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One-Step Generation of Mice Carrying Reporter and Conditional Alleles by CRISPR/Cas-Mediated Genome Engineering

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SUMMARY

The type II bacterial CRISPR/Cas system is a novel genome-engineering technology with the ease of multiplexed gene targeting. Here, we created reporter and conditional mutant mice by coinjection of zygotes with Cas9 mRNA and different guide RNAs (sgRNAs) as well as DNA vectors of different sizes. Using this one-step procedure we generated mice carrying a tag or a fluorescent reporter construct in the *Nanog*, the *Sox2*, and the *Oct4* gene as well as *Mecp2* conditional mutant mice. In addition, using sgRNAs targeting two separate sites in the *Mecp2* gene, we produced mice harboring the predicted deletions of about 700 bps. Finally, we analyzed potential off-targets of five sgRNAs in gene-modified mice and ESC lines and identified off-target mutations in only rare instances.

INTRODUCTION

Mice with specific gene modification are valuable tools for studying development and disease. Traditional gene targeting in embryonic stem (ES) cells, although suitable for generating sophisticated genetic modifications in endogenous genes, is complex and time-consuming (Capecchi, 2005). The production of genetically modified mice and rats has been greatly accelerated by novel approaches using direct injection of DNA or mRNA of site-specific nucleases into the one-cell-stage embryo, generating DNA double-strand breaks (DSB) at specified sequences leading to targeted mutations (Carbery et al., 2010; Geurts et al., 2009; Shen et al., 2013; Sung et al., 2013; Tesson et al., 2011; Wang et al., 2013). Coinjection of a single-stranded or double-stranded DNA template containing homology to the sequences flanking the DSB can produce mutant alleles with precise point mutations or DNA inserts (Brown et al., 2013; Cui et al., 2011; Meyer et al., 2010; Wang et al., 2013; Wefers et al., 2013). Recently, pronuclear injection of two pairs of ZFNs and two double-stranded donor vectors into rat fertilized eggs produced rat containing loxP-flanked (floxed) alleles (Brown et al., 2013). However, the complex and time-consuming design and generation of ZFNs and double-stranded donor vectors limit the application of this method.

CRISPR (clustered regularly interspaced short palindromic repeat) and Cas (CRISPR-associated) proteins function as the RNA-based adaptive immune system in bacteria and archaea (Horvath and Barrangou, 2010; Wiedenheft et al., 2012). The type II bacterial CRISPR/Cas system has been demonstrated as an efficient gene-targeting technology that facilitates multiplexed gene targeting (Cong et al., 2013; Wang et al., 2013). Because the binding of Cas9 is guided by the simple base-pair complementarities between the engineered single-guide RNA (sgRNA) and a target genomic DNA sequence, it is possible to direct Cas9 to any genomic locus by providing the engineered sgRNA (Cho et al., 2013; Cong et al., 2013; Gilbert et al., 2013; Hwang et al., 2013; Jinek et al., 2012; Jinek et al., 2013; Mali et al., 2013b; Qi et al., 2013; Wang et al., 2013).

Previously, we used the type II bacterial CRISPR/Cas system as an efficient tool to generate mice carrying mutations in multiple genes in one step (Wang et al., 2013). However, this study left a number of issues unresolved. For example, neither the efficiency of using the CRISPR/Cas gene-editing approach for the insertion of DNA constructs into endogenous genes nor its utility to create conditional mutant mice was clarified. Here, we report the one-step generation of mice carrying reporter constructs in three different genes as well as the derivation of conditional mutant mice. In addition, we performed an extensive off-target cleavage analysis and show that off-target mutations are rare in targeted mice and ES cells derived from CRISPR/Cas zygote injection.

RESULTS

Targeted Insertion of Short DNA Fragments

In previous work, we introduced precise base pair mutations into the *Tet1* and *Tet2* genes through homology directed repair (HDR)-mediated genome editing following coinjection of single-stranded mutant DNA oligos, sgRNAs, and Cas9 mRNA (Wang et al., 2013). To test whether a larger DNA construct could be inserted at the same DSBs at *Tet1* exon 4 and *Tet2* exon 3, we

Table 1. Mice with Reporters in the Endogenous Genes

Donor	Blastocysts/Injected Zygotes	Targeted Blastocysts/Total		Targeted ESCs/Total	Transferred embryos (recipients)	Knockin pre- and postnatal mice/Total
Tet1-loxP + Tet2-loxP	65/89	Tet1-loxP	6/15	N/A	N/A	N/A
		Tet2-loxP	8/15			
		Both	3/15			
Sox2-V5	414/498	ND		7/16	200(10)	12/35
Nanog-mCherry	936/1262	86/936		ND	415 (21)	7/86
Oct4-GFP	254/345	47/254		3/9	100(4)	3/10

Cas9 mRNA, sgRNAs targeting *Tet1*, *Tet2*, *Sox2*, *Nanog*, or *Oct4*, and single-stranded DNA oligos or double-stranded donor vectors were injected into fertilized eggs. Targeted blastocysts were identified by RFLP or fluorescence of reporters. The blastocysts derived from injected embryos were derived ES cell lines or transplanted into foster mothers and E13.5 embryos and postnatal mice were obtained and genotyped.
ND, not determined.

designed oligos containing the 34 bp loxP site and a 6 bp EcoRI site flanked by 60 bps sequences on each side adjoining the DSBs (Figure S1A available online). We coinjected Cas9 mRNA, sgRNAs, and single-stranded DNA oligos targeting both *Tet1* and *Tet2* into zygotes. The restriction fragment length polymorphism (RFLP) assay shown in Figure S1B identified 6 out of 15 tested embryos carrying the loxP site at the *Tet1* locus, 8 carrying the loxP site at the *Tet2* locus, and 3 had at least one allele of each gene correctly modified. The correct integration of loxP sites was confirmed by sequencing (Figure S1C). These results demonstrate that HDR-mediated repair can introduce targeted integration of 40 bp DNA elements efficiently through CRISPR/Cas-mediated genome editing (summarized in Table 1).

Mice with Reporters in the Endogenous *Nanog*, *Sox2*, and *Oct4* Genes

Because the study of many genes and their protein products are limited by the availability of high-quality antibodies, we explored the potential of fusing a short epitope tag to an endogenous gene. We designed a sgRNA targeting the stop codon of *Sox2* and a corresponding oligo to fuse the 42 bp V5 tag into the last codon (Figure 1A). After injection of the sgRNA, Cas9 mRNA, and the oligo into zygotes, in vitro differentiated blastocysts were explanted into culture to derive ES cells. PCR genotyping and sequencing identified 7 out of 16 ES cell lines carrying a correctly targeted insert (Figures 1B and 1C). Western blot analysis revealed a protein band at the predicted size using V5 antibody in targeted ES cells but not in the control cells (Figure 1D). As expected from a correctly targeted and functional allele, *Sox2* expression was seen in targeted blastocysts and ES cells using V5 antibody (Figures 1E and 1F). Twelve of 35 E13.5 embryos and live-born mice derived from injected zygotes carried the V5 tag correctly targeted into the *Sox2* gene as indicated by PCR genotyping and sequencing (data not shown, Table 1).

To assess whether a marker transgene could be inserted into an endogenous locus, we coinjected Cas9 mRNA, sgRNA, and a double-stranded donor vector that was designed to fuse a p2A-mCherry reporter with the last codon of the *Nanog* gene (Figure 2A). A circular donor vector was used to minimize random integrations. To assess toxicity and to optimize the concentration of donor DNA, we microinjected different amounts of Nanog-2A-mCherry vector. Injection with a high concentration of donor DNA (500 ng/μl) yielded mCherry-positive embryos with high efficiency, with most blastocysts being retarded, whereas injection with a lower donor DNA concentration (10 ng/μl) yielded mostly healthy blastocysts, most of which were mCherry-negative. When 200 ng/μl donor DNA was used, 75% (936/1,262) of the injected zygotes developed to blastocysts, 9% (86/936) of which were mCherry-positive (Figure 2C; Table S1). mCherry was mainly expressed in the inner cell mass (ICM), consistent with targeted integration of the mCherry transgene into the *Nanog* gene. We derived six ES cell lines from mCherry-positive blastocysts, four of which uniformly expressed mCherry with the signal disappearing upon cellular differentiation (Figure 2C). The other two lines showed variegated mCherry expression, with some colonies being mCherry positive and others negative (Figure S2A, Table S2) consistent with mosaic donor embryos, which would be expected if transgene insertion occurred later than the zygote stage, as has been previously observed with ZNF and TALEN-mediated targeting (Brown et al., 2013; Cui et al., 2011; Wefers et al., 2013). Correct transgene integration in ES cell lines was confirmed by Southern blot analysis (Figure 2B). We also generated mice from injected zygotes. Southern blot analysis (Figures S2B and S2C) revealed that 7 out of 86 E13.5 embryos and live-born mice carried the mCherry transgene in the *Nanog* locus. One targeted mouse was mosaic (Table S2), because the intensity of targeted allele was lower than the wild-type allele (Figure S2B, #6). Two of the mice carried an additional randomly integrated transgene (Figure S2C, #3). As summarized in Tables 1 and S1, the efficiency of targeted insertion of the transgene was about 10% in blastocysts and mice derived from injected zygotes.

Finally, we designed sgRNA targeting the *Oct4* 3′ UTR, which was coinjected with a published donor vector designed to integrate the 3 kb transgene cassette (IRES-eGFP-loxP-Neo-loxP; Figure 2D) at the 3′ end of the *Oct4* gene (Lengner et al., 2007). Blastocysts were derived from injected zygotes, inspected for GFP expression, and explanted to derive ES cells. About 20% (47/254) of the blastocysts displayed uniform GFP expression in the ICM region. Three of nine derived ES cell lines expressed GFP (Figure 2E), including one showed mosaic expression (Table S2). Three out of ten live-born mice contained the targeted allele (Table 1). Correct targeting in mice and ES cell lines was confirmed by Southern blot analysis (Figure 2F).

