Cell

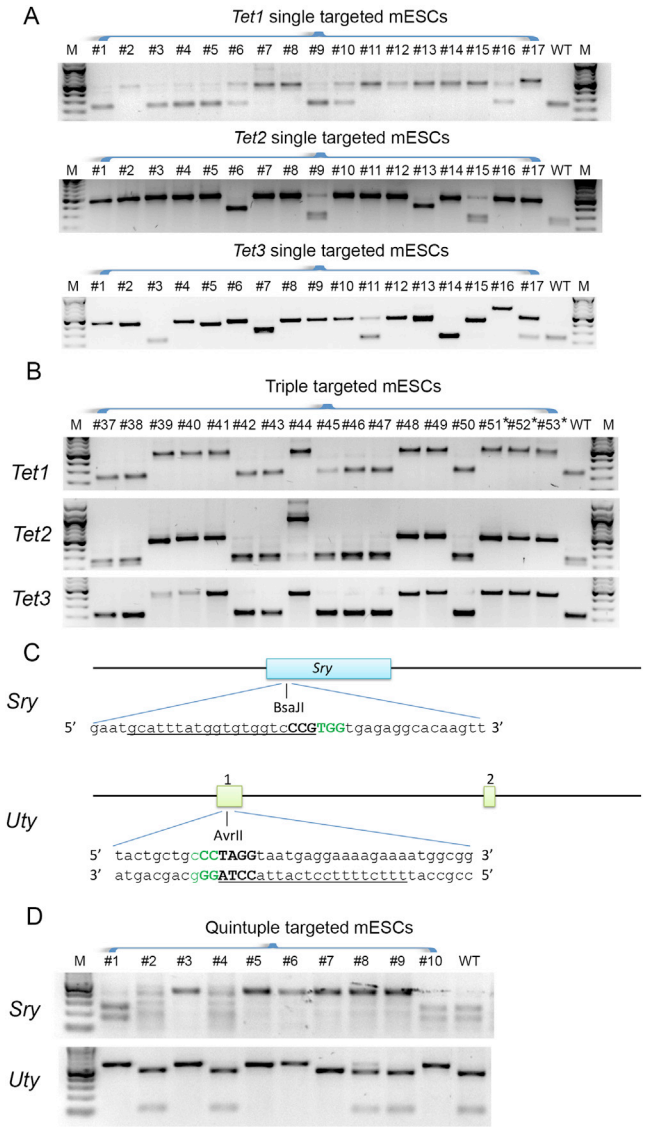

Figure S1. Single-, Triple-, and Quintuple-Gene Targeting in mES Cells, Related to Figure 1

(A) RFLP analysis of clones from each single-targeting experiment (1 to 17 are shown).

(B) RFLP analysis of triple-gene-targeted clones (37 to 53 are shown). *Tet1* PCR products were digested with SacI, *Tet2* PCR products were digested with EcoRV, and *Tet3* PCR products were digested with XhoI. WT control is shown in the last lane. Genotyping of clone 51, 52, and 53 are also shown in Figure 1C.

(C) Schematic of the Cas9/sgRNA-targeting sites in *Sry* and *Uty*. 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 analysis are shown.

(D) RFLP analysis of quintuple-gene-targeted clones (1 to 10 are shown). *Sry* PCR products were digested with BsaJI, *Uty* PCR products were digested with AvrII. WT control is shown in the last lane. RFLP analysis of *Tet1*, *2*, *3* loci are not shown.

