

## Topic Introduction

# Editing the Mouse Genome Using the CRISPR–Cas9 System

Adam Williams,<sup>1,6,7</sup> Jorge Henao-Mejia,<sup>2,3,6,7</sup> and Richard A. Flavell<sup>4,5,7</sup>

<sup>1</sup>The Jackson Laboratory for Genomic Medicine, Department of Genetics and Genome Sciences, University of Connecticut Health Center, Farmington, Connecticut 06032; <sup>2</sup>Institute for Immunology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104; <sup>3</sup>Division of Transplant Immunology, Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia, University of Pennsylvania, Philadelphia, Pennsylvania 19104; <sup>4</sup>Department of Immunobiology, Yale University School of Medicine, New Haven, Connecticut 06520; <sup>5</sup>Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 06520

The ability to modify the murine genome is perhaps one of the most important developments in modern biology. However, traditional methods of genomic engineering are costly and relatively clumsy in their approach. The use of programmable nucleases such as zinc finger nucleases and transcription activator-like effector nucleases significantly improved the precision of genome-editing technology, but the design and use of these nucleases remains cumbersome and prohibitively expensive. The CRISPR–Cas9 system is the next installment in the line of programmable nucleases; it provides highly efficient and precise genome-editing capabilities using reagents that are simple to design and inexpensive to generate. Furthermore, with the CRISPR–Cas9 system, it is possible to move from a hypothesis to an in vivo mouse model in less than a month. The simplicity, cost effectiveness, and speed of the CRISPR–Cas9 system allows researchers to tackle questions that otherwise would not be technically or financially viable. In this introduction, we discuss practical considerations for the use of Cas9 in genome engineering in mice.

## Cas9 BACKGROUND AND PRINCIPAL COMPONENTS

Genetically modified mice are a cornerstone of biomedical research as they provide essential tools to understand gene function and to model complex human diseases. Until recently, genetically engineered mice were generated through genetic modification of mouse embryonic stem (ES) cells by homologous recombination. Targeted ES cells are expanded and injected into wild-type mouse blastocysts with the expectation that they will contribute to the germline of chimeric mice. Chimeric mice are then bred to wild-type mice to generate progeny containing the targeted locus (Thomas and Capecchi 1987). This process is extremely costly, time-consuming and, in some cases, uncertain. Although, this procedure usually takes 9–12 mo, the generation of mice carrying multiple targeted loci or challenging targeting locations can substantially add more time, effort, and economic cost.

In the last decade, different methods have been developed to generate mutant mice in a rapid and efficient manner. The most successful approaches use programmable nucleases such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Boch et al. 2009) injected directly into mouse one-cell embryos, a procedure that greatly accelerates the process of

<sup>6</sup>These authors contributed equally to this work.

<sup>7</sup>Correspondence: adam.williams@jax.org, jhena@mail.med.upenn.edu, richard.flavell@yale.edu

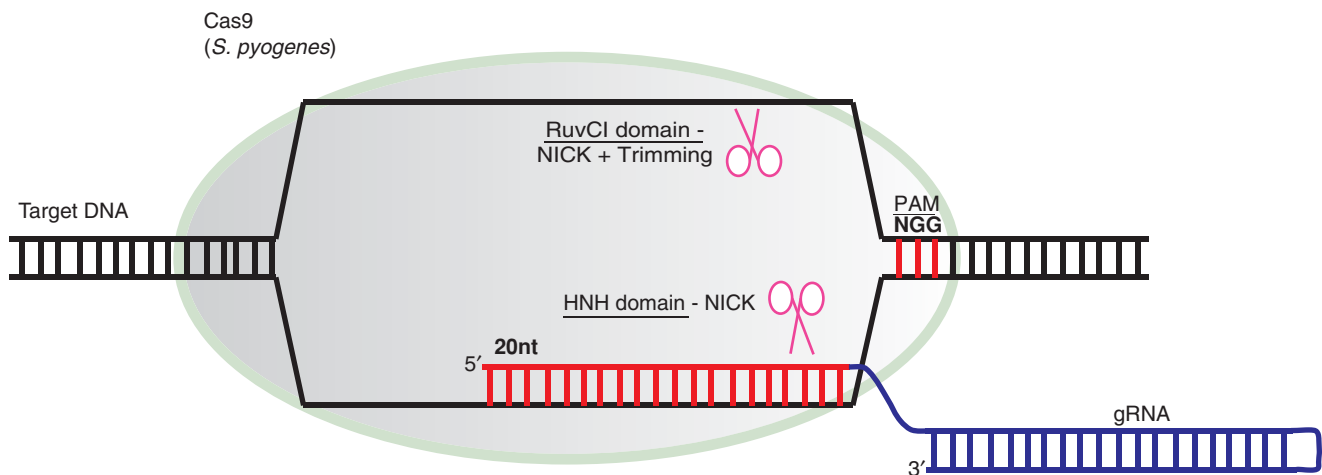
© 2016 Cold Spring Harbor Laboratory Press