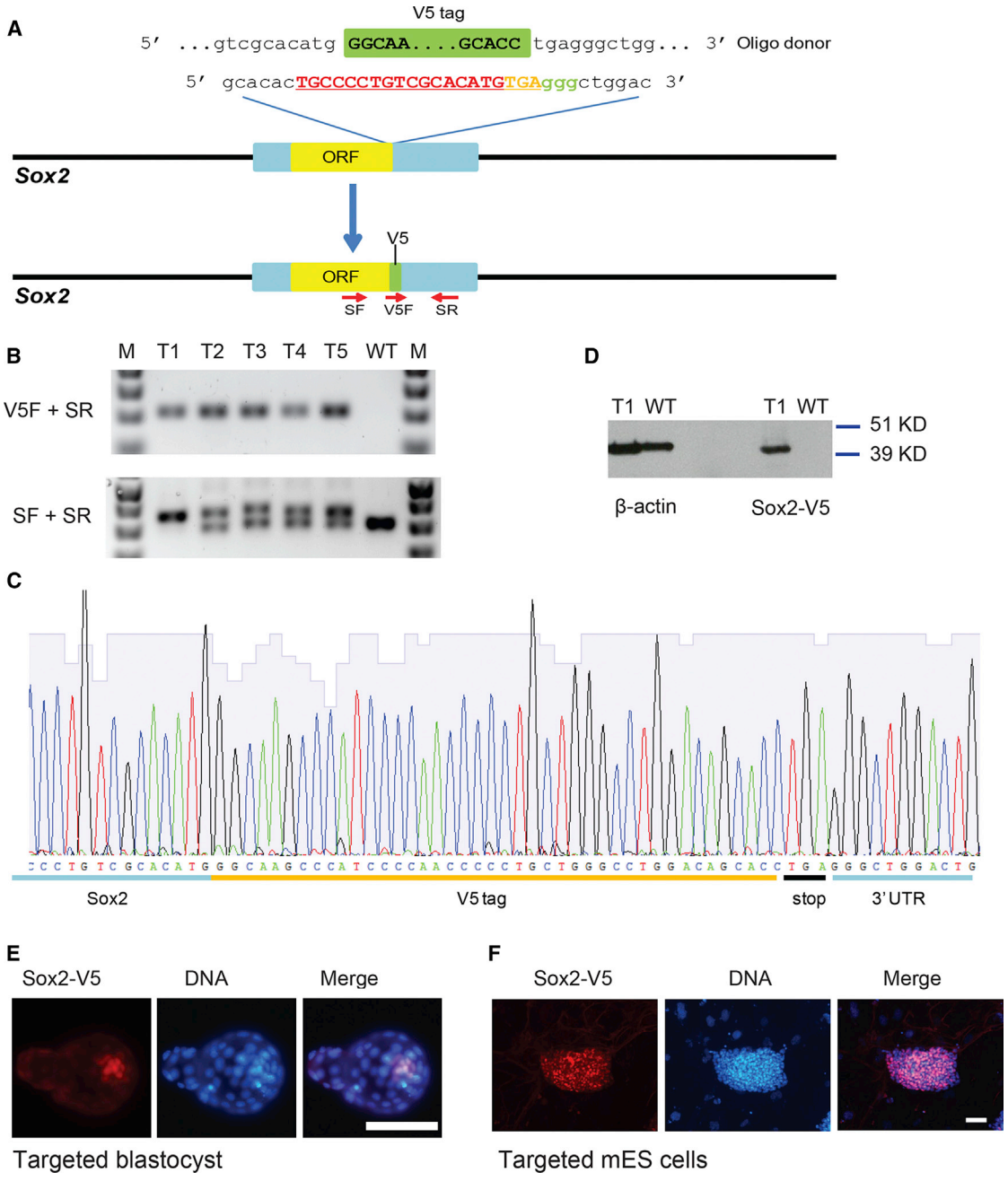


Figure 1. One-Step Generation of the *Sox2-V5* Allele

(A) Schematic of the Cas9/sgRNA/oligo-targeting site at the *Sox2* stop codon. The sgRNA coding sequence is underlined, capitalized, and labeled in red. The protospacer-adjacent motif (PAM) sequence is labeled in green. The stop codon of *Sox2* is labeled in orange. The oligo contained 60 bp homologies on both sides flanking the DSB. In the oligo donor sequence, the V5 tag sequence is labeled as a green box. PCR primers (SF, V5F, and SR) used for PCR genotyping are shown as red arrowheads.

(B) Top: PCR genotyping using primers V5F and SR produced bands with correct size in targeted ES samples T1 to T5, but not in WT sample. Bottom: PCR genotyping using primers SF and SR produced slightly larger products, indicating the 42 bp V5 tag sequence was integrated. T1 only contain larger product, suggesting either both alleles were targeted, or one allele failed to amplify.

(C) PCR products using primers SF and SR were cloned into plasmid and sequenced. Sequence across the targeting region confirmed correct fusion of V5 tag to the last codon of *Sox2*.

(D) Western blot analysis identified Sox2-V5 protein using V5 antibody in ES cells containing *Sox2-V5* allele. Beta-actin was shown as the loading control. Because beta-actin and Sox2-V5 run at the same size, the samples for the V5 signal and beta-actin were run in parallel on the same gel.

(E) Immunostaining of targeted blastocyst using V5 antibody showed signal in ICM. Scale bar, 50 μm.

(F) Immunostaining of targeted ES cells using V5 antibody showed uniform Sox2 expression. Scale bar, 100 μm.

Conventionally, transgenic mice are generated by pronuclear instead of cytoplasmic injection of DNA. To optimize the generation of CRISPR/Cas9-targeted embryos, we compared different concentrations of RNA and the Nanog-mCherry or the Oct4-GFP DNA vectors as well as three different delivery modes: (1) simultaneous injection of all constructs into the cytoplasm, (2) simultaneous injection of the RNA and the DNA into the pronucleus, and (3) injection of Cas9/sgRNA into the cytoplasm followed 2 hr later by pronuclear injection of the DNA vector. Table S1 shows that simultaneous injection of all constructs into the cytoplasm at a concentration of 100 ng/μl Cas9 RNA, 50 ng/μl of sgRNA and 200 ng/μl of vector DNA was optimal, resulting in 9% (86/936) to 19% (47/254) of targeted blastocysts. Similarly, the simultaneous injection of 5 ng/μl Cas9 RNA, 2.5 ng/μl of sgRNA, and 10 ng/μl of DNA vector into the pronucleus yielded between 9% (7/75) and 18% (13/72) targeted blastocysts. In contrast, the two-step procedure with Cas9 and sgRNA simultaneous injected into the cytoplasm followed 2 hr later by pronuclear injection of different concentrations of DNA vector yielded no or at most 3% (1/34) positive blastocysts. Thus, our results suggest that simultaneous injection of RNA and DNA into the cytoplasm or nucleus is the most efficient procedure to achieve targeted insertion.

Conditional *Mecp2* Mutant Mice

We investigated whether conditional mutant mice can be generated in one step by insertion of two loxP sites into the same allele of the *Mecp2* gene. To derive conditional mutant mice similar to those previously described using traditional homologous recombination methods in ES cells (Chen et al., 2001), we designed two sgRNAs targeting *Mecp2* intron 2 (L1, L2) and two sgRNAs targeting intron 3 (R1, R2), as well as the corresponding loxP site oligos with 60 bp homology to sequences on each side surrounding each sgRNA-mediated DSB (Figure S3A). To facilitate detection of correct insertions, the oligos targeting intron 2 were engineered to contain a NheI restriction site and the oligos targeting intron 3 to contain an EcoRI site in addition to the loxP sequences (Figures 3A and S3A). To determine the efficiency of single loxP site integration at the *Mecp2* locus, we injected Cas9 mRNA and each single sgRNA and corresponding oligo into zygotes, which were cultured to the blastocyst stage and genotyped by the RFLP assay. As shown in Figure S3B, the L2 and R1 sgRNAs were more efficient in integrating the oligos with four out of eight embryos carrying the L2 oligo and two out of six embryos carrying the R1 oligo. Therefore, L2 and R1 sgRNAs and the corresponding oligos were chosen for the generation of a floxed allele (Figure 3A).

A total of 98 E13.5 embryos and mice were generated from zygotes injected with Cas9 mRNA, sgRNAs, and DNA oligos targeting the L2 and R1 sites. Genomic DNA was digested with both NheI and EcoRI, and analyzed by Southern blot using exon 3 and exon 4 probes (Figures 3A and 3B). The L2 and R1 oligos contained, in addition to the loxP site, different restriction sites (NheI or EcoRI). Thus, single loxP site integration at L2 or R1 will produce either a 3.9 kb or a 2 kb band, respectively, when hybridized with the exon3 probe (Figures 3A and B). We found that about 50% (45/98) of the embryos and mice carried a loxP site at the L2 site and about 25% (25/98) at the R1 site. Importantly, integration of both loxP sites on the same DNA molecule, generating a floxed allele, produces a 700 bp band as detected by exon 3 probe hybridization (Figures 3A and 3B). RFLP analysis, sequencing (Figures S4A and S4B) and Southern blot analysis (Figure 3B) showed that 16 out of the 98 mice tested contained two loxP sites flanking exon 3 on the same allele. Table 2 summarizes the frequency of all alleles and shows that the overall insertion frequency of an L2 or R1 insertion was slightly higher in females (21/38) than in males (28/60) consistent with the higher copy number of the X-linked *Mecp2* gene in females. To confirm that the floxed allele was functional, we used genomic DNA for in vitro Cre-mediated recombination. Upon Cre treatment, both the deletion and circular products were detected by PCR in targeted mice, but not in DNA from wild-type mice (Figure 3C). The PCR products were sequenced and confirmed the precise Cre-loxP-mediated recombination (Figure S4C).

