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Article: **Gene editing in mouse zygotes using the CRISPR/Cas9 system**

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Abstract

The generation of targeted mouse mutants is a key technology for biomedical research. Using the CRISPR/Cas9 system for induction of targeted double-strand breaks, gene editing can be performed in a single step directly in mouse zygotes. This article covers the design of knockout and knockin alleles, preparation of reagents, microinjection or electroporation of zygotes and the genotyping of pups derived from gene editing projects. In addition we include a section for the control of experimental settings by targeting the Rosa26 locus and PCR based genotyping of blastocysts.

1. Introduction

Engineering of the mouse germline is a key technology in biomedical research for studying the function of genes in health and disease. In the first two decades of this technology, gene targeting was based on the use of embryonic stem (ES) cell lines, relying on the spontaneous recombination of vector encoded sequences with the ES cell genome, followed by the injection of engineered ES cells into blastocysts to obtain germline chimaeric mice. This approach was displaced in recent years by the ascent of sequence-specific, programmable nucleases which are introduced directly into mouse zygotes. In such one-cell embryos nucleases are used for creating targeted, highly recombinogenic double-strand breaks (DSBs) in genes of interest. These targeted DSBs are processed by DNA repair enzymes, often resulting in a variety of sequence modifications. The two step procedure of nuclease induced DSB induction followed by mutagenic DNA repair is commonly referred to as `gene editing´. Since gene editing is directly applied in mouse zygotes it has become the preferred standard procedure for generating knockout and knockin mice which saves time and efforts as compared to classical gene targeting in ES cells.

1.1. Gene editing in mouse zygotes

Nuclease mediated gene editing in zygotes was demonstrated using zinc-finger and TAL nucleases between 2009-2013 [1]. However, these types of nucleases require the recoding of protein sequences for each new target site and the construction of two molecules which act as dimers. Therefore, it was a breakthrough that in 2013 the CRISPR/Cas9 nuclease system of *Streptococcus pyogenes*, a defense mechanism against pathogenic DNA, was adapted for generating double strand breaks in the genome of mammalian cells, enabling targeted genome editing at high efficiency [2]. Using this system, two basic components are introduced into cells to achieve gene editing: the Cas9 protein, harboring two nuclease domains, and a pair of short RNAs, a crRNA and tracrRNA which hybridize to each other and associate with Cas9. If desired, a DNA molecule serving as repair template can be added as a third component. For many applications the crRNA and tracrRNA are conveniently fused into a single guide (sg) RNA molecule. The first twenty nucleotides of sgRNA direct Cas9 to a specific complementary DNA target sequence via RNA-DNA hybridization. If the target sequence is located upstream of an invariant PAM sequence ('protospacer adjacent motif'; 5'-NGG-3' in case of Cas9 from *S. pyogenes*), the Cas9 nuclease function is activated and creates a DSB located 3 bp upstream of the PAM site. Using this system Cas9 induced DSBs can be directed to any DNA sequence of the formula N_{1-20}-NGG by simply altering the first 20 nucleotides of the sgRNA which correspond to the target DNA sequence (**Fig. 1**). The advantage of CRISPR/Cas9 is that new target sites can be easily addressed by modification of the short target RNA sequence whereas the Cas9 protein component is invariant. Since

2013 numerous studies confirmed the utility and efficacy of CRISPR/Cas9 gene editing in mouse zygotes enabling the direct production of knockout and knockin mutants in a single step [3] (**Fig. 2**). Initially Cas9 mRNA and sgRNAs were generated by *in vitro* transcription and delivered into zygotes by pronuclear or cytoplasmic microinjection as used for the production of transgenic mice or nuclear transfer. The procedure can be further streamlined by the use of recombinant Cas9 protein and chemically synthesized RNAs which are commercially available. Furthermore, the electroporation of zygotes in batches has been recently developed as an alternative to the tedious microinjection procedures [4].

- Figure 1-

- Figure 2-

1.2. Gene editing by repair of targeted double-strand breaks

Gene editing occurs by targeted induction of DSBs which is followed by DNA repair. Two main DNA repair pathways exist in mammalian cells. In most cases DSBs are repaired by the non-homologous end joining (NHEJ) pathway that religates open DNA ends by DNA ligase IV without the use a repair template [5]. DSBs which are processed by NHEJ repair frequently exhibit the random deletion and/or insertion of nucleotides (indels), leading to frameshift (knockout) mutations within coding regions. NHEJ repair is active in all phases of the cell cycle. Alternatively DSBs can be repaired by homology directed repair (HDR), which requires a DNA molecule as repair template and is restricted to the S and G2 phases of the cell cycle [6]. For the repair of spontaneously occurring DSBs, cycling cells use the intact region of the sister chromatid as HDR template. To achieve gene editing of targeted DSBs, HDR can be hijacked by the addition of artificial DNA template molecules which include homology regions identical to the sequences located up- and downstream of the DSB, flanking a heterologous sequence modification or insertion. In the repair process sequence conversion extends from the template`s homology regions into the heterologous sequence and transfers the genetic modification into the target gene (knockin), enabling the introduction of preplanned mutations such as codon replacement or the insertion of a reporter gene. Large sequence insertions require the construction of plasmid-based gene targeting vectors which include homology regions of several thousand basepairs, whereas small sequence modifications can be introduced using synthetic single-stranded DNA oligonucleotides with lengths of 100-150 nt. In cycling cells both mechanisms work side by side, but mostly DSBs are repaired by prevailing NHEJ pathway whereas HDR occurs less frequently. Therefore, the induction of DSBs in the pronuclei of zygotes leads to a variety of individually repaired alleles at the target locus and to a group of heterogeneous founder mutants (F_0 generation; **Fig. 2**) which must be screened for the presence of desired mutant alleles. Furthermore, in some cases gene editing occurs late in individual nuclei of the 2-cell stage, resulting into mosaic founder mutants harboring two or more mutant alleles. Modified alleles identified in ear or tail biopsies are also present in the founder's germ cells. Upon mating of selected founder mutants to wildtype mice their F_1 progeny must be genotyped again to verify the presence of the founder`s mutation (**Fig. 2**). Next, heterozygous F_1 pups must be intercrossed in order to establish a homozygous mutant line.