Cite this introduction as *Cold Spring Harb Protoc*; doi:10.1101/pdb.top087536

generating genetically modified mice by avoiding the use of ES cells (for a recent review, see Kim and Kim 2014). Once injected, these nucleases have the capability to generate double-strand breaks (DSBs) at predefined sites in the genome that are then repaired by error-prone nonhomologous end joining (NHEJ), resulting in either insertion or deletion (indel) mutations; indels located within protein-coding exons can cause frameshifts resulting in a knockout allele (Kim and Kim 2014). Alternatively, if a single-stranded DNA (ssDNA) or a circular donor plasmid with homology regions flanking, the DSB is introduced into the one-cell embryo in combination with these nucleases, a defined DNA sequence can be inserted into the genome by high-fidelity homology-directed repair (HDR), allowing the generation of knock-in mice carrying point mutations, tags, conditional alleles, or fluorescent proteins (Kim and Kim 2014).

The most recently developed genome-editing tool is the CRISPR-associated protein 9 (Cas9) nuclease. The CRISPR–Cas system functions as an RNA-based adaptive immune system in bacteria and archaea (Barrangou et al. 2007). In *Streptococcus pyogenes*, a type II CRISPR–Cas system composed of Cas9, CRISPR RNAs (crRNAs), and a *trans*-activating crRNA (tracrRNA) target and degrade nucleic acids from foreign plasmids or bacteriophages (Deltcheva et al. 2011; Jinek et al. 2012). In this system, the Cas9 nuclease is guided to invading foreign nucleic acids by crRNAs that are partially complementary to the target sequence, and the tracrRNA plays a pivotal structural role for the proper activity of the Cas9 nuclease. The repurposing of Cas9 to generate site-specific DSBs in mammalian genomes was a turning point in genome editing (Cho et al. 2013; Cong et al. 2013; Mali et al. 2013b; Wang et al. 2013; Yang et al. 2013). Part of this repurposing was the fusion of the crRNA and tracrRNA to form a single-guide RNA (sgRNA) (Jinek et al. 2012). To direct Cas9 to a specific genomic region, the sgRNA is designed so that the 20 nucleotides at its 5' end are homologous to the genomic target sequence. In addition, the genomic sequence must be immediately followed by a protospacer adjacent motif (PAM) sequence, a 3-bp (NGG) motif present in the target sequence but not the sgRNA (Fig. 1).

Like previous programmable nucleases, CRISPR–Cas9 provides highly efficient and precise genome editing capabilities in mice (Wang et al. 2013; Yang et al. 2013). However, the significant advantage that Cas9 offers is that it uses reagents that are simple, inexpensive, and quick to design and generate. Furthermore, it is also significantly more efficient than ZFNs and TALENs (Yasue et al. 2014). In addition, the CRISPR–Cas9 system allows many targeting applications, such as the targeting of multiple loci simultaneously, the generation of conditional alleles, and the production of mice carrying endogenous reporters (Wang et al. 2013; Yang et al. 2013). Moreover, these modifications



**FIGURE 1.** Basic components of the Cas9 system. The Cas9 nuclease generates DSBs by using its two catalytic domains (HNH and RuvCI) to cleave each strand of a DNA target site next to a PAM sequence (red) and matching the 20-nucleotide sequence of the guide RNA (gRNA). The sgRNA includes a fused RNA sequence derived from CRISPR RNA and the *trans*-activating crRNA that binds and stabilizes the Cas9 nuclease.

can be made in pure inbred strains of mice (e.g., C57BL/6) as well as directly in established mutant strains, dramatically reducing the time and cost required to generate/modify complex animal models.