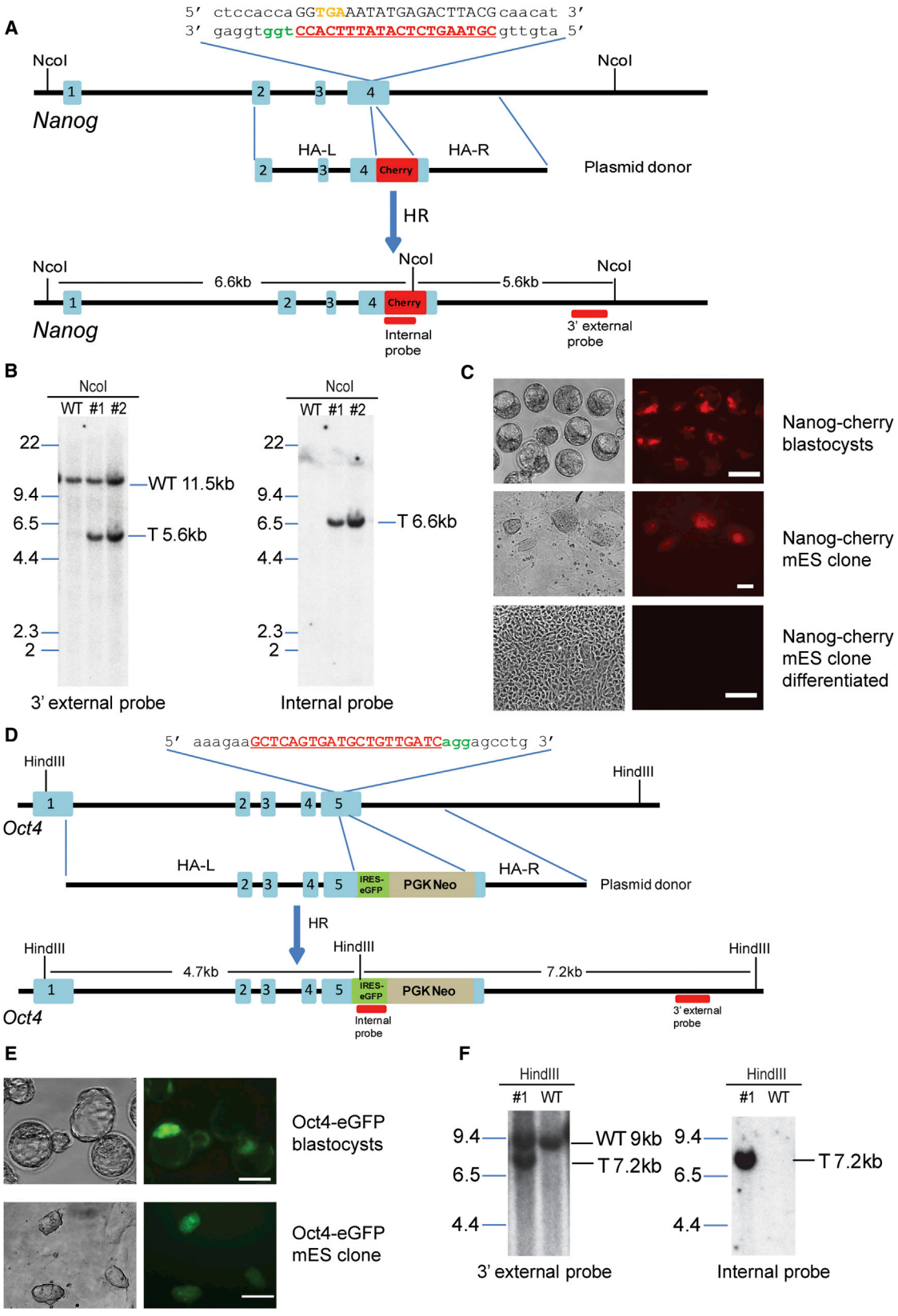
We noticed that some pups carried large deletions but no loxP insertions, raising the possibility that two cleavage events may generate defined deletions. To confirm this notion, we coinjected Cas9 mRNA, Mecp2-L2, and R1 sgRNAs but without oligos. PCR genotyping and sequencing (Figures 3D and 3E) revealed that 8 out of 23 mice carried deletions of about 700 bp spanning the L2 and R1 sites removing exon 3. This was confirmed by Southern analysis (data not shown). Because DNA breaks are repaired through the nonhomologous end joining (NHEJ) pathway, the ends of the breaks are different in different deletion alleles (Figure 3E).

Mosaicism

As mentioned above, we noted that some animals were mosaic for the targeted insertion. We decided to characterize the frequency of mosaicism in *Mecp2*-targeted mice by Southern blot analysis. Because *Mecp2* is an X-linked gene, in males more than one allele and in females more than two different alleles suggest mosaicism, which would be expected if integration occurred after the zygote stage. For example, as shown in Figure 3B, female mouse #2 contained three different alleles (one WT allele, one floxed allele, and one L2-loxP allele), and female mouse #4 contained four different alleles (one WT allele, one floxed allele, one L2-loxP allele, and one R1-loxP allele). Male mouse #5 contained two different alleles, with each allele carrying a single loxP site (Figure 3B). We identified eight mosaics out of 16 mice containing a *Mecp2* floxed allele. The frequency of mosaicism among 49 embryos and mice containing loxP site was about 40% (20/49) (Table S2). Because Southern blot analysis cannot detect small in-del mutations caused by NHEJ repair, it is possible that this underestimates the overall mosaicism frequency.

Off-Target Analysis

Two recent studies identified a high level of off-target cleavage in human cell lines using the CRISPR/Cas system, with Cas9-targeting specificity being shown to tolerate small numbers of mismatches between sgRNA and target DNA in a sequence and position-dependent manner (Fu et al., 2013; Hsu et al., 2013). Similarly, using a transcription-based method, Mali et al. (2013a) reported that Cas9/sgRNA binding could tolerate up to three mismatches.



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We characterized potential off-target (OT) mutations in mice and ES cell lines derived from zygotes injected with Cas9 and sgRNAs targeting the *Sox2*, the *Nanog*, the *Oct4*, and the *Mecp2* gene. We identified all genomic loci containing up to three or four base pair mismatches compared to the 20 bp sgRNA coding sequence (Table S3). We amplified all 13 potential OT sites of *Sox2* sgRNA in six mice and four ES cell lines carrying the *Sox2-V5* allele and tested for potential off target mutations using the Surveyor assay. No mutation was detected in any locus. When nine *Nanog* sgRNA potential OT sites (including seven genomic loci containing four base pair mismatches in the PAM distal region) were tested in five correctly targeted mice and four targeted ES cell lines, mutations were found in seven samples at OT1 (Table S3). Since *Nanog* OT1 has only one base pair difference at the very 5′ end of the sgRNA (position 20, numbered 1–20 in the 3′ to 5′ direction of sgRNA target site), it may not be surprising to find such a high frequency of mutations at this locus. In contrast, no off-target mutation was seen in any other *Nanog* OTs, which contain three or four base pair difference. For *Oct4*, we tested all 11 OT sites containing up to three base pair mismatches in three targeted mice and three targeted ES cell lines. Mutations were found in four out of six samples at OT1, which has only one base pair mismatch at position 19, whereas no off-target mutation was identified in any other *Oct4* OTs, which contain two or three base pair mismatches (Table S3). Finally, four potential off-targets sites for *Mecp2* L2, and ten sites for *Mecp2* R1 were analyzed in ten mice carrying a *Mecp2* floxed allele. Only one off-target mutation was identified in one mouse at the *Mecp2* R1 OT2 (Table S3). In summary, we tested all potential off-target sites differing by up to three or four base pairs in 35 mice or ES cell lines and only identified mutations in one off-target site for *Nanog* (OT1 7/9 samples, one mismatch at position 20), *Oct4* (OT1 4/6 samples, 1 mismatch at position 19), and *Mecp2* (R1-OT2 1/10 samples, two mismatches at positions 7 and 20). No off target mutation was identified in any genomic locus containing three base pair mismatches. Thus, although the off-target mutation rate was lower than what had been observed in the previous studies using cultured human cancer cell lines, our results are consistent with the conclusion that two or more interspaced mismatches dramatically reduce Cas9 cleavage (Fu et al., 2013; Hsu et al., 2013).

DISCUSSION

In this study, we demonstrate that CRISPR/Cas technology can be used for efficient one-step insertions of a short epitope or longer fluorescent tags into precise genomic locations, which will facilitate the generation of mice carrying reporters in endogenous genes. Mice and embryos carrying reporter constructs in the *Sox2,* the *Nanog* and the *Oct4* gene were derived from zygotes injected with Cas9 mRNA, sgRNAs, and DNA oligos or vectors encoding a tag or a fluorescent marker. Moreover, microinjection of two *Mecp2*-specific sgRNAs, Cas9 mRNA, and two different oligos encoding loxP sites into fertilized eggs allowed for the one-step generation of conditional mutant mice. In addition, we show that the introduction of two spaced sgRNAs targeting the *Mecp2* gene can produce mice carrying defined deletions of about 700 bp. Though all RNA and DNA constructs were injected into the cytoplasm or nucleus of zygotes, the gene modification events could happen at the one-cell stage or later. Indeed, Southern analyses revealed mosaicism in 17% (1/6) to 40% (20/49) of the targeted mice and ES cell lines indicating that the insertion of the transgenes had occurred after the zygote stage (Table S2).

Our previous experiments (Wang et al., 2013) demonstrated an efficiency of CRISPR/Cas sgRNA-mediated cleavage that was high enough to allow for the one-step production of engineered mice up to 90% of which carried homozygous mutations in two genes (four mutant alleles). The results reported here show that the sgRNA-mediated DSBs occur at a significantly higher frequency than insertion of exogenous DNA sequences. Therefore, the allele not carrying the insert will likely be mutated as a consequence of NHEJ-based gene disruption. Thus, the reporter allele would need to be segregated away from the mutant allele in order to produce mice carrying a reporter as well as a wild-type allele.

Two recent studies reported a high off-target mutation rate in CRISPR/Cas9-transfected human cell lines (Fu et al., 2013; Hsu et al., 2013). We analyzed the off-target rate for five different sgRNAs and identified cleavage of *Nanog* OT1, *Oct4* OT1, and *Mecp2* R1 OT2. *Nanog* OT1 has only one base pair difference from the targeting sequence at the extreme 5′ end (position 20), whereas *Oct4* OT1 contains one base pair mismatch at position 19; and *Mecp2* R1 OT2 has one base pair mismatch

Figure 2. One-Step Generation of an Endogenous Reporter Allele

(A) Schematic overview of strategy to generate a *Nanog-mCherry* knockin allele. The sgRNA coding sequence is underlined, capitalized, and labeled in red. The protospacer-adjacent motif (PAM) sequence is labeled in green. The stop codon of *Nanog* is labeled in orange. The homologous arms of the donor vector are indicated as HA-L (2 kb) and HA-R (3 kb). The restriction enzyme used for Southern blot analysis is shown, and the Southern blot probes are shown as red boxes.

(B) Southern analysis of *Nanog-mCherry* targeted allele. NcoI-digested genomic DNA was hybridized with 3′ external probe. Expected fragment size: WT (wild-type) = 11.5 kb, T (targeted) = 5.6 kb. The blot was then stripped and hybridized with mCherry internal probe. Expected fragment size: WT = N/A, T = 6.6 kb.

(C) *Nanog-mCherry* targeted blastocysts showed expression in ICM. Mouse ES cell lines derived from targeted blastocysts remain mCherry positive, and the mCherry expression disappear upon differentiation. Scale bar, 100 μm.

(D) Schematic overview of strategy to generate an *Oct4-eGFP* knockin allele. The sgRNA coding sequence is underlined, capitalized, and labeled in red. The PAM sequence is labeled in green. The homologous arms of the donor vector are indicated as HA-L (4.5 kb) and HA-R (2 kb). The IRES-eGFP transgene is indicated as a green box, and the PGK-Neo cassette is indicated as a gray box. The restriction enzyme used for Southern blot analysis is shown, and the Southern blot probes are shown as red boxes.

(E) *Oct4-eGFP* targeted blastocysts showed expression in ICM. Scale bar, 50 μm. Mouse ES cell lines derived from targeted blastocysts remain GFP positive. Scale bar, 100 μm.

