas9 mRNAs (from 20 ng/ μ l to 200 ng/ μ l) and sgRNA (from 20 ng/ μ l to 50 ng/ μ l) was injected into the cytoplasm of fertilized eggs with well recognized pronuclei in M2 medium (Sigma). For oligos injection, Cas mRNA (100 ng/ μ l), sgRNA (50 ng/ μ l), and donor oligos (100 ng/ μ l) were mixed and injected into zygotes at the pronuclei stage. The injected zygotes were cultured in KSOM with amino acids at 37°C under 5% CO₂ in air until blastocyst stage by 3.5 days. Thereafter, 15–25 blastocysts were transferred into uterus of pseudopregnant ICR females at 2.5 dpc.

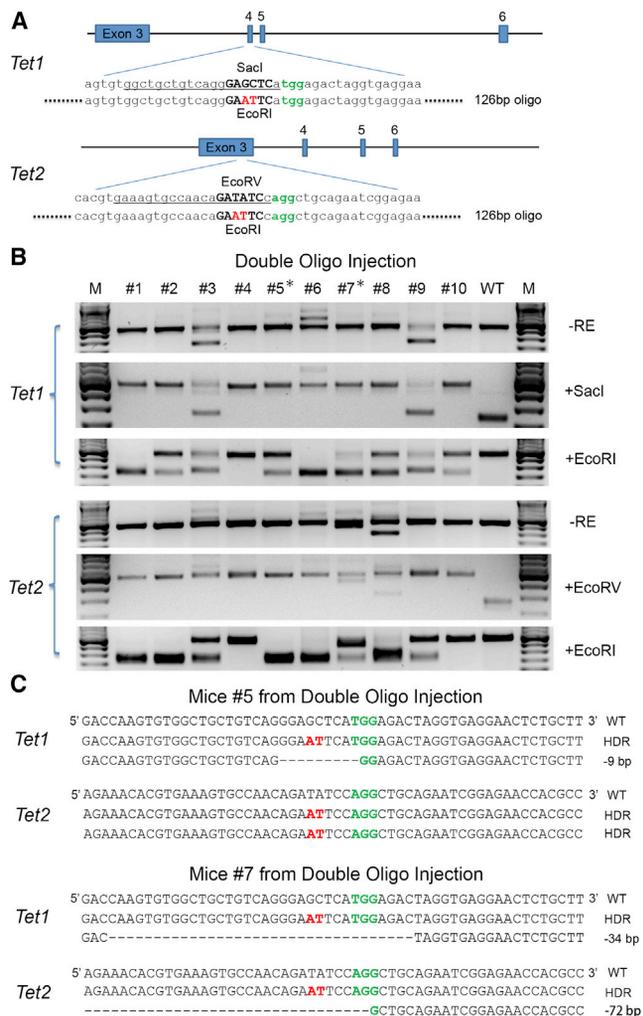


Figure 3. Multiplexed HDR-Mediated Genome Editing In Vivo
 (A) Schematic of the oligo-targeting sites at *Tet1* and *Tet2* loci. The sgRNA-targeting sequence is underlined, and the PAM sequence is labeled in green. Oligo targeting each gene is shown under the target site, with 2 bp changes labeled in red. Restriction enzyme sites used for RFLP analysis are bold and capitalized.
 (B) RFLP analysis of double oligo injection mice with HDR-mediated targeting at the *Tet1* and *Tet2* loci.
 (C) The sequences of both alleles of *Tet1* and *Tet2* in mouse 5 and 7 show simultaneously HDR-mediated targeting at one allele or two alleles of each gene, and NHEJ-mediated disruption at the other alleles. See also Figure S4.

Southern Blotting

Genomic DNA was separated on a 0.8% agarose gel after restriction digests with the appropriate enzymes, transferred to a nylon membrane (Amersham) and hybridized with ³²P random primer (Stratagene)-labeled probes.

Prediction of Potential Off Targets

Potential targets of CRISPR sgRNAs were found using the rules outline in (Mali et al., 2013). For a 20 nt sgRNA targeting sequence of nnnn nMMMM MMMMM MMMMM, where M are the seed bases preceding the PAM sequence NGG, four search sequences (MMM MMMMM MMMMM AGG; MMM MMMMM MMMMM CGG; MMM MMMMM MMMMM GGG; MMM MMMMM MMMMM TGG) were generated. Exact matches to these search

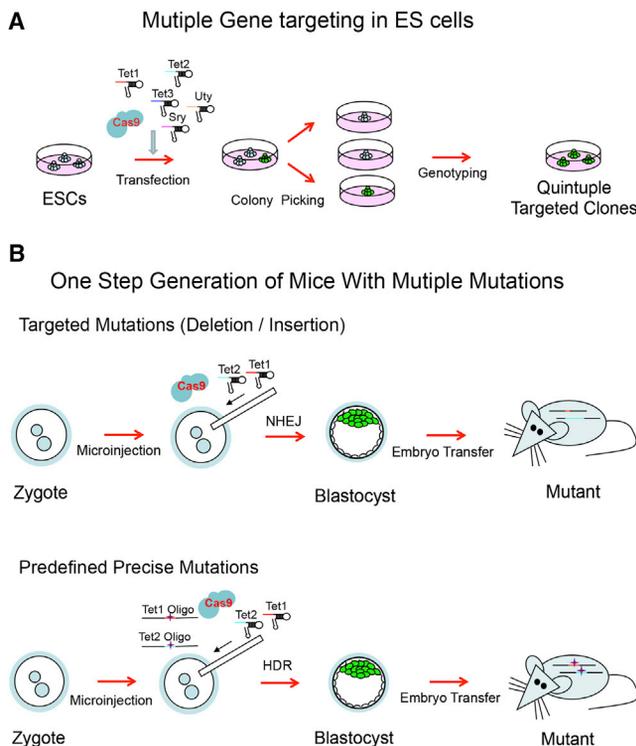


Figure 4. Multiplexed Genome Editing in ES Cells and Mouse
 (A) Multiple gene targeting in ES cells.
 (B) One-step generation of mice with multiple mutations. Upper: multiple targeted mutations with random indels introduced through NHEJ. Lower: multiple predefined mutations introduced through HDR-mediated repair.

sequences in the mouse genome (mm9) were found using bowtie and reported as potential targets of the CRISPR sgRNA.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2013.04.025>.

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