Since adopting this revolutionary technology in late 2012, we have generated more than one hundred novel genetically engineered mouse strains. In concordance with previous reports, we have observed high success rates in all the potential types of genome targeting events. In Protocol: **Generation of Genetically Modified Mice Using the CRISPR–Cas9 Genome-Editing System** (Henao-Mejia et al. 2016), we describe in detail the optimal conditions to generate mice carrying point mutations, chromosomal deletions, conditional alleles, fusion tags, or endogenous reporters.

## Cas9 GENOME-EDITING APPLICATIONS

In this section we will discuss general considerations for the main Cas9-mediated genome-editing applications, as outlined below (Fig. 2).

### Gene Knockout through Indel Generation

One main use of genome modification has been in the generation of gene knockout animals. DSBs generated by Cas9 are repaired by the error-prone NHEJ pathway, resulting in indel generation. When targeted within the coding region of a gene indels frequently results in a frameshift and loss of function. This is the most simple and efficient form of Cas9-mediated genome editing, requiring only injection of Cas9 and a single sgRNA.

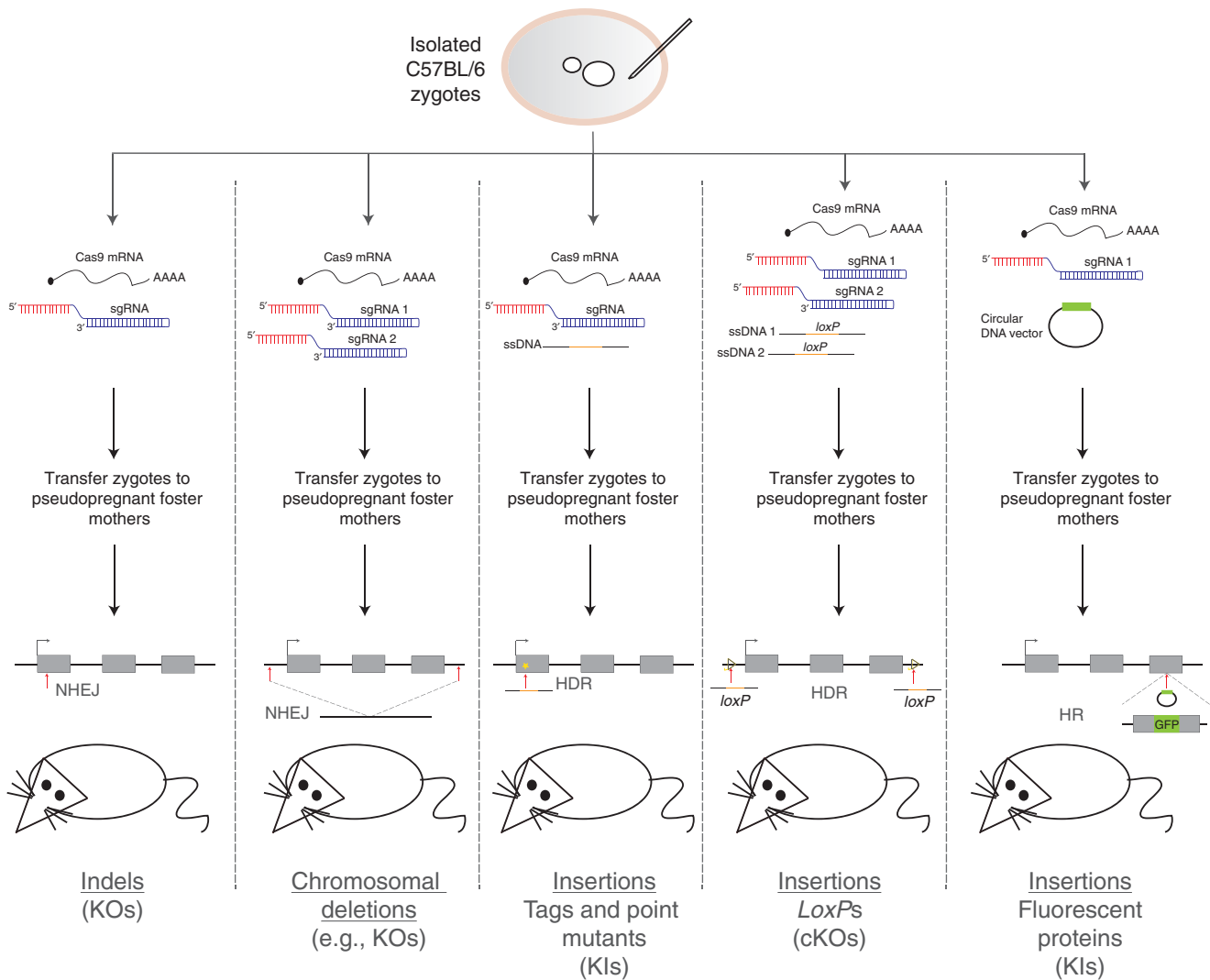
### Point Mutations/Small Insertions

A powerful use of the Cas9 system is the precise editing of the mouse genome to introduce specific nucleotide changes (Wang et al. 2013). This enables disease modeling by allowing exact nucleotide changes engineered to mimic human disease mutations. In addition, the creation/destruction of specific genomic sequences, such as transcription factor binding sites, allows interrogation of their function. Cas9 can also be used to introduce short artificial sequences such as hemagglutinin tags or *loxP* sites into the genome at precise locations (Yang et al. 2013). Precise genome-editing requires three components: Cas9, an sgRNA, and an ssDNA oligo containing the desired nucleotide modifications. As described above, Cas9 is directed by an sgRNA to generate a DSB at a specific location in the genome. In the presence of an ssDNA oligo with homology flanking the DSB, the host DNA repair machinery is able to perform HDR using the donor oligo as a template; any mutation/artificial sequence included in the donor oligo will be copied into the genome at this exact