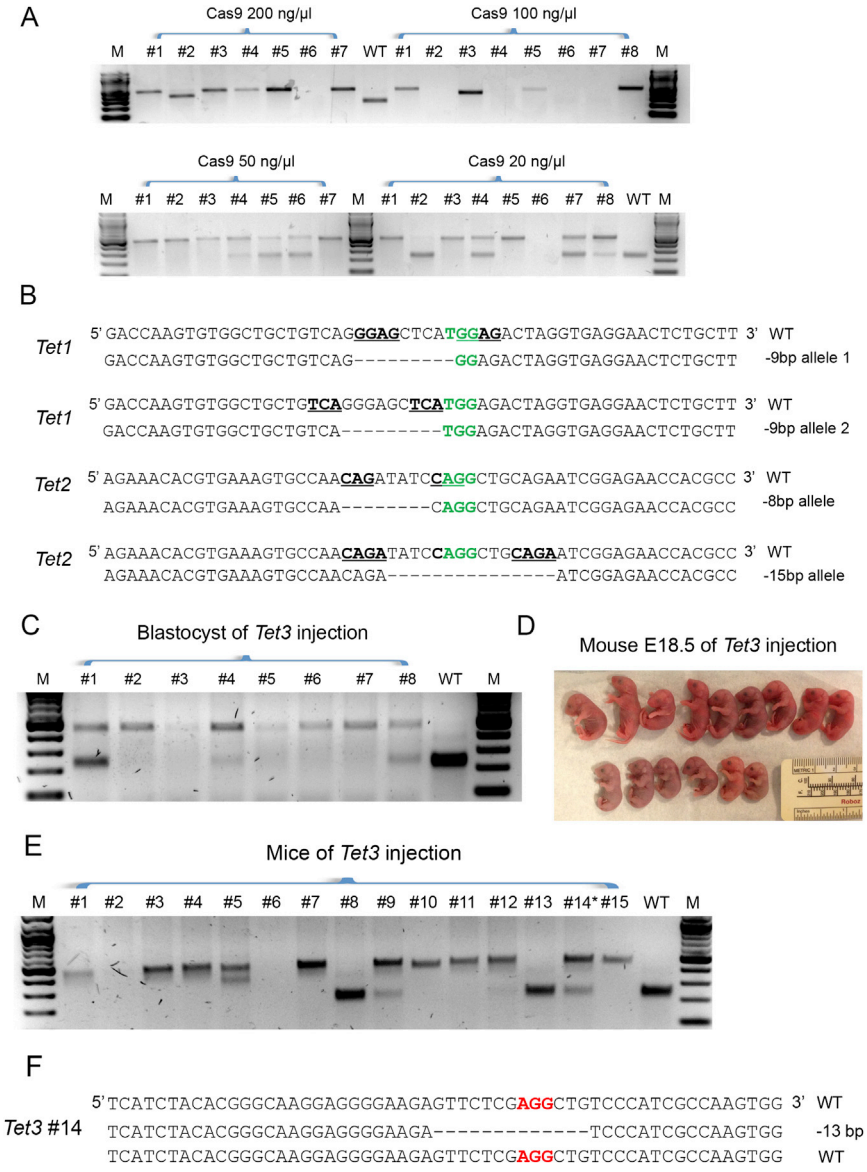

Figure S2. One-Step Generation of Single-Gene Mutant Mice by Zygote Injection, Related to Figure 2

(A) RFLP analysis of blastocysts injected with different concentration of Cas9 mRNA and *Tet1* sgRNA at 20 ng/μl. *Tet1* PCR products were digested with SacI.

(B) Commonly recovered *Tet1* and *Tet2* alleles resulted from MMEJ. PAM sequence of each targeting sequence is labeled in green. Microhomology flanking the DSB is bold and underlined in WT sequence.

(C) RFLP analysis of eight *Tet3*-targeted blastocysts demonstrated high targeting efficiency (embryo 3 and 5 failed to amplify). *Tet3* PCR products were digested with XhoI.

(D) Some *Tet3*-targeted mice show smaller size and all homozygous mutants died within 1 day after birth.

(E) RFLP analysis of *Tet3* single-targeted newborn mice. Mouse 8 and 14 survived after birth. Sample 2 and 6 failed to amplify.

(F) Sequences of both *Tet3* alleles of surviving *Tet3*-targeted mouse 14. PAM sequences are labeled in red.