Up to 70% of the pups derived from microinjections of CRISPR/Cas9 reagents exhibit small sequence deletions at the target site, such that one or a few days of microinjection are

sufficient to obtain a variety of knockout alleles. In contrast, HDR repair events occur less frequently in zygotes and are found in 5-10% of pups obtained from microinjections. The overall mutagenesis rate depends on several critical parameters such as the concentration and volume of the injected RNA/DNA reagents, the activity of individual crRNAs and the differential susceptibility of target loci for nucleases and repair proteins.

1.3. Timing of CRISPR/Cas9 gene editing projects

Overall a CRISPR/Cas9 project can be divided into four steps which are represented by protocols in the methods section: 1. target sequence selection and the design of mutant alleles, 2. preparation of reagents, 3. microinjection or electroporation of zygotes and embryo transfer, and 4. the genotyping of founder mice. The first two steps are required for preparation and may need only a few days for ordering crRNA or more time if a HDR template vector needs to be constructed. Standard times apply for steps 3 and 4. Female mice are treated with hormones for superovulation starting three days before zygote microinjections (**Fig. 3A**). Upon the mating of superovulated females to males, zygotes are collected at the next day, microinjected with CRISPR/Cas9 reagents and transferred into foster mothers (**Fig. 3B**) which give rise to birth after 3 weeks. Biopsies can be taken from the pups derived from microinjected zygotes at the age of 4 weeks and used for genotyping 7 weeks after microinjection (**Fig. 3C**). Mutant F_0 founders reach fertility at 7-8 weeks and can be further mated to obtain F_1 progeny which can be genotyped 11 weeks after mating (**Fig. 3D**). In many cases a few days of CRISPR/Cas9 microinjections result into desired mutant founders such that mutant F_1 progeny is available within 5 months. However, the total number of F_0 pups recovered from manipulated zygotes is variable and sometimes insufficient, requiring time consuming repeat experiments.

For the delivery of CRISPR/Cas9 reagents into zygotes, various options with regard to the nature, concentration and delivery are available. The protocols described in this article are the standard procedure we presently use for our in-house mutant production. It is based on the pronuclear zygote microinjection using Cas9 protein with synthetic crRNA and tracrRNA and optionally a DNA oligonucleotide or plasmid vector as HDR template. Using this approach, we routinely obtain in F_0 mice derived from microinjections mutant alleles harboring small deletions at a frequency of 20-70% and HDR alleles at a frequency of 5-30%. In addition, we began to explore the electroporation of zygotes which yielded first positive results. Anticipating that this method may become a new routine procedure we also include our protocol for zygote electroporation.

- Figure 3 -

2. Design of guide RNAs and HDR template molecules

The positioning of guide RNA target sites is defined by the aim of a specific experiment, which may require the generation of knockout alleles or precise sequence insertions, replacements or deletions at predefined sequence positions. It has been shown that CRISPR/Cas9-mediated gene editing in one-cell embryos can be used for the induction of small deletions leading to frameshift mutations within coding regions and to achieve sequence insertions & replacements by recombination with targeting vectors or single-stranded oligodeoxynucleotides (ssODNs) [3].

An elementary application is the generation of knockout alleles by the induction of undirected, small sequence indels occurring at the DSB site by error-prone NHEJ repair. To achieve gene disruption, the target site has to be located within an exon of the targeted gene, so that the majority of randomly sized nucleotide indels (often in the range of 1-50 bp) cause reading frameshift mutations and premature termination.

Targeted mutations by HDR are guided by repair templates, being either ssODNs or plasmid gene targeting vectors. To generate targeted mutations by sequence insertion/replacement, the guide RNA target site should be located close to the position of the intended mutation as the frequency of sequence conversion by HDR decreases with distance. In particular when using ssODNs we recommend selecting target sites that are less than 30 bp distant to the planned mutation. In the most simple configuration the selected recognition sequence should cover the position of the intended mutation, bridging the homology arms of the targeting vector to prevent its processing and the potential reprocessing of targeted alleles by Cas9, being specific to the wild-type sequence.

ssODNs contain a short sequence modification (deletion, insertion or substitution) flanked by two homology sequences. ssODNs are beneficial as they are quickly synthetized for a reasonable price so that no molecular cloning work is necessary. Most important, as their length is limited, a desired mutation should be located at the center of an ssODN, resulting in similarly-sized homology regions (usually 40-60 bp each) that flank the desired mutation symmetrically on both sides. For the generation of directed deletions using two guide RNAs, the ssODN should consist of the two homology regions that flank the excised segment, such that the homology regions are directly fused together by recombination. Many commercial manufacturer of custom ssODNs offer lengths up to 150 or more, enabling the insertion of sequences of up to ~50bp. ssODNs should be HPLC purified, but chemical end-modification to improve stability of the ssODN using phosphorothioate bonds [7] is not necessary for PNI on mouse zygotes. Recent studies from Jacob Corn's lab suggest that asymmetric ssODN can increase the knockin efficiency in mammalian cell lines [8], however, it is not validated whether this applies to zygotes as well.