(F) Southern analysis of *Oct4-eGFP* targeted allele. Southern analysis of *Oct4-eGFP* targeted allele. HindIII-digested genomic DNA was hybridized with 3′external probe. Expected fragment size: WT = 9 kb, Targeted = 7.2 kb. The blot was then stripped and hybridized with eGFP internal probe. Expected fragment size: WT = N/A, Targeted = 7.2 kb. See also Figure S2.

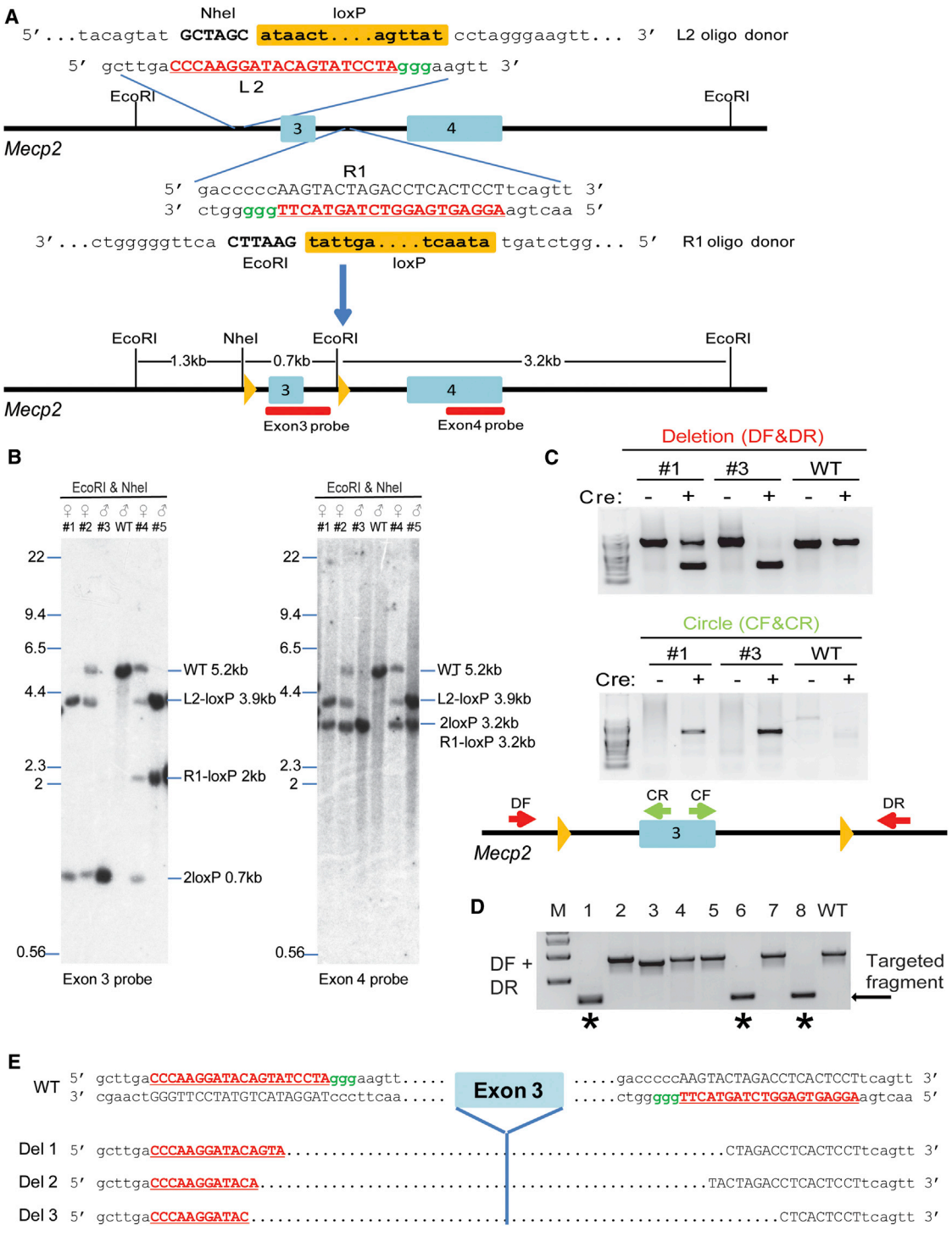

Figure 3. One-Step Generation of a *Mecp2* Floxed Allele

(A) Schematic of the Cas9/sgRNA/oligo targeting sites in *Mecp2* intron 2 and intron 3. The sgRNA coding sequence is underlined, capitalized, and labeled in red. The PAM sequence is labeled in green. In the oligo donor sequence, the loxP site is indicated as an orange box, and the restriction site sequences are in bold and capitalized. The oligo contained 60 bp homologies on both sides flanking the DSB. Restriction enzymes used for RFLP and Southern blot analysis are shown, and the Southern blot probes are shown as red boxes.

(B) Southern analysis of targeted alleles. Data of five mice are shown. EcoRI/NheI-digested genomic DNA was hybridized with the exon3 probe. Expected fragment size: WT = 5.2 kb, 2loxP = 0.7 kb, L2-loxP = 3.9 kb, and R1-loxP = 2 kb. The blot was then stripped and hybridized with the exon 4 probe. Expected fragment size: WT = 5.2 kb, 2loxP = 3.2 kb, L2-loxP = 3.9 kb, and R1-loxP = 3.2 kb. The sequence of the floxed allele is shown in Figure S4B.

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Table 2. Conditional *Mecp2* Mutant Mice

Donor	Blastocyst/Injected Zygotes	Transferred Embryos (Recipients)	Sex	Pre- and Postnatal Mice with loxP/Total				
				Total[a]	L2[b]	R1[c]	Two loxP in Two Alleles	Two loxP in One Allele
Mecp2-L2 + Mecp2-R1	367/451	360(18)	Male	28/60	26/60	12/60	2[d]/60	8/60
			Female	21/38	19/38	13/38	3/38	8/38
			Total	49/98	45/98	25/98	5/98	16/98

Cas9 mRNA, sgRNAs targeting *Mecp2*-L2 and *Mecp2*-R1, and single-stranded DNA oligos were injected into fertilized eggs. The blastocysts derived from the injected embryos were transplanted into foster mothers and pre- and postnatal mice were genotyped.
[a]Total mice containing loxP site integration in the genome.
[b]Mice containing loxP site integrated at L2 site.
[c]Mice containing loxP site integrated at R1 site.
[d]These male mice were mosaic.

at position 20, and one mismatch at position 7. Thus, the only off-target mutations in 2 bp mismatch targets were seen when one of the mismatches was at the distal 5′ end. This result is consistent with previous findings that Cas9 can catalyze DNA cleavage in the presence of single-base mismatches in the PAM distal region (Cong et al., 2013; Hsu et al., 2013; Jiang et al., 2013; Jinek et al., 2012). No mutations were detected in 42 potential OTs of *Sox2*, *Nanog*, *Oct4*, or *Mecp2* containing 3 or 4 bp mismatches in a total of 35 mice and ES cell lines tested, consistent with the observation that three or more interspaced mismatches dramatically reduce Cas9 cleavage (Hsu et al., 2013). Thus, for designing the most suitable target sequences for generating Cas9 cleavage-mediated, genetically modified mice, it is important to avoid targeting sites that have only one or two mismatches in other genomic loci. This is particularly important for mismatches that are at the PAM distal region.

We consider several possibilities to explain the lower off-target cleavage rate seen in animals derived from manipulated zygotes and the results reported for CRISPR/Cas-treated human cell lines (Fu et al., 2013; Hsu et al., 2013). (1) In our study, the off-target mutagenesis was based on the analysis of a "clonal genome" in animals derived from a single manipulated zygote in contrast to the previous reports that analyzed heterogeneous cell populations. The surveyor assay, based upon extensive PCR amplification, may identify any mutation, even very rare alleles that may be present in the heterogeneous population. (2) The transformed human cell lines may have different DNA damage responses resulting in a different mutagenesis rate than the normal one-cell embryo. (3) In our experiments CRISPR/Cas was injecting as short-lived RNA in contrast to Fu et al. (2013) and Hsu et al. (2013), who used DNA plasmid transfection, which may express the Cas9/sgRNA for longer time periods leading to more extensive cleavage. Thus, our data suggest high specificity of the CRISPR/Cas9 system for gene editing in early embryos aimed at generating gene-modified mice. Nevertheless, future characterization of off-target mutagenesis of CRISPR/Cas system using whole-genome sequencing would be highly informative and may allow designing sgRNAs with higher specificity.

In summary, CRISPR/Cas-mediated genome editing represents an efficient and simple method of generating sophisticated genetic modifications in mice such as conditional alleles and endogenous reporters in one step. The principles described in this study could be directly adapted to other mammalian species, opening the possibility of sophisticated genome engineering in many species where ES cells are not available.

EXPERIMENTAL PROCEDURES

Production of Cas9 mRNA and sgRNA

Bicistronic expression vector px330 expressing Cas9 and sgRNA (Cong et al., 2013) was digested with BbsI and treated with Antarctic Phosphatase, and the linearized vector was gel purified. A pair of oligos (Table S4) for each targeting site was annealed, phosphorylated, and ligated to the linearized vector.

T7 promoter was added to Cas9 coding region by PCR amplification using primer Cas9 F and R (Table S4). T7-Cas9 PCR product was gel purified and used as the template for in vitro transcription (IVT) using mMESSAGE mMACHINE T7 ULTRA kit (Life Technologies). T7 promoter was added to sgRNAs template by PCR amplification using primer listed in Table S4. 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.

Single-Stranded and Double-Stranded DNA Donors

All single-stranded oligos were ordered as Ultramer DNA oligos from Integrated DNA Technologies. Nanog-2A-mCherry vector was modified from previously published targeting vector Nanog-2A-mCherry-PGK-Neo (Faddah et al., 2013). Nanog-2A-mCherry-PGK-Neo was digested with PacI and AscI to drop out the PGK-Neo cassette; the 9.7 kb fragment was gel purified and blunt-ended using T4 DNA polymerase (New England Biolabs) then self-ligated using T4 DNA ligase (New England Biolabs). Oct4-IRES-eGFP-PGK-Neo vector is previously published (Lengner et al., 2007).

(C) In vitro Cre-mediated recombination of the floxed *Mecp2* allele. The genomic DNA of targeted mice #1 and #3 was incubated with Cre recombinase, and used as PCR template. Primers DF and DR flanking the floxed allele produce shorter products upon Cre-dependent excision. Primers CF and CR detect the circular molecule, which only form upon Cre-loxP recombination. The position of each primer is shown at the bottom cartoon. The deletion and circular PCR products were sequenced and the sequences are shown in Figure S4C.
(D) Injection of Cas9 mRNA and both L2 and R1 sgRNA generated *Mecp2* mutant allele with deletion of exon 3. PCR genotyping using primers DF and DR identified defined deletion events in mice #1, #6, and #8 (indicated by stars).
(E) Sequences of three mutant alleles with exon 3 deletions in three mice. R2 and L1 sgRNA coding sequences were underlined, capitalized, and labeled in red. The PAM sequence is labeled in green. See also Figures S3 and S4.