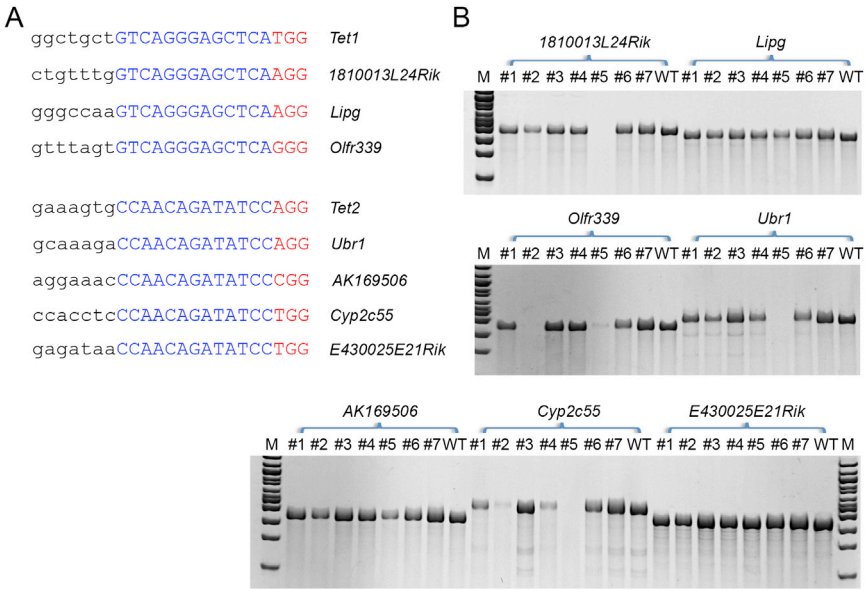

Figure S3. Off-Target Analysis of Double-Mutant Mice, Related to Figure 2

(A) Three potential off targets of *Tet1* sgRNA and four potential off targets of *Tet2* sgRNA are shown. The 12 bp perfect matching seed sequence is labeled in blue, and NGG PAM sequence is labeled in red.

(B) Surveyor assay of all seven potential off-target loci in seven double-mutant mice derived with high concentration of Cas9 mRNA (100 ng/μl) injection. WT control is included as the eighth sample. The weak cleavage activity at *Ubr1* locus is not due to off-target effect because sequences of these PCR products show no mutations.

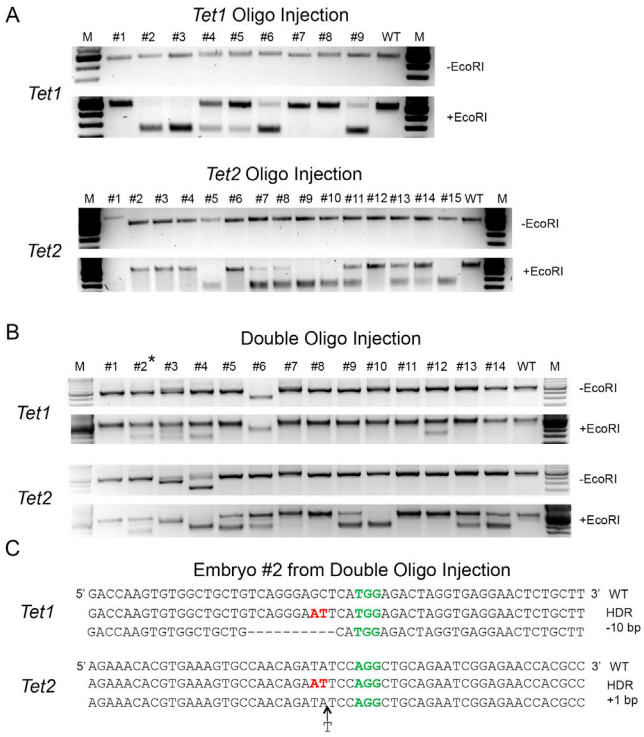

Figure S4. Multiplexed Precise HDR-Mediated Genome Editing In Vivo, Related to Figure 3

(A) RFLP analysis of single oligo injection embryos with HDR-mediated targeting at *Tet1* and *Tet2* locus.

(B) RFLP analysis of double oligo injection embryos with multiplexed HDR-mediated targeting at both *Tet1* and *Tet2* loci.

(C) Sequences of both alleles of *Tet1* and *Tet2* in embryo 2 show simultaneously HDR-mediated targeting at one allele of both genes, and NHEJ-mediated gene disruption at the other allele of each gene.