For larger and more sophisticated modifications, like insertion of cDNA or reporter cassettes, plasmid targeting vectors with long homology arms are required. Such vectors include the desired insertion (or deletion) which is flanked by two sequence regions up- and downstream of the DSB, being able to initiate sequence conversion by HDR. For the design and construction of gene targeting vectors, adhere to protocols developed for gene targeting in ES cells [9, 10] except of two adjustments: firstly, for the pronuclear injection, no antibiotic selection cassette is required; secondly, the homology arms used are often shorter (1 – 2 kb) as compared to ES cell targeting vectors to facilitate cloning by PCR or gene synthesis. Nevertheless, the exact relation between the length of homology regions and the resulting targeting frequency in mouse embryos needs to be further established. For vector construction, homology arms can be PCR-amplified from an isogenic genomic template and

ligated into a cloning plasmid backbone. Alternatively, the insert can be generated by gene synthesis using a commercial provider.

2.1. Guide RNA selection tools

Due to their simple structure Cas9 target sites can be easily manually identified by inspection of a DNA sequence for NGG PAM motifs. However, previous studies have demonstrated that the sequence composition of the 20 nt target sequence (such as GC content) influences the nuclease activity of Cas9 at the target site (on-target activity), as well as the likelihood that Cas9 cuts at other related sites within the genome (off-target activity). Since the advent of the CRISPR/Cas9 system, many labs have developed online tools that offer the design of specific and efficient guide RNAs (**Table 1**). Most of them offer to enter gene accession numbers, genomic coordinates of the gene of interest, or to paste custom sequences as source. Besides the analysis for off-target activities some of these tools further predict the on-target activity of the proposed guide RNAs. However, different algorithms generate inconsistent results and it is not yet clear which algorithm delivers the most realistic results. Therefore, we use for each target gene whenever possible two alternative sgRNAs side-by-side which are each injected into half of the number of zygotes to minimize the risk of a complete experimental failure. The CRISPOR webpage delivers the results of all published activity ranking tools in a single view. To date, for newcomers we recommend using either CRISPOR or ChopChop, which are both very fast and intuitive, and offer all relevant options for the design and evaluation of guide RNAs.

Table 1: Guide RNA design and evaluation tools

	Target site search	On-target prediction	Off-target prediction	URL	Reference
Breaking Cas	o	-	+	http://bioinfogp.cnb.csic.es/tools/breakingcas	[11]
Cas-Designer	o	+	+	http://www.rgenome.net/cas-designer/	[12]
CCTop	o	-	+	http://crispr.cos.uni-heidelberg.de/	[13]
ChopChop	#	+	+	http://chopchop.cbu.uib.no/	[14]
COSMID	-	-	+	https://crispr.bme.gatech.edu/	[15]
CRISPOR	o	+	+	http://crispor.tefor.net/	[16]
CRISPR design	o	-	+	http://crispr.mit.edu/	[17]
CRISPR Finder	#	-	+	http://www.sanger.ac.uk/htgt/wge/find_crisprs	[18]
CRISPRdirect	#	-	+	http://crispr.dbcls.jp/	[19]
CRISPR-ERA	#	+	+	http://crispr-era.stanford.edu/	[20]
CRISPRscan	#	+	+	http://www.crisprscan.org/	[21]
CROP-IT	o	-	+	http://adlilab.org/CROP-IT/	[22]
E-CRISP	#	+	+	http://www.e-crisp.org/	[23]
sgRNA designer	#	+	+	http://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design	[24]
sgRNA Scorer	o	+	+	https://crispr.med.harvard.edu/sgRNAScorer/	[25]
ZiFiT	o	-	+	http://zifit.partners.org/ZiFiT/	[26]

#: target search on gene IDs, genomic coordinates or custom sequences
o: target search on custom sequences only

2.2. Processing at off-target sites

Since 20 nt target sequences are not necessarily unique within mammalian genomes and Cas9 has been shown to tolerate one or more mismatched nucleotides, CRISPR/Cas9 mutagenesis has the potential to introduce undesired mutations at genomic off-target sites. Various studies suggest that off-target sites of RNA-guided Cas9 nucleases can be variable in frequency, challenging to predict and it is not possible to predict how many mismatches can be tolerated. Nevertheless, a careful selection among the target sequences addressing a given genomic region of interest may reduce the risk of creating off-target mutations.