Suveryor Assay and RFLP Analysis for Genome Modification

Suveryor assay was performed as described (Guschin et al., 2010). Genomic DNA from targeted and control mice or embryos was extracted and PCR was performed using gene-specific primers (Table S4) under the following conditions: 95°C for 5 min; 35 × (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. For RFLP analysis, 10 μl of *Tet1*, *Tet2*, *Mecp2*-R1, R2 PCR product was digested with EcoRI, 10 μl of *Mecp2*-L1, and L2 PCR product was digested with NheI. 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.

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. Different concentrations of Cas mRNA, sgRNA, and oligos or plasmid vectors were mixed and injected into the cytoplasm or pronucleus of fertilized eggs with well-recognized pronuclei in M2 medium (Sigma). 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.

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 ^{32}P random primer (Stratagene)-labeled probes. Between hybridizations, blots were stripped and checked for complete removal of radioactivity before rehybridization with a different probe.

In Vitro Cre Recombination

A 20 μl reaction containing 1 μg of genomic DNA and 10 units of recombinant Cre recombinase (New England Biolabs) in 1× buffer was incubated at 37°C for 1 hr. For all targets, 1 μl of the Cre reaction mix was used as template for PCR reactions with gene-specific primers. For each target, primers DF and DR were used for detecting the deletion products, and primers CF and CR were used to detect the circle product. All products were sequenced.

Immunostaining and Western Blot Analysis

For immunostaining, cells in 24-well were fixed in PBS supplemented with 4% paraformaldehyde for 15 min at room temperature (RT). The cells were then permeabilized using 0.2% Triton X-100 in PBS for 15 min at RT. The cells were blocked for 30 min in 1% BSA in PBS. Primary antibody against V5 (ab9137, abcam) was diluted in the same blocking buffer and incubated with the samples overnight at 4°C. The cells were treated with a fluorescently coupled secondary antibody and then incubated for 1 hr at RT. The nuclei were stained with Hoechst 33342 (Sigma) for 5 min at RT.

For western blot, Cell pellets were lysed on ice in Laemmli buffer (62.5 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate, 5% beta-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue) for 30 min in presence of protease inhibitors (Roche Diagnostics), boiled for 5–7 min at 100°C, and subjected to western blot analysis. Primary antibodies: V5 (1:1,000, ab9137, Abcam), beta-actin (1:2,000). Blots were probed with anti-goat, or anti-rabbit IgG-HRP secondary antibody (1:10,000) and visualized using ECL detection kit (GE Healthcare).

ESC Derivation and Differentiation

Morulas or blastocysts were selected to generate ES cell lines. The zona pellucida was removed using acid Tyrode solution. Each embryo was transferred into one well of a 96-well plate seeded with ICR embryonic fibroblast feeders in ESC medium supplemented with 20% knockout serum replacement, 1,500 U/ml leukemia inhibitory factor (LIF), and 50 μM of the MEK1 inhibitor (PD098059). After 4–5 days in culture, the colonies were trypsinized and transferred to a 96-well plate with a fresh feeder layer in fresh medium. Clonal expansion of the ESCs proceeded from 48-well plates to 6-well plates with feeder cells and then to 6-well plates for routine culture.

For ESC differentiation, cells were harvested by trypsinization and transferred to bacterial culture dishes in the ES medium without or LIF. After 3 days, aggregated cells were plated onto gelatin-coated tissue culture dishes and incubated for another 3 days.

Prediction of Potential Off-Targets

Potential off-targets were predicted by searching the mouse genome (mm9) for matches to the 20 nt sgRNA sequence allowing for up to four mismatches (*Nanog*) or three mismatches (*Sox2*, *Oct4*, *Mecp2*-L2, and *Mecp2*-R1) followed by NGG PAM sequence. Matches were ranked first by ascending number of mismatches then by ascending distance from the PAM sequence.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.08.022.

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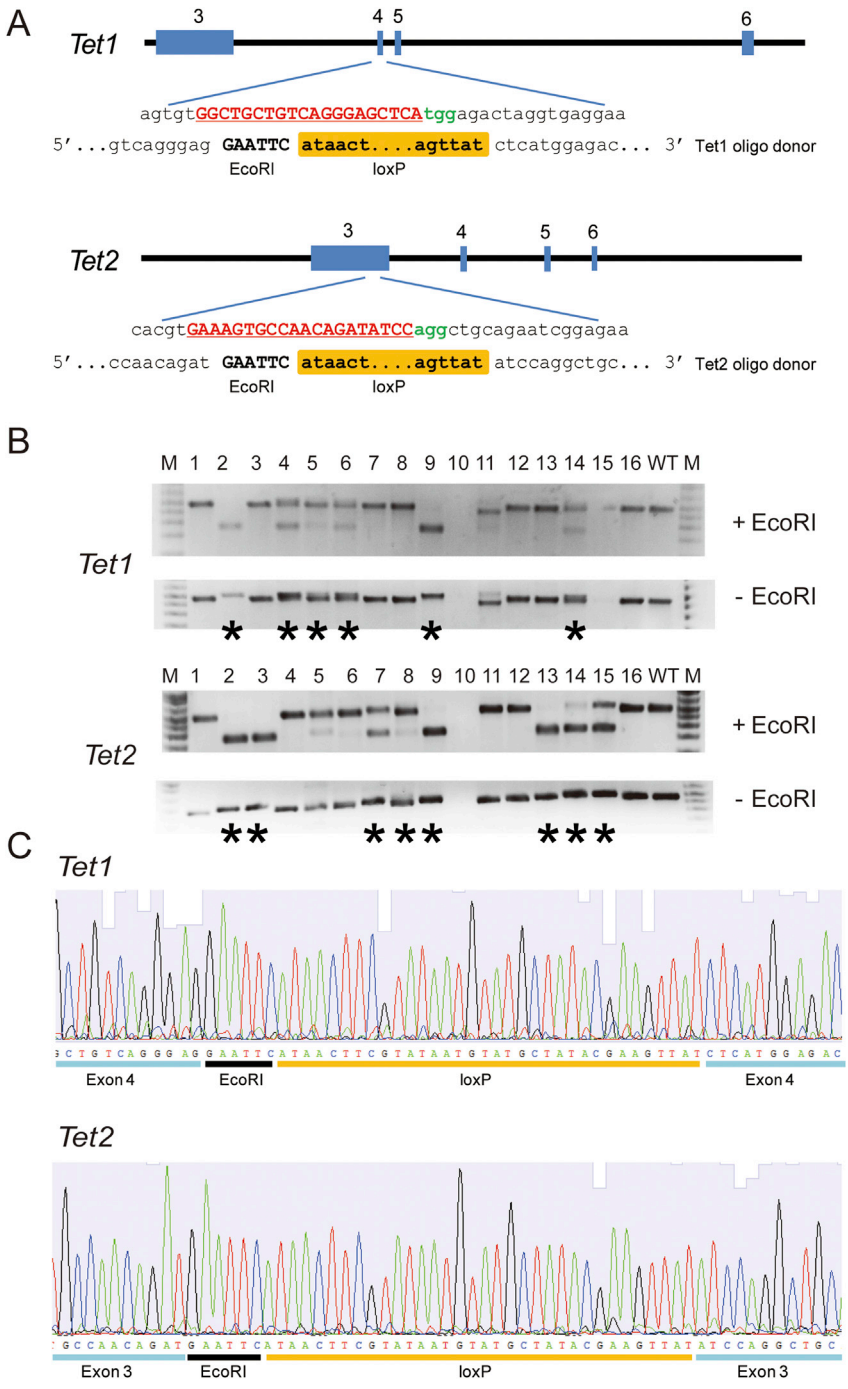

Figure S1. Integration of loxP Sites at *Tet1* and *Tet2* Loci, Related to Table 1

(A) Schematic of the Cas9/sgRNA/oligo targeting sites in *Tet1* exon 4 and *Tet2* exon 3. The sgRNA coding sequence is underlined, capitalized, and labeled in red. The PAM sequence is labeled in green. In oligo donor sequence, the loxP site is indicated by an orange box, and the restriction site sequence is in bold and capitalized.

(B) RFLP analysis of double sgRNA/oligo injection mice with HDR-mediated targeting at the *Tet1* and *Tet2* loci. About 500 bp regions around the targeting sites at *Tet1* and *Tet2* were amplified from 16 embryos and digested with EcoRI. The #10 embryo failed to be amplified. A corrected targeted allele is identified as a cleaved fragment. Samples containing targeted alleles are indicated by stars.

(C) The sequences of targeted alleles of *Tet1* and *Tet2* in sample #2 and #9 confirmed precise integration of loxP sites at both loci.

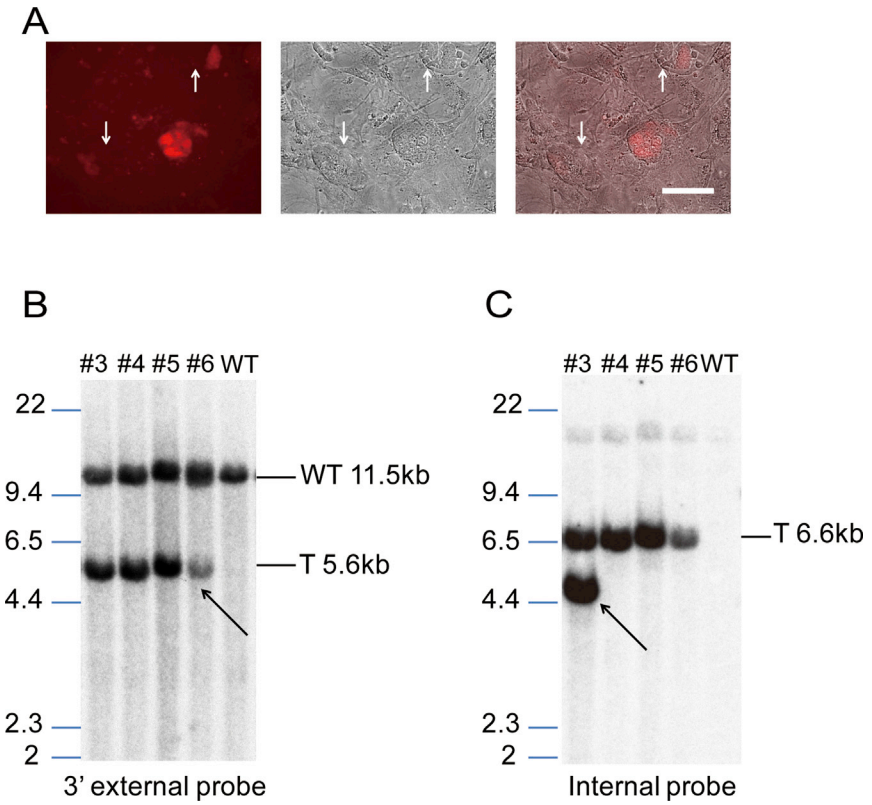

Figure S2. Characterization of *Nanog-mCherry* Alleles, Related to Figure 2 and Tables 1, S1, and S2

(A) ES cell line with mosaic expression of mCherry. The mCherry-negative colony is indicated by arrow.