location. Donor ssDNA oligos typically contain 50–60 bases either side of the region to be edited. The addition of phosphorothioate linkages at the 5' and 3' terminal nucleotides can increase in vivo stability of the oligos, potentially increasing the efficiency of the reaction. Using this approach we have successfully inserted an artificial 100-nt sequence into the genome.

To prevent Cas9 from recutting the edited sequence and introducing an indel, sgRNAs should be designed to place the modified region as close to the PAM sequence as possible. This increases the chance that the modification is correctly incorporated, as well as reducing the probability of Cas9 recutting (by altering the sgRNA-binding site). When making modifications at multiple sites, such as flanking an exon with *loxP* sequences, a separate sgRNA and donor oligo is required for each modification site. It is important to remember that these are independent targeting events and might not occur on the same copy of the chromosome and therefore can segregate on breeding.

### Large Deletions

For some applications, the ablation of large chromosomal regions is desired. Such deletions are difficult to achieve with classical targeting methodologies, often requiring multiple rounds of recombination in ES cells (Hacisuleyman et al. 2014). However, large deletions are relatively



**FIGURE 2.** Procedures to generate genetically modified mice using the CRISPR–Cas9 genome-editing system. Isolated zygotes are co-injected with Cas9 mRNA and sgRNAs to generate mice carrying indel mutations or targeted chromosomal deletions (i.e., knockouts [KOs]). Alternatively, the Cas9 mRNA and sgRNAs are co-injected in combination with donor ssDNAs or circular plasmids to generate mice harboring point mutations, tags, *loxP* sites, or large DNA fragments such as a fluorescent protein (KIs, knock-ins; cKOs, conditional knockouts.)

simple to achieve using the CRISPR–Cas9 system. By using two sgRNAs to generate DSBs flanking a region of interest, it is possible to efficiently delete the intervening sequence through the NHEJ repair process (Yang et al. 2013; Krishnaswamy et al. 2015). Although efficiency is likely influenced by the linear distance between the sgRNAs (and potentially the three-dimensional structure of the genome), we have deleted genomic regions of up to 200-kb using this approach.