The occurrence of these off-target mutations and their frequency has been studied intensively in cell culture models using sensitive next-generation sequencing techniques [27, 28]. Various modifications of the CRISPR/Cas9 system to reduce the off-target frequencies have then been developed, like Cas9 nickases [29, 30], Cas9-FokI fusions [31], high-fidelity variants eCas9(1.1) [32] and Cas9-HF [33], truncated guide RNAs [34], orthologous or inducible Cas proteins [35-38]. Validation of such alternative nucleases in mouse zygotes has been reported for SpCas9 D10A nickase [39, 40], AsCpf1 [41, 42], LbCpf1 [42], St1Cas9 [43], Fok-dCas9 [40, 44], and SaCas9 [45]. These improvements are very helpful for animal or cell culture models in which even rare off-target mutations are deleterious, like the generation of isogenic cell lines or in somatic gene therapy. For the generation of mutant mouse lines by gene targeting in the zygote, however, off-target mutations are not that troublesome as unlinked mutations can be easily segregated through breeding. Moreover, whole-genome sequencing of a CRISPR/Cas9-generated mutant mouse line showed no unwanted modifications throughout the genome beside a single known bona fide off-target site [46]. For these reasons, and as the prediction tools for specific guide RNAs become better and better, we recommend focusing in the design of highly-specific guide RNAs. Still, in every newly-generated mouse line we verify the sequence integrity of the most putative off-target sites (as predicted using one or several of the web tools listed in Table 1) by simple Sanger sequencing of the amplified genomic loci surrounding the potential cutting sites. By using only guide RNAs that have been rated as highly-specific, we never observed any off-target event. For the rare case that no highly specific guide RNA is located near the desired sequence region, targeting can also be performed using medium- or low-specific guide RNAs. In this case, use guide RNAs with off-target sites that are located on a different chromosome than the on-target locus, so that off-target mutations (identified by Sanger sequencing) can be segregated by backcrossing of founder mutants to wild-type mice.

2.3. Choice of CRISPR/Cas9 reagents

With regard to the specification of Cas9 nuclease reagents, the number of options increased in recent years. In the first phase of gene editing in zygotes, Cas9 and single guide RNAs were exclusively delivered as sgRNAs prepared by *in vitro* transcription from plasmid templates. With the growing number of CRISPR/Cas9 reagent and service providers, the use of Cas9 protein and of chemically synthesized RNAs provides additional options. Since these reagents are becoming more cost effective and entirely eliminate own hands on time, we meanwhile routinely use nuclease protein (from IDT or NEB) together with the dual RNA system (IDT) for projects involving gene editing by SpCas9. The dual RNA system follows the natural function of CRISPR/Cas9 in bacteria consisting of short, target specific crRNAs which hybridize to the longer tracrRNA scaffold molecule. By this means only economic,

short crRNAs are required for recoding of Cas9 towards new target sites whereas the invariant tracrRNA can be used for all targets. However, reagents for alternative Cas9 enzymes are presently not commercially available and still require home-made preparation. See Table 2 for the pros and cons of the different reagent formats.

Table 2:

	Advantages	Disadvantages
Pronuclear injection of Cas9 mRNA	- Pronuclear injection method is widely established - Any Cas9 ortholog or variant applicable	- In-house production by *in vitro* transcription laborious and expensive - Commercial mRNA expensive - Easily degraded due to RNase contamination - Higher rate of mosaicism due to prolonged Cas9 expression
Pronuclear injection of Cas9 protein	- Pronuclear injection method is widely established - No Cas9 production hands-on time - Cas9 protein more stable than mRNA - Commercial SpCas9 protein cost-effective - Reduced mosaicism	- In-house production sophisticated, laborious, and expensive - Only SpCas9 wildtype and nickase variants commercially available to date
Electroporation of Cas9 protein	- No microinjection setup necessary - Ease of use - High throughput possible	- High amounts of Cas9 components needed - Limited experiences (not widely-used) - Not validated for delivery of plasmid targeting vectors

3. Methods

3.1. In vitro Cas9 nuclease assay

To control for the activity of the reagents used for microinjections we routinely perform an in vitro nuclease assay. As nuclease substrate we use PCR products (300 - 400 bp) derived from the target locus which include the Cas9 target and PAM sequence in the center.

Materials:

- 2x PCR master mix (e.g. OneTaq 2X Master Mix, NEB, M0482)

- locus-specific forward and reverse PCR primers (10 µM)

- PCR purification kit

- Cas9 protein (10 µg/µl, IDT, 1074181), prepare 1 µg/µl solution with enzyme diluent buffer B (NEB #B8002S; 300 mM NaCl, 10 mM Tris-HCl, 0.1 mM EDTA, 1 mM DTT, 500 µg/ml BSA, 50% glycerol. pH 7.4)

- target-specific crRNA (IDT, custom Alt-R™ crRNA), prepare 20 µM stock solution

- generic tracrRNA (IDT, 1072532), prepare 20 µM stock solution

- 10x NEB 3.1 restriction enzyme buffer

- RNAse A (4 µg/µl)

- Stop solution (30% glycerol, 1.2% SDS, 250 mM EDTA)

- agarose gel electrophoresis (setup and consumables)

Protocol:

1. In a 25 µl-PCR assay, use 12.5 µl 2x PCR master mix, 0.5 µl of each primer, and 0.5-2.0 µl genomic DNA to amplify the targeted genomic region.

2. Check 5 µl of the PCR product on an agarose gel to confirm successful and specific amplification.

3. Purify the remaining PCR product using a PCR purification kit according to the manufacturer's instructions.

4. For each crRNA set up a 15 µl nuclease reaction:

    - 150 ng purified PCR product

    - 0.5 µl crRNA (20 µM stock, equals 120 ng)

    - 0.5 µl tracrRNA (20 µM stock, equals 220 ng)

    - 1.5 µl 10x NEB 3.1 restriction enzyme buffer

    - fill up to 14 µl with Nuclease free water

    - add 1.0 µl Cas9 protein (equals 1 µg)

5. Mix by pipetting and incubate at 37°C for 1 h

6. Add 1 µl of RNAse A, incubate at 37°C for 15 min.

7. Add 1 µl of Stop solution, incubate at 37°C for 15 min.

8. Add gel loading buffer and run the sample on a 1.5 % agarose gel

For direct comparison with the nuclease reaction include a control sample without cr/tracrRNA. Together with cr/tracrRNA and Cas9 at least 50% of the PCR product should be cut and result into two smaller fragments. Reagents exhibiting less or no nuclease activity should not be used for microinjections.