(B) Southern analysis of *Nanog-mCherry* targeted allele identified mosaic animal. NcoI-digested genomic DNA was hybridized with 3′external probe. Expected fragment size: WT = 11.5 kb, T = 5.6 kb. Mouse #6 is identified as mosaic, because the targeted band (indicated by arrow) is weaker than WT band.

(C) The blot was then stripped and hybridized with mCherry internal probe. Expected fragment size: WT = N/A, Targeted = 6.6 kb. In addition to the targeted allele, one extra band (indicated by arrow) is present in mouse #3, indicating a random insertion of the donor vector.

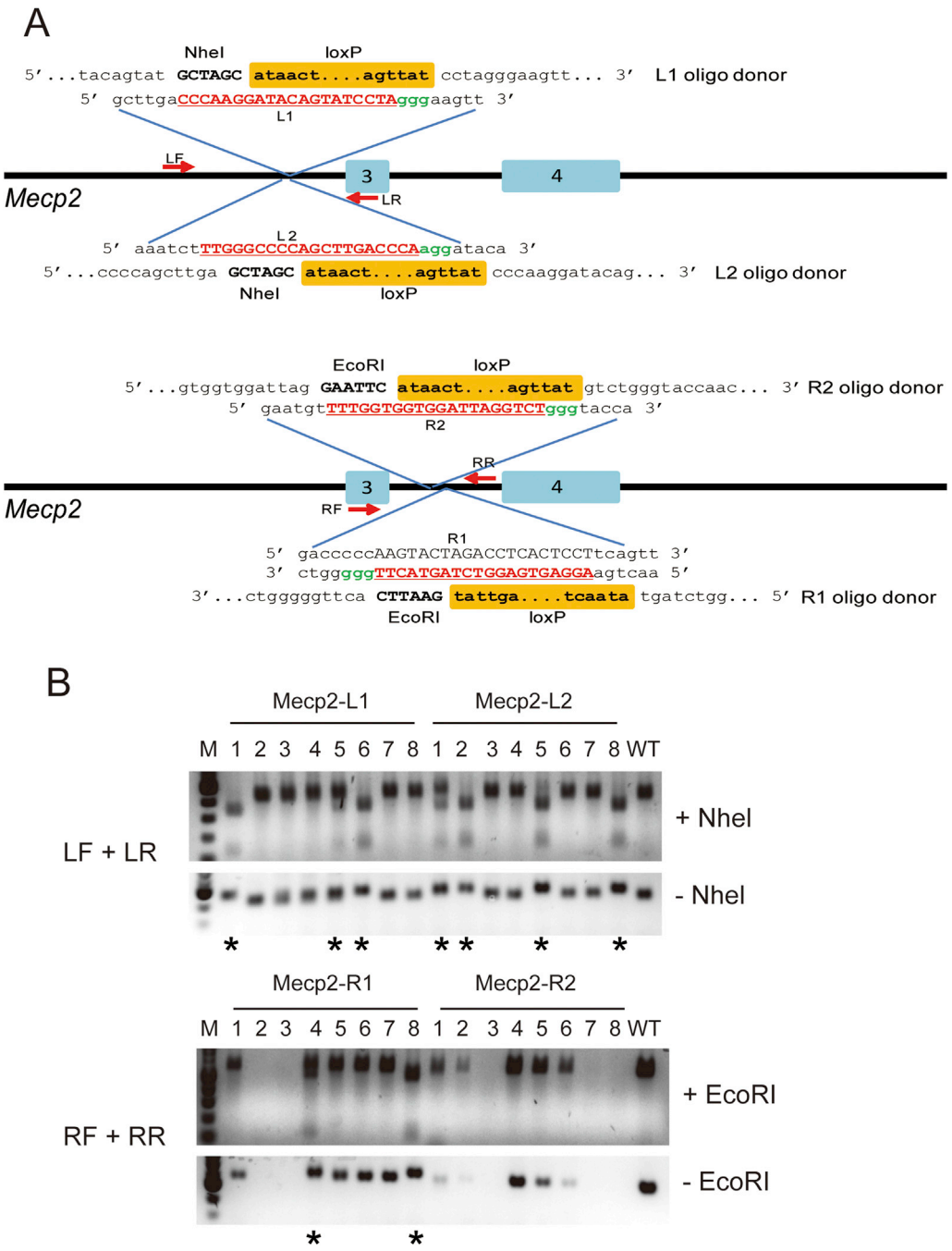

Figure S3. Integration of loxP Sites at *Mecp2* Intron 2 and 3, Related to Figure 3 and Table 2

(A) Schematic of the Cas9/sgRNA/oligo targeting sites. The sgRNA coding sequence is underlined, capitalized, and labeled in red. The PAM sequence is labeled in green. In oligo donor sequence, the loxP site is labeled as an orange box, and the restriction site sequence is in bold and capitalized. PCR primers used for RFLP analysis are shown as red arrows. For intron 2, two sgRNA coding sequences L1 and L2 are shown, and their corresponding oligos are named accordingly. For intron 3, R1, R2 and their targeting oligos are shown. PCR primers LF and LR are used to amplify the intron 2 region, while RF and RR are used to amplify the intron 3 region.

(B) RFLP analysis of single sgRNA/oligo injection mice with HDR-mediated targeting at *Mecp2* intron 2 or intron 3. Cleavage of PCR product upon NheI or EcoRI digestion indicates loxP integration at intron 2 or intron 3 respectively. LoxP integration efficiency at L1, L2, R1, and R2 sites are compared. Samples containing loxP site are labeled by stars. Three out of eight samples contained a loxP site at the L1 site, and four out of eight contained a loxP site at the L2 site. Two out of six samples contained loxP site at R1 site, while none was detected at the R2 site. Primers used for each PCR are labeled.

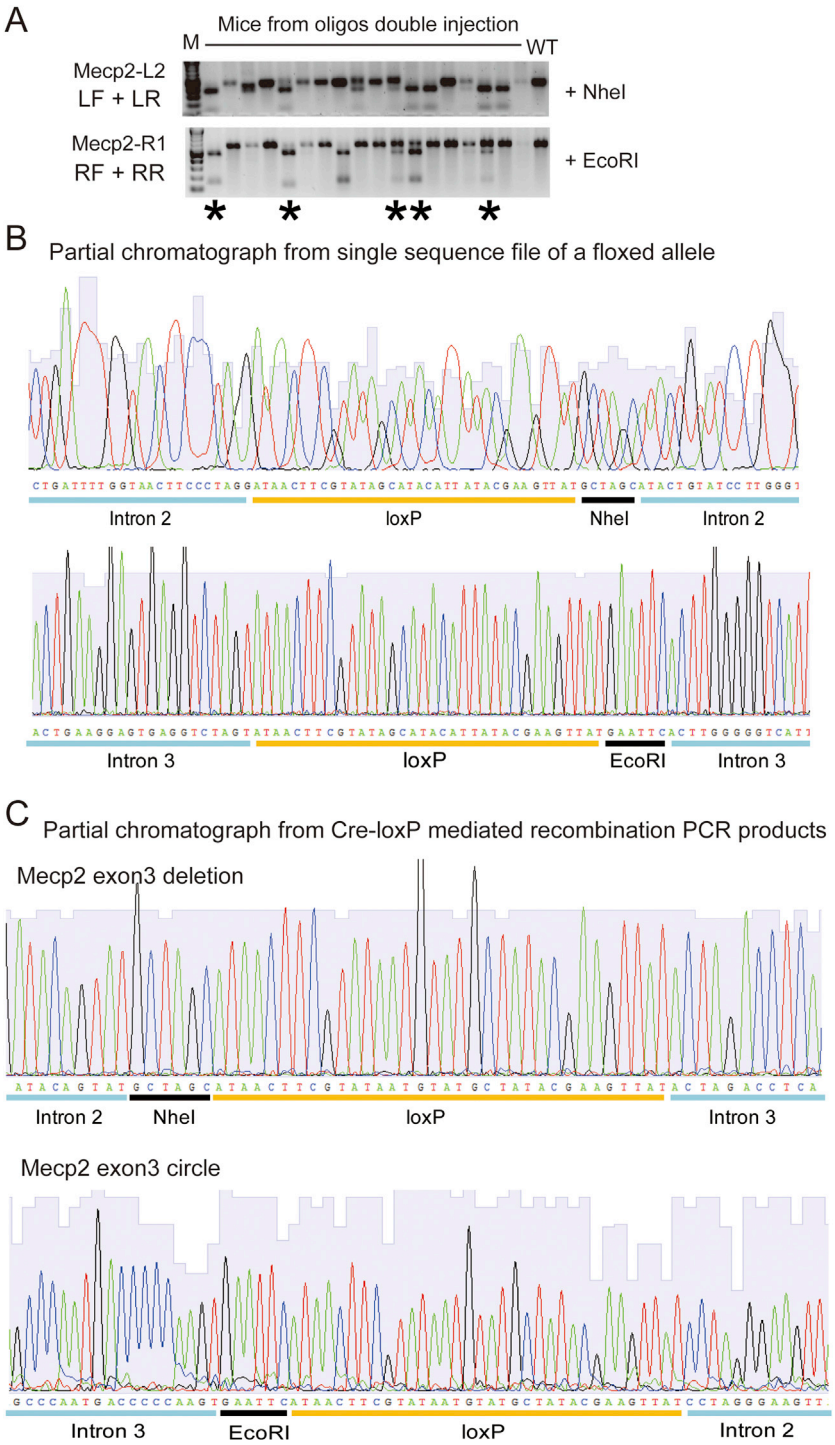

Figure S4. Analysis of *Mecp2* Floxed Allele, Related to Figure 3 and Table 2

(A) RFLP analysis detected loxP integration at intron 2 (Mecp2-L2) and intron 3 (Mecp2-R1) in mice derived from L2 and R1 double sgRNA/oligo injections. Primers LF and LR were used to amplify intron 2 region, and RF and RR were used to amplify intron 3 region. Mice containing loxP sites in both introns are marked by stars.

(B) Partial chromatograph from one single sequencing file crossing both loxP sites, exon 3, and flanking intron sequences.