## Large Insertions

To introduce large DNA sequences (e.g., fluorescent proteins) at precise locations, a plasmid that encodes the DNA sequence to be inserted flanked by >2 kb of homology is general used (Yang et al. 2013). However, we have been able to introduce large fragments of DNA with smaller homology regions (~500 bp). The DNA sequence of interest should be inserted as close as possible to the generated DSB, and if possible the sgRNA target sequence should be modified

in the targeting vector to prevent cutting of the donor DNA or recutting of the genome after HDR. We have had success using both cytoplasmic and pronuclear injections for large insertions. However, to reduce random insertion, it is best to use circular plasmids rather than linearized DNA.

## DESIGN OF sgRNAs TO MAXIMIZE CUTTING AND MINIMIZE OFF-TARGETS

---

The design of sgRNAs is relatively simple, requiring only that the 20-nt homology be immediately followed in the genome by an NGG PAM sequence. However, certain nucleotides are favored/disfavored at different positions along the sgRNA and this should be considered during the sgRNA design (Doench et al. 2014). Most importantly, careful selection of sgRNAs is paramount to minimize potential off-target cutting; candidate sgRNAs should be blasted against the genome: an ideal sgRNA would only be present as a single site within the genome and should have at least five mismatches to any similar sites in the genome. A number of websites offer free tools to assist with the sgRNA design. The use of truncated sgRNA has been shown to reduce off-target cutting; however, we have little experience with this approach (Fu et al. 2014). Alternatively, off-target effects can be reduced by using the nickase Cas9 mutant, which can only generate DSBs when two sgRNA targets are close to each other (Mali et al. 2013a). Nickase can be used in place of regular Cas9 in any of the applications described above; however, this requires replacing every sgRNA with two sgRNAs (Lee and Lloyd 2014; Rong et al. 2014; Shen et al. 2014). We have had similar success rates using the nickase Cas9 mutant when compared with the wild-type Cas9 in the generation of mice with indel mutations or small deletions.

## CONSIDERATIONS FOR SCREENING

---

For screening indels or small insertions, a simple PCR across the targeted region followed by a Surveyor assay can be used for initial screening. To confirm sequence of mutated alleles, cloning of the genotyping product followed by sequencing is often required. For genotyping large deletions, primers spanning the excised region provide a simple assay for deletion. However, NHEJ across large deletions can result in loss of additional nucleotides proximal to each DSB; therefore, PCR primers should be placed at least 100-bp outside of the expected cut sites. Although founder animals may sometimes carry homozygous modifications, they are frequently mosaic, with different cells in the same animal carrying different modifications; we have detected founders carrying five to six unique alleles. It is therefore important to cross founder animals to wild-type mice and then screen the various alleles after segregation. This is especially important when screening to select animals in which *loxP* sites have integrated on the same copy of the chromosome.

## CONCLUSIONS AND FUTURE PERSPECTIVES

---

The CRISPR–Cas9 system has revolutionized genome engineering, overcoming many of the problems associated with previous programmable nucleases. However, Cas9-mediated targeting is still limited to sites containing a PAM sequence (NGG). An expanding toolbox of CRISPR–Cas from different bacterial species, with alternative PAM sequence requirements, will provide greater targeting flexibility (Hou et al. 2013). Finally, the current bottleneck in Cas9 targeting is the highly technical and time-consuming process of microinjection. In the future, high-throughput methods of delivery will replace microinjection, opening up this technology to even more labs.



A. Williams et al.

## ACKNOWLEDGMENTS

We would like to thank Jon Alderman for his technical assistance and discussions. We would also like to thank Caroline Lieber for her assistance in the preparation and submission of the manuscript. This work was supported in part by the National Institutes of Health National Institute of Allergy and Infectious Diseases (NIAID), grant 1R21AI110776-01 (A.W. and R.A.F).