3.2. Superovulation and embryo collection

Materials:

- PMSG (Pregnant Mare's Serum Gonadotrophin) stock solution: dissolve at 50 IU/ml PMSG in sterile water. Prepare single-use aliquots and store at -20 °C no longer than 2 months.

- hCG (human Chorionic Gonadotropin) stock solution: dissolve at 50 IU/ml hCG in sterile water. Prepare single-use aliquots and store at -20 °C no longer than 2 months

- female donor mice (3-6 weeks old)

- stud males (2-6 month old; ideally of same genetic background than female donors)

- M2 medium (Sigma, M7167)

- Hyaluronidase (Sigma, H4272)

- Hyaluronidase stock solution: 10 mg/ml Hyaluronidase in M2 medium. Prepare 50 µl aliquots and store at -20 °C no longer than 6 months.

- M2/Hya: 0.3 mg/ml Hyaluronidase in M2 medium, prepare freshly

- Transfer pipets

- Surgical instruments

- Stereomicroscope

Protocol:

1. Three days before microinjection (day -3): inject donor females with 5 IU PMSG at 9 am – 10 am (Note: time points vary depending on genetic background and light-dark cycle and therefore have to be adjusted empirically)

2. One day before microinjection (day -1): inject donor females with 5 IU hCG at 9 am – 10 am (Note: time points vary depending on genetic background and light-dark cycle and therefore have to be adjusted empirically)

3. After hCG injection, mate each donor female with a stud male overnight

4. On the day of injection, identify plug-positive donor females and sacrifice them at 8 am using an approved method (Note: time points vary depending on genetic background and light-dark cycle and therefore have to be adjusted empirically).

5. Dissect out the oviducts and place each into a drop of 50 µl of M2/Hya in a 6-cm Petri dish.

6. Dissect the cumulus complex using a pair of fine forceps and incubate it until the cumulus cells dissociate.

7. Transfer the one-cell embryos to a fresh drop of 50 µl prewarmed M2 medium (without Hyaluronidase) for removal of cumulus cells.

8. Pool all embryos in a drop of 200 µl prewarmed M2 medium and keep them in a humidified 37°C, 5% CO_2 incubator until injection/electroporation.

3.3. Treatment of zygote

Delivery of CRISPR/Cas9 into zygotes can be achieved either by microinjection of a Cas9/guideRNA solution into the pronucleus or by electroporation of batches of zygotes using defined electric pulses (protocol adopted from Chen et al. [4]). Selection of a technique depends mainly on the available instrumental setup and technical skills. Furthermore, the delivery of targeting vectors for HDR has yet only been successfully shown by pronuclear microinjection.

3.3.1. Pronuclear microinjection

Materials:

- Embryo-tested water (Sigma, W1503)

- $T_{10}E_{0.1}$ injection buffer (10 mM Tris-HCl, 0.1 mM EDTA; prepare with embryo-tested water and sterilize using a 0.22 µm filter unit)

- target-specific crRNA (IDT, custom Alt-R™ crRNA, resuspend in $T_{10}E_{0.1}$ injection buffer; 20 µM stock solution)

- generic tracrRNA (IDT, 1072532, resuspend in $T_{10}E_{0.1}$ injection buffer; 20 µM stock solution)

- Cas9 protein (10 µg/µl, IDT, 1074181), prepare 1 µg/µl solution with enzyme diluent buffer B (NEB #B8002S; 300 mM NaCl, 10 mM Tris-HCl, 0.1 mM EDTA, 1 mM DTT, 500 µg/ml BSA, 50% glycerol. pH 7.4); Note: Cas9 should contain at least one nuclear localization signal (NLS) peptide

- *If applicable*: Targeting ssODN (IDT, custom Ultramer® Oligo) or targeting vector

- *Optional*: centrifugal filter column (Millipore, UFC30LG25)

- M2 medium (Sigma, M7167)

- Mineral oil (Sigma, M8410)

- Inverted microscope equipped with 5x, 20x, and 40x objective with phase-contrast and Differential Interference Contrast (DIC, only for 40x)

- Micromanipulators for holding and microinjection devices (e.g. Eppendorf TransferMan 4r)

- Microinjection device for holding embryos (e.g. Eppendorf CellTram)

- Microinjection device for pronuclear injection (e.g. Eppendorf Femtojet)

- Transfer pipets

- Holding pipets

- Pronucleus injection capillaries (Eppendorf Femtotips, 5242952008)

- Capillary loading tips (Eppendorf, 5242956003)

Preparation of microinjection aliquot:

1. Preparation of targeting molecule (if appropriate): for preparation of ssODNs follow a), for preparation of targeting vectors follow b)

   a. ssODN:

      i. Dissolve the lyophilized ssODN in embryo-tested water to a concentration of 1 µg/µl.

      ii. *Optional*: dialyse ssODN solution against embryo-tested water to remove impurities and salts.

      iii. Denature ssODN by incubation at 80°C for 10 min.

      iv. Determine the DNA concentration using a UV spectrophotometer and store on ice until the preparation of the injection aliquot (or -20°C for long-term storage).

   b. targeting vector:

      i. Precipitate 10 µg plasmid DNA by adding 0.1 volumes of 3 M Sodium acetate (pH 5.2) and 2.5 volumes of 100% EtOH.

      ii. Mix by flicking the tube and pellet the DNA by centrifugation at 16.000g for 15 min.

      iii. Carefully discard the supernatant and wash the pellet with 500 µl 70% EtOH.

      iv. Centrifuge at 16.000g for 2 min and carefully discard the supernatant.

      v. Let the pellet air-dry and then resuspend it in 20 µl $T_{10}E_{0.1}$ buffer.

      vi. Measure concentration using a UV spectrophotometer and store on ice until the preparation of the injection aliquot (or -20°C for long-term storage).