(C) Partial chromatograph from sequences of Cre-mediated recombination PCR products (deletion and circular products from Figure 3C).

Table S1. Efficiency of Generation of Reporter Embryos by Cytoplasm and Pronuclear Injection, Related to Figure 2 and Table 1

Donor	Dose of Cas9/sgRNA (ng/μl)	Dose of Donor vector (ng/μl)	Injected zygotes	Targeted Blastocysts / Total
One-step injection				
Nanog-mCherry	100/50 (Cyto)	500(Cyto)	186	1/81
Nanog-mCherry	100/50 (Cyto)	200(Cyto)	1262	86/936
Nanog-mCherry	100/50 (Cyto)	50(Cyto)	402	7/308
Nanog-mCherry	100/50 (Cyto)	10(Cyto)	333	1/278
Oct4-GFP	100/50 (Cyto)	200(Cyto)	345	47/254
Nanog-mCherry	5/2.5 (Nuc)	10 (Nuc)	98	7/75
Oct4-GFP	5/2.5 (Nuc)	10 (Nuc)	105	13/72
Two-step injection				
Nanog-mCherry	100/50 (Cyto)	50 (Nuc)	45	0/0
Nanog-mCherry	100/50 (Cyto)	10 (Nuc)	91	1/34
Nanog-mCherry	100/50 (Cyto)	2 (Nuc)	85	1/68

Cas9 mRNA, sgRNAs targeting *Nanog*, or *Oct4*, and double stranded donor vectors were injected into cytoplasm or pronuclei of zygotes. In one-step injection, the RNA and the DNA were simultaneously injected into the cytoplasm or pronucleus. In two-step injection, Cas9/sgRNA were injected into the cytoplasm followed 2 hours later by pronuclear injection of the DNA vector. Targeted blastocysts were identified by fluorescence of reporters.

Cyto, cytoplasm; Nuc, nucleus.

Table S2. Mosaicism in Targeted Mice, Related to Figure S2 and Tables 1 and 2

Donor		Mosaic / Total targeted
Nanog-Cherry	Mice	1/7
	ESCs	2/6
	Total	3/13
Oct4-EGFP	Mice	0/3
	ESCs	1/3
	Total	1/6
Mecp2-L2 + Mecpe-R1	Male	11/28
	Female	9/21
	Total	20/49[a]

Targeted mice or ESCs were identified by RFLP, Southern bolt or Sequencing. The frequency of mosaicism in targeted mice was determined by fluorescent reporter or Southern blot analysis.

[a]These 49 mice contain at least one loxP integration.

Table S3. Off-Target Analysis, Related to Figures 1, 2, 3 and Tables 1 and 2

Site name	Sequence	Indel mutation frequency (Mutant/Total)	Coordinate
Target_Sox2_Stop	TGCCCCTGTCGCACATGTGAGGG	/	chr3: 34550278-34550300
OT1_Sox2_Stop	T**a**CCC**t**TGT**t**GCACATGTGAAGG	0/10	chr4: 126636377-126636399
OT2_Sox2_Stop	T**t**CCC**a**TGT**a**GCACATGTGAGGG	0/10	chr14: 58830941-58830963
OT3_Sox2_Stop	T**c**CCCCTGTC**a**CACATGTG**g**TGG	0/10	chr1: 136641174-136641196
OT4_Sox2_Stop	TGC**a**CCTGT**g**GCACATGTG**g**GGG	0/10	chr9: 69081892-69081914
OT5_Sox2_Stop	TGCC**a**C**a**GT**t**GCACATGTGAGGG	0/10	chr1: 130633965-130633987
OT6_Sox2_Stop	TGCC**a**CTGT**t**GCA**a**ATGTGAGGG	0/10	chr18: 61611640-61611662
OT7_Sox2_Stop	TGCC**t**CTGTC**a**CA**g**ATGTGACGG	0/10	chr5: 136841014-136841036
OT8_Sox2_Stop	TGCC**t**CTGTC**t**CACATGTG**c**TGG	0/10	chr4: 141162434-141162456
OT9_Sox2_Stop	TGCC**t**CTGTCGC**t**CATG**g**GATGG	0/10	chr9: 48224429-48224451
OT10_Sox2_Stop	TGCCC**a**TGTC**c**CACATG**g**GATGG	0/10	chr7: 72596616-72596638
OT11_Sox2_Stop	TGCCCCT**c**T**gt**CACATGTGAAGG	0/10	chr18: 56473819-56473841
OT12_Sox2_Stop	TGCCCCTGTC**at**ACATGTG**g**AGG	0/10	chr6: 98776389-98776411
OT13_Sox2_Stop	TGCCCCTGTC**t**C**c**CATGTG**c**TGG	0/10	chr4: 148868089-148868111
Target_Nanog_Stop[a]	CGTAAGTCTCATATTTCACCTGG	/	chr6: 122663559-122663581
OT1_Nanog_Stop	**t**GTAAGTCTCATATTTCACCTGG	7/9[b]	chrX: 87128718-87128740
OT2_Nanog_Stop	C**cc**AAGTCTCAT**t**TTTCACCAGG	0/9	chr14: 21598653-21598675
OT3_Nanog_Stop	**ga**TAAG**ga**TCATATTTCACCCGG	0/9	chrX:88301349-88301371
OT4_Nanog_Stop	**t**G**c**AA**t**T**t**TCATATTTCACCTGG	0/9	chr12:71841888-71841910
OT5_Nanog_Stop	**g**GT**c**A**tc**CTCATATTTCACCAGG	0/9	chr11:13346951-13346973
OT6_Nanog_Stop	**t**GT**t**A**t**TC**a**CATATTTCACCTGG	0/9	chr6:13888078-13888100
OT7_Nanog_Stop	**t**GT**g**AGT**ag**CATATTTCACCTGG	0/9	chr18:41037112-41037134
OT8_Nanog_Stop	**t**GTAA**a**T**aa**CATATTTCACCCGG	0/9	chrX:70168441-70168463
OT9_Nanog_Stop	C**a**TA**gag**CTCATATTTCACCAGG	0/9	chr4:80388067-80388089
Target_Oct4_Stop	GCTCAGTGATGCTGTTGATCAGG	/	chr17:35647634-35647656
OT1_ Oct4_Stop	G**t**TCAGTGATGCTGTTGATCTGG	4/6[b]	chr3:129137779-129137801
OT2_ Oct4_Stop	G**tc**CAGTGATGCTGTTGATCAGG	0/6	chr14:17938758-17938780
OT3_ Oct4_Stop	**c**CTCA**c**TGA**a**GCTGTTGATCAGG	0/6	chr16:77843697-77843719
OT4_ Oct4_Stop	**a**CTCAGTGAT**t**CTG**c**TGATCTGG	0/6	chr11:52953191-52953213
OT5_ Oct4_Stop	G**gg**CAGTGATGCTGTTGA**c**CAGG	0/6	chr14:119915346-119915368
OT6_ Oct4_Stop	G**g**TCAG**a**GATGCTG**a**TGATCAGG	0/6	chr1:100129231-100129253
OT7_ Oct4_Stop	GCT**t**AGTGATGCTGT**ac**ATCTGG	0/6	chr3:39458512-39458534
OT8_ Oct4_Stop	GCTCA**a**TGAT**a**CT**t**TTGATCTGG	0/6	chr5:16781367-16781389
OT9_ Oct4_Stop	GCTCA**a**TGAT**t**CTGTTGAT**t**GGG	0/6	chr4:35112775-35112797
OT10_ Oct4_Stop	GCTCAG**g**GATGC**a**GTTG**t**TCTGG	0/6	chr4:25918134-25918156
OT11_ Oct4_Stop	GCTCAGTGATGCTGT**ctg**TCTGG	0/6	chr6:138212621-138212643

Target_Mecp2_L2	CCCAAGGATACAGTATCCTAGGG	/	chrX: 71282802-71282824
OT1_Mecp2_L2	CCCAAGGAT**g**C**tt**TATCCTAAGG	0/10	chr8: 121441299-121441321
OT2_Mecp2_L2	CCCA**t**GGATA**g**AGTA**g**CCTAAGG	0/10	chr15: 55526927-55526949
OT3_Mecp2_L2	CCCAAG**a**ATACAGT**g**T**g**CTAAGG	0/10	chr4: 83371755-83371777
OT4_Mecp2_L2	CCCAAGGA**c**ACAG**g**ATCC**c**AAGG	0/10	chr17: 27887352-27887374
Target_Mecp2_R1	AGGAGTGAGGTCTAGTACTTGGG	/	chrX: 71282103-71282125
OT1_Mecp2_R1	AGG**g**GTGAG**t**TCTAGTACTTCGG	0/10	chr13: 48912459-48912481
OT2_Mecp2_R1	**t**GGAGTGAGGTCT**t**GTACTTGGG	1/10[b]	chr12: 15404584-15404606
OT3_Mecp2_R1	AGGAGT**ct**GG**g**CTAGTACTTGGG	0/10	chr6: 115474148-115474170
OT4_Mecp2_R1	A**a**GAGTGAGG**g**CTA**c**TACTTTGG	0/10	chr9: 74128922-74128944
OT5_Mecp2_R1	AGGAGTGAGGT**g**T**t**GT**g**CTTAGG	0/10	chr9: 69065264-69065286
OT6_Mecp2_R1	AGGAGTGAGGTCT**g**G**at**CTTGGG	0/10	chr5: 137155304-137155326
OT7_Mecp2_R1	AGGAGTGAGG**aa**TAGTA**a**TTGGG	0/10	chr5: 53385487-53385509
OT8_Mecp2_R1	AGGAG**g**GAGGTCT**c**GTA**a**TTTGG	0/10	chr10: 94407719-94407741
OT9_Mecp2_R1	AGGAGTG**g**GGTCTAGT**c**CT**c**AGG	0/10	chr1: 38087440-38087462
OT10_Mecp2_R1	AGGAGT**a**AGGTCTAGTAC**ca**AGG	0/10	chr4: 13635404-13635426

Mismatches from the on-target sequence are lower-case, boldface and underlined. Indel mutation frequencies in targeted mice or ESCs were calculated by Suveryor assay. Coordinate in which sites were located are shown. OT, off-target; /, not tested.

[a]Nanog OT1 and 2 contain 3bp mismatches; OT3 to 9 contain 4bp mismatches lying in PAM distal region.

[b]PCR products were cloned and sequenced to confirm off-target mutations.