## REFERENCES

- Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P. 2007. CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315: 1709–1712.
- Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, Lahaye T, Nickstadt A, Bonas U. 2009. Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 326: 1509–1512.
- Cho SW, Kim S, Kim JM, Kim JS. 2013. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat Biotechnol* 31: 230–232.
- Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, et al. 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339: 819–823.
- Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E. 2011. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 471: 602–607.
- Doench JG, Hartenian E, Graham DB, Tothova Z, Hegde M, Smith I, Sullender M, Ebert BL, Xavier RJ, Root DE. 2014. Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nat Biotechnol* 32: 1262–1267.
- Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK. 2014. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol* 32: 279–284.
- Hacisuleyman E, Goff LA, Trapnell C, Williams A, Henao-Mejia J, Sun L, McClanahan P, Hendrickson DG, Sauvageau M, Kelley DR, et al. 2014. Topological organization of multichromosomal regions by the long intergenic noncoding RNA Firre. *Nat Struct Mol Biol* 21: 198–206.
- Henao-Mejia J, Williams A, Rongvaux A, Stein J, Hughes C, Flavell R. 2016. Generation of genetically modified mice using the CRISPR–Cas9 genome-editing system. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot090704.
- Hou Z, Zhang Y, Propson NE, Howden SE, Chu LF, Sontheimer EJ, Thomson JA. 2013. Efficient genome engineering in human pluripotent stem cells using Cas9 from *Neisseria meningitidis*. *Proc Natl Acad Sci* 110: 15644–15649.
- Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. A programmable dual-RNA-guided RNA endonuclease in adaptive bacterial immunity. *Science* 337: 816–821.
- Kim H, Kim JS. 2014. A guide to genome engineering with programmable nucleases. *Nat Rev Genet* 15: 321–334.
- Krishnaswamy JK, Singh A, Gowthaman U, Wu R, Gorrepati P, Sales Nascimento M, Gallman A, Liu D, Rhebergen AM, Calabro S, et al. 2015. Coincidental loss of DOCK8 function in NLRP10-deficient and C3H/HeJ mice results in defective dendritic cell migration. *Proc Natl Acad Sci* 112: 3056–3061.
- Lee AY, Lloyd KC. 2014. Conditional targeting of Ispd using paired Cas9 nickase and a single DNA template in mice. *FEBS Open Bio* 4: 637–642.
- Mali P, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S, Yang L, Church GM. 2013a. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat Biotechnol* 31: 833–838.
- Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. 2013b. RNA-guided human genome engineering via Cas9. *Science* 339: 823–826.
- Rong Z, Zhu S, Xu Y, Fu X. 2014. Homologous recombination in human embryonic stem cells using CRISPR/Cas9 nickase and a long DNA donor template. *Protein Cell* 5: 258–260.
- Shen B, Zhang W, Zhang J, Zhou J, Wang J, Chen L, Wang L, Hodgkins A, Iyer V, Huang X, et al. 2014. Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. *Nat Methods* 11: 399–402.
- Thomas KR, Capecchi MR. 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* 51: 503–512.
- Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, Jaenisch R. 2013. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153: 910–918.
- Yang H, Wang H, Shivalila CS, Cheng AW, Shi L, Jaenisch R. 2013. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* 154: 1370–1379.
- Yasue A, Mitsui SN, Watanabe T, Sakuma T, Oyadomari S, Yamamoto T, Noji S, Mito T, Tanaka E. 2014. Highly efficient targeted mutagenesis in one-cell mouse embryos mediated by the TALEN and CRISPR/Cas systems. *Sci Rep* 4: 5705.



# Cold Spring Harbor Protocols

## Editing the Mouse Genome Using the CRISPR–Cas9 System

Adam Williams, Jorge Henao-Mejia and Richard A. Flavell

*Cold Spring Harb Protoc*; doi: 10.1101/pdb.top087536

---

### Email Alerting Service

Receive free email alerts when new articles cite this article - [click here](#).

---

### Subject Categories

Browse articles on similar topics from *Cold Spring Harbor Protocols*.

[Molecular Biology, general](#) (1314 articles)  
[Mouse](#) (465 articles)  
[Mutagenesis](#) (57 articles)  
[Transgenic Mice](#) (163 articles)

---

---

To subscribe to *Cold Spring Harbor Protocols* go to:  
<http://cshprotocols.cshlp.org/subscriptions>

---