2. For each day of microinjection, prepare one single-use aliquot of 20 µl as follows (prepare a master mix for several aliquots): Mix 0.6 pmol/µl crRNA (final concentration) and 0.6 pmol/µl tracrRNA (final concentration) and incubate 10 min at RT for the assembly of the crRNA:tracrRNA heteroduplex. Then add the ssODN (12.5 ng/µl final) or targeting vector (50 ng/µl final) and fill to 19 µl with $T_{10}E_{0.1}$ buffer.

3. *Optional*: Purify the injection mix by filtering through a centrifugal filter column

4. Store the microinjection aliquot at -80°C until the day of injection.

5. On the day of injection, thaw one aliquot and add 1.0 µl Cas9 protein (50 ng/µl final, using the stock diluted to 1 µg/µl; diluted glycerol does not affect embryo development). Incubate at RT for 10 min for the RNP assembly.

6. Centrifuge at 16.000g for 2 min, transfer supernatant to fresh tube, and keep the aliquot on ice until injection.

Microinjection

1. Load the injection capillary with 3 µl of microinjection aliquot using capillary loading tips.

2. Mount the loaded injection capillary on the capillary holder of the microinjection device and set the injection conditions to 240 hPa as injection pressure and to 140 hPa as compensation pressure (Note: parameters dependent on the microinjection device and capillaries, and have to be adjusted empirically).

3. Pipet 100 µl M2 medium in the middle of a 6-cm Petri dish and cover the drop with mineral oil.

4. Transfer the one-cell embryos (from section 3.2 step 8) to the upper part of the Petri dish.

5. Fix a single embryo with the holding pipet and move it to the center of the Petri dish.

6. Penetrate the zona pellucida with the injection capillary and move the tip of the capillary to the center of the larger (male) pronucleus.

7. Leave the capillary in the pronucleus for 1-3 sec until a swelling of the pronucleus becomes visible (the microinjection aliquot is injected by an over-pressure of the microinjection device).

8. Slowly pull the injection capillary out of the embryo.

9. Move the injected embryo to the lower part of the M2 drop.

10. Repeat injection with all other embryos.

11. Finally, transfer all injected embryos into a fresh drop of 200 µl prewarmed M2 medium covered with mineral oil and incubate them in a humidified 37°C, 5% CO_2 incubator until implantation.

3.3.2. Electroporation of mouse zygotes

Materials:

- HEPES, 1 M, pH 7.5 (ThermoFisher, 15630-049)

- KCl (Sigma, P9333), prepare 1 M stock solution

- $MgCl_2$ hexahydrate (Sigma, M9272), prepare 100 mM stock solution

- Glycerol (Sigma, G6279)

- Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Sigma, C4706), prepare 50 mM stock solution

- Nuclease free water (IDT, 11-05-01-14)

- M2 medium (Sigma, M7167)

- BSA (Sigma, A3311)

- M2/BSA: M2 medium supplemented with 4 mg/ml BSA

- Tyrode's Solution, Acidic, embryo-tested (Sigma, T1788)

- OptiMEM reduced serum medium (ThermoFisher, 31985-062)

- KSOM/AA (Merck Millipore, MR-106D)

- KSOM/BSA: KSOM/AA medium supplemented with 4 mg/ml BSA

- Cas9 protein (10 µg/µl, IDT, 1074181); Note: Cas9 should contain at least one nuclear localization signal (NLS) peptide

- target-specific crRNA (IDT, custom Alt-R™ crRNA), prepare 100 µM stock solution

- generic tracrRNA (IDT, 1072532), prepare 100 µM stock solution

- crRNA:tracrRNA heteroduplex: mix crRNA and tracrRNA stocks in equimolar ratio and incubate 10 min at RT to obtain 50 µM heteroduplex; store on ice until use.

- alternatively to crRNA:tracrRNA: *in vitro* transcribed sgRNA (50 µM stock solution)

- *If applicable*: Targeting ssODN (IDT, custom Ultramer® Oligo)

- 1-mm electroporation cuvette (BioRad, 1652089)

- Electroporator (BioRad Gene Pulser XCell, or similar)

Preparation of the electroporation mix:

1. Prepare a 2x pre-mix:

Compound	Stock Conc.	Final conc.	2x Pre-Mix (350 µl)
HEPES	1 M	20 mM	20 µl
KCl	1 M	150 mM	150 µl
MgCl$_2$	100 mM	1 mM	10 µl
Glycerol	100 %	10 %	100 µl
TCEP	50 mM	1 mM	20 µl
Nuclease-free water			50 µl