Table S4. Oligonucleotides Used In This Study

Oligonucleotides used for cloning sgRNA expression vector

Gene target	Direction	Sequence (5’ to 3’)
Tet1	F	caccggctgctgtcagggagctca
	R	aaactgagctccctgacagcagcc
Tet2	F	caccgaaagtgccaacagatatcc
	R	aaacggatatctgttggcactttc
Sox2	F	caccgtgcccctgtcgcacatgtga
	R	aaactcacatgtgcgacaggggcac
Nanog	F	caccgcgtaagtctcatatttcacc
	R	aaacggtgaaatatgagacttacgc
Oct4	F	caccgctcagtgatgctgttgatc
	R	aaacgatcaacagcatcactgagc
Mecp2 L1	F	caccgttgggccccagcttgaccca
	R	aaactgggtcaagctggggcccaac
Mecp2 L2	F	caccgcccaaggatacagtatccta
	R	aaactaggatactgtatccttgggc
Mecp2 R1	F	caccgaggagtgaggtctagtactt
	R	aaacaagtactagacctcactcctc
Mecp2 R2	F	caccgtttggtggtggattaggtct
	R	aaacagacctaatccaccaccaaac

Oligonucleotides used for RFLP analysis and PCR genotyping.

Gene target	Direction	Sequence (5’ to 3’)
Tet1	F	ttgttctctcctctgactgc
	R	tgattgatcaaataggcctgc
Tet2	F	cagatgcttaggccaatcaag
	R	agaagcaacacacatgaagatg
Sox2	SF	acatgatcagcatgtacctcc
	SR	taatttggatgggattggtgg
V5	V5F	acatgggcaagcccatcc
Mecp2 L1,L2	LF	aatgtgccactttaacagcac
	LR	ttctgatgtttctgctttgcc
Mecp2 R1,R2	RF	aagcatgagccactacaacc
	RR	cttgctcagaagccaaaacag

Oligonucleotides used for making template for in vitro transcription

Template	Direction	Sequence (5’ to 3’)
Cas9	F	taatacgactcactatagggagaatggactataaggaccacgac
	R	gcgagctctaggaattcttac
Tet1	F	ttaatacgactcactataggctgctgtcagggagctc

sgRNA	R	aaaagcaccgactcggtgcc
Tet2 sgRNA	F	ttaatacgactcactataggaaagtgccaacagatatcc
	R	aaaagcaccgactcggtgcc
Sox2 sgRNA	F	ttaatacgactcactatagtgcccctgtcgcacatgtga
	R	aaaagcaccgactcggtgcc
Nanog sgRNA	F	ttaatacgactcactatagcgtaagtctcatatttcacc
	R	aaaagcaccgactcggtgcc
Oct4 sgRNA	F	ttaatacgactcactataggctcagtgatgctgttgatc
	R	aaaagcaccgactcggtgcc
Mecp2-L1 sgRNA	F	ttaatacgactcactatagttgggccccagcttgaccca
	R	aaaagcaccgactcggtgcc
Mecp2-L2 sgRNA	F	ttaatacgactcactatagcccaaggatacagtatccta
	R	aaaagcaccgactcggtgcc
Mecp2-R1 sgRNA	F	ttaatacgactcactatagaggagtgaggtctagtactt
	R	aaaagcaccgactcggtgcc
Mecp2-R2 sgRNA	F	ttaatacgactcactatagtttggtggtggattaggtct
	R	aaaagcaccgactcggtgcc

Oligonucleotides used for HDR-mediated repair through embryo injection

Gene target	**Sequence (5’ to 3’)**
Tet1-loxP	gaaaaaggcccatattatacacaccttggggcaggaccaagtgtggctgctgtcaggg agGAATTCataacttcgtataatgtatgctatacgaagttatctcatggagactaggt gaggaactctgcttcccgctaacccattcttcccggtgacctgg
Tet2-loxP	ctctgtgactataaggctctgactctcaagtcacagaaacacgtgaaagtgccaacag atGAATTCataacttcgtataatgtatgctatacgaagttatatccaggctgcagaat cggagaaccacgcccgagctgcagagcctcaagcaaccaaaagc
Sox2-v5	taccagagcggcccggtgcccggcacggccattaacggcacactgcccctgtcgcaca tgGGCAAGCCCATCCCCAACCCCCTGCTGGGCCTGGACAGCACCtgagggctggactg cgaactggagaaggggagagatttcaaagagatacaagggaattg
Mecp2-L1 -loxP	tgtttgaccaatatcaccagcaacctaaagctgttaagaaatctttgggccccagctt gaGCTAGCataacttcgtataatgtatgctatacgaagttatcccaaggatacagtat cctagggaagttaccaaaatcagagatagtatgcagcagccagg
Mecp2-L2 -loxP	ccagcaacctaaagctgttaagaaatctttgggccccagcttgacccaaggatacagt atGCTAGCataacttcgtataatgtatgctatacgaagttatcctagggaagttacca aaatcagagatagtatgcagcagccaggggtctcatgtgtggca
Mecp2-R1 -loxP	ccactcctctgtactccctggcttttccacaatccttaaactgaaggagtgaggtcta gtataacttcgtatagcatacattatacgaagttatGAATTCacttgggggtcattgg gctagactgaatatctttggttggtacccagacctaatccacca
Mecp2-R2 -loxP	ccaaaaaggctggacaccatgccttggttaaaatggaggaatgttttggtggtggatt agGAATTCataacttcgtataatgtatgctatacgaagttatgtctgggtaccaacca aagatattcagtctagcccaatgacccccaagtactagacctca

Oligonucleotides used for off-targeted analysis

Gene target	Direction	Sequence (5’ to 3’)
OT1_Sox2_Stop	F	atgacatgacctaagtaaaccc
	R	ctccactctgtactaggcac
OT2_Sox2_Stop	F	tgatggtttttggtgactgcc
	R	gacagatcatagatagaaaattg
OT3_Sox2_Stop	F	aaactgaggcacagagtctg
	R	gtgacgaagccactttgacc
OT4_Sox2_Stop	F	caccttaggttcatggcattc
	R	gatggatcagtgattaagagc
OT5_Sox2_Stop	F	accatgatggactgtaccatc
	R	catggacgtcattactagatg
OT6_Sox2_Stop	F	ttcctcgaagatgaaatgatt
	R	cagtgtgcagactctgagag
OT7_Sox2_Stop	F	atgtgccacacaaggcaggc
	R	gcaaaacctctgaaagttgac
OT8_Sox2_Stop	F	ttcctgtcctggcttccttc
	R	gcactagttgtcacgtgatg
OT9_Sox2_Stop	F	gactcagatttccaagccatg
	R	acatctctgagctctaagcc
OT10_Sox2_Stop	F	tgccatgtgctgtgttcacc
	R	ttgatatttaagacagggtctc
OT11_Sox2_Stop	F	gtaaggaatgtaagaactcttg
	R	aattctcaactgaggaatactg
OT12_Sox2_Stop	F	tctcagacagaaacgctgtg
	R	gacttgatatgccaggatgag
OT13_Sox2_Stop	F	agctgacagaagacgatgag
	R	taaacccaagcaaaggtcatg
OT1_Nanog_Stop	F	gctggtgagatggctcagtg
	R	gtcttaacctgcttatagcaac
OT2_Nanog_Stop	F	agatcccattacggatggttg
	R	ggacactcaccaatgtttgg
OT3_Nanog_Stop	F	tagattatctagtgtgttccac
	R	agtttcagtgctcagagcac
OT4_Nanog_Stop	F	gacactttctaagtgggcttg
	R	gttaagggacagtgaatatcc
OT5_Nanog_Stop	F	tcccatctaccctctgactg
	R	gcctgaagaaaagaaggtcc
OT6_Nanog_Stop	F	tctgaggtgagcaaagcatg
	R	aatccaccatgtcttccgtg
OT7_Nanog_Stop	F	caattttctcagtgaggtagg

	R	cttgttcagtgcattgctgc
OT8_Nanog_Stop	F	tctcttcagaaaagagtaggc
	R	gttggcaactgcactctgtg
OT9_Nanog_Stop	F	agctcatgcatgctgagctg
	R	aacttcaagtggaactgcttg
OT1_Mecp2_Left2	F	cacacacacactgaataaaatg
	R	aagctggctttgagcaggac
OT2_Mecp2_Left2	F	tagtcacttatgtttactcctc
	R	gtgatgccagcagttggcag
OT3_Mecp2_Left2	F	tcactttccctcagtactcc
	R	caagtatcattctctgaacaag
OT4_Mecp2_Left2	F	gaactttgagacagggtctc
	R	gacagagcagcttggccttc
OT1_Mecp2_Right1	F	gcagcaccagtggaatattac
	R	gcctattgatgaatctgccc
OT2_Mecp2_Right1	F	acagatgcagccactcacag
	R	gtccaagtcacttctcccac
OT3_Mecp2_Right1	F	tccgacaatggtttatgtctg
	R	agatactagcagtgcagctg
OT4_Mecp2_Right1	F	gttcctgctggttttgtttcg
	R	tagaccaatctacaaccacag
OT5_Mecp2_Right1	F	tgctgtgaaactcaggcatg
	R	cttctaagacaagccagaaag
OT6_Mecp2_Right1	F	cggcataaacctcccattag
	R	ctctgtgcttgtaaggcaaac
OT7_Mecp2_Right1	F	gccagacaataattcccaag
	R	ctgatattgctactgctaacc
OT8_Mecp2_Right1	F	ccattgtgaaagtgggatgc
	R	ggctgctctcgtaaacaaaac
OT9_Mecp2_Right1	F	gtcactctcatgtgcaggtg
	R	ctagcacttgggaagcaaatg
OT10_Mecp2_Right1	F	ctaatcacacttctacaagctg
	R	agagaggctccaattgttag
OT1_Oct4_Stop	F	accacactactcgatacctg
	R	gggtaatgcgctgagtggac
OT2_Oct4_Stop	F	atgatgtgaactaaggcaagg
	R	ccaagtaatacacctgcaatg
OT3_Oct4_Stop	F	gatctttccatcttctgagatc
	R	aatcagggactagacaaggc
OT4_Oct4_Stop	F	tggcaatgccagacactaag
	R	ttggatgctgcctcactgtc
OT5_Oct4_Stop	F	tatccgtggtatcataggttg

	R	tgtttgccaacatgctaaacg
OT6_Oct4_Stop	F	acaagtggattactaagggtg
	R	agtaggcttcttaccgatttc
OT7_Oct4_Stop	F	accatctgatgattgagtgag
	R	atttcaactacaaacttaatggc
OT8_Oct4_Stop	F	ttggcggagctagccttgag
	R	cctccctgtgcactaggaac
OT9_Oct4_Stop	F	ctttgtgttccattgtcaagc
	R	cagttcatccagtcttcttag
OT10_Oct4_Stop	F	aatccaacttgaccatttaagc
	R	caccttgccagctgtagac
OT11_Oct4_Stop	F	cactgcatgacatatagagag
	R	catggacttatgcacaaaagg