2. Prepare the electroporation mix (Note: always prepare mix freshly):

Compound	Stock Conc.	Final conc.	Volume
2x Pre-Mix			3.5 µl
Cas9 protein	61 µM (≈10 µg/µl)	8 µM[#] (≈1.3 µg/µl)	1.3 µl
crRNA:tracrRNA *or* sgRNA	50 µM (≈1.8 µg/µl)	12 µM[#] (≈0.4 µg/µl)	2.4 µl
opt.: ssODN (140nt)	100 µM (≈4.3 µg/µl)	20 µM (≈0.9 µg/µl)	2.0 µl
Nuclease-free water			fill up to 10 µl

#: corresponds to 8 µM Cas9/crRNA/tracrRNA RNP

3. Incubate the electroporation mix at 37°C for 10 min before electroporation.

Electroporation:

1. Equilibrate KSOM/BSA medium (3-4h before electroporation)

2. Wash the zygotes from section 3.2 step 8 five times in M2/BSA and for each electroporation transfer about 30-40 zygotes into a drop of 200 µl Tyrode's solution for 30-60 sec until 20% of zona is digested.

3. Wash the zygotes four times in M2/BSA and keep them in the incubator until electroporation.

4. For electroporation wash the zygotes once in OptiMEM and transfer them into 10 µl OptiMEM. Add 10 µl of the electroporation mix (step 3).

5. Using a P20 pipette transfer the mix into a 1 mm Biorad cuvette.

6. Insert the cuvette into the BioRad Xcell device and apply two square wave pulses (30 V amplitude, 3 ms duration) with a 100 ms interval (30V/3ms—100ms—30V/3ms).

7. Recover embryos from the cuvette by flushing with 100 µl KSOM/AA.

8. Wash embryos once in pre-equilibrated KSOM/BSA.

9. Culture the zygotes overnight in 20 µl pre-equilibrated KSOM/BSA in 35 mm dish in a humidified 37°C, 5% CO_2 incubator.

10. On the next day, use successfully developed 2-cell embryos for embryo transfer.

3.4. Embryo transfer

Materials:

- Pseudo-pregnant females

- Vasectomized males (2-6 months old)

- Anesthesia, Analgesia (in accordance with local regulations)

- M2 medium (Sigma, M7167)

- Surgical instruments

- Stereomicroscope

Protocol:

1. The day before the embryo transfer, mate the foster females with vasectomized males.

2. On the day of embryo transfer, identify plug-positive foster females.

3. Anesthetize a plug-positive foster female and make a 5-mm skin incision parallel to the dorsal midline above the position of the left or right oviduct. The ovarian fat pad should become visible.

4. Pull out the ovary and fix the fat pad.

5. Locate the infundibulum and tear the bursa using two fine forceps.

6. Transfer all injected embryos to a drop of 200 µl prewarmed M2 medium without mineral oil (this avoids the coating of the transfer capillary).

7. Load 12-16 embryos in the transfer capillary.

8. Insert the tip of the transfer capillary into the infundibulum and carefully release the embryos.

9. Relocate the ovary and fat pad into the abdomen and sew the peritoneum and skin.

10. Repeat the procedure with the next plug-positive foster female until all embryos are transferred.

3.5. *In vitro* blastocyst assay (optional)

In order to analyze the efficiency of CRISPR/Cas9-mediated gene targeting in mice, a PCR-based assay on mouse blastocysts can be performed. Here, after delivery of CRISPR/Cas9 reagents by microinjection or electroporation, the zygotes are grown in culture into morulas or blastocysts. Then DNA is extracted from individual embryos and the genome region of interest is amplified by nested PCR followed by sequencing or restriction fragment length polymorphism (RFLP).

Materials:

- DNA quick extraction solution (Epicentre, 09050)

- 2x PCR master mix (e.g. OneTaq 2X Master Mix, NEB, M0482)

- locus-specific outer forward and reverse PCR primers (10 µM)

- locus-specific inner forward and reverse PCR primers (10 µM)

- PCR purification kit

- agarose gel electrophoresis (setup and consumables)

- suitable restriction enzyme

Protocol:

1. Pick each morula/blastocyst and transfer it into 10 µl DNA quick extraction solution in PCR tubes. Under a Stereomicroscope, a P10 micropipette or an Embryo Transfer pipet can be used for this purpose.

2. Incubate the tubes at 68°C for 15 min followed by 95°C for 15 min in a thermocycler.

3. In a first 25 µl-PCR assay, use 12.5 µl 2x PCR master mix, 0.5 µl of each outer primer, and 2 µl or 5 µl DNA (depending on blastocyst or morula) to amplify the targeted genomic region.

4. In a second 25 µl-PCR assay, use 12.5 µl 2x PCR master mix, 0.5 µl of each inner primer, and 0.5 µl of the first PCR amplicon to amplify the targeted genomic region.

5. Check 5 µl of the PCR product on an agarose gel to confirm successful and specific amplification.

6. Purify the remaining PCR product using a PCR purification kit according to the manufacturer's instructions.

7. In a 20 μl-restriction assay, mix 500 ng of purified PCR product with 5 U restriction enzyme and 2 μl of the respective 10x digestion buffer.

8. Incubate restriction assay for 1 h at the enzyme's recommended temperature.

9. Visualize the digested samples by running a gel electrophoresis and check for the expected fragment sizes. Use wildtype samples for comparison.

10. Alternatively to restriction digest, the purified PCR product can also be Sanger-sequenced using the inner forward or inner reverse primer.

3.6. Isolation of genomic DNA from founder animals

At the age of 3 weeks, F_0 founder animals are weaned and biopsies of ear-punch, tail-tip, or toe-clip are taken for isolation of genomic DNA (Note: tail-tip and toe-clip biopsies may be excluded by local regulations). Many different protocols and commercial kits are available for the isolation of genomic DNA; here we present two of them: one very quick and reliable protocol which gives high yield in a crude lysate and a more elaborate one that ends with a very clean genomic DNA solution.

4.5.1 Isolation of genomic DNA (quick protocol)

Materials:

- Alkaline Lysis Reagent: 25 mM NaOH, 0.2 mM EDTA

- Neutralization Buffer: 40 mM Tris-HCl

Protocol:

1. Add 50 μl of Alkaline Lysis Buffer to each biopsy.

2. Incubate for 30 min at 95°C with agitation.

3. Add 50 μl Neutralization Buffer.

4. Centrifuge at 16,000g for 5 min.

5. Transfer the supernatant to a fresh tube.

6. Store the genomic DNA at +4°C for short-term until genotyping or at -20°C for long-term.

4.5.2 Isolation of genomic DNA (clean protocol)

Materials:

- Genomic DNA purification kit (Promega, A1120)

- EDTA (Sigma, E4884)

- Proteinase K (20 mg/ml) (Serva Electrophoresis, 33755)

- Isopropanol

- Ethanol p.a.

- DNA storage buffer (TE or similar)

Protocol:

1. Add 500 µl of Nuclei Lysis Solution to each biopsy.

2. Add 120 µl of 0.5 M EDTA (pH 8) and 17.5 µl of proteinase K.

3. Incubate for 3 h at 55°C with agitation.

4. Add 200 µl of Protein Precipitation solution, mix vigorously, and incubate on ice for 5 min.

5. Centrifuge at 16,000g for 10 min and transfer supernatant into a new 1.5 ml tube containing 600 µl isopropanol.

6. Mix by inverting the tube several times and centrifuge at 16,000g for 1 min.

7. Discard the supernatant and wash pellet with 600 µl of 70% EtOH.

8. Mix tube several times and centrifuge at 16,000g for 1 min.

9. Aspirate the ethanol and let the pellet dry in air for 15 min.

10. Resuspend the DNA pellet in 50-100 µl of DNA storage buffer for either 2 h at 55 °C or overnight at 4°C.

11. Store the genomic DNA at +4°C for short-term until genotyping or at -20°C for long-term.

3.7. Genotyping of founder mice

It is important to establish a PCR genotyping protocol before biopsies of founder mice are obtained. The selection of a suitable strategy depends on the type of mutation. In most cases, the DNA region of interest is amplified using PCR and then analyzed by means of sequencing, restriction digestion, or subcloning. You should pretest your genotyping method with genomic DNA from wildtype mice to avoid time delays later. PCR primer pairs and reaction conditions for genotyping should be already optimized when you get your first injection-derived offspring. We also recommend confirming the correct identity of the PCR amplicon by direct sequencing.

Here we present these most common genotyping strategies for founder mice, however, other strategies like Southern blotting, melting curve analysis, or even next generation sequencing approaches might be suitable, but will not be discussed here.

3.7.1. Genotyping by direct Sanger sequencing of PCR amplicons

Materials:

- 2x PCR master mix (e.g. OneTaq 2x Master Mix, NEB, M0482)

- locus-specific forward and reverse PCR primers (10 µM)

- agarose gel electrophoresis (setup and consumables)

- PCR purification kit

Protocol:

1. In a 25 µl-PCR assay, use 12.5 µl 2x PCR master mix, 0.5 µl of each primer, and 0.5-2.0 µl genomic DNA to amplify the targeted genomic region.

2. Check 5 µl of the PCR product on an agarose gel to confirm successful and specific amplification.

3. Purify the remaining PCR product using a PCR purification kit according to the manufacturer's instructions.

4. Use either the forward or the reverse PCR primer for Sanger sequencing.

5. Check the chromatogram for mixed sequencing peaks (indicating heterozygous substitutions by HDR) or continuous mixed peaks (indicating Indel mutations caused by NHEJ).

6. Deconvulation tools, like Tide (https://tide.nki.nl/, [47]) or CRISP-ID (http://crispid.gbiomed.kuleuven.be/, [48]) can help to identify the actual mutation.

3.7.2. Genotyping by restriction fragment length polymorphism (RFLP) analysis

This strategy can be applied if an enzymatic restriction site is depleted or introduced by either NHEJ-mediated deletion or a HR-mediated knock-in at the target site.

Materials:

- 2x PCR master mix (e.g. OneTaq 2X Master Mix, NEB, M0482)

- locus-specific forward and reverse PCR primers (10 µM)

- PCR purification kit

- agarose gel electrophoresis (setup and consumables)

- suitable restriction enzyme

Protocol:

1. Prepare PCR products following section 3.1, steps 1. -3.

2. In a 20 µl-restriction assay, mix 500 ng of purified PCR product with 5 U restriction enzyme and 2 µl of the respective 10x digestion buffer.

3. Incubate restriction assay for 1 h at the enzyme's recommended temperature.

4. Visualize the digested samples by running a gel electrophoresis and check for the expected fragment sizes. Use wildtype samples for comparison.

3.7.3. Genotyping by subcloning of PCR products

Materials:

- 2x PCR master mix (e.g. OneTaq 2X Master Mix, NEB, M0482)

- locus-specific forward and reverse PCR primers (10 µM)

- PCR purification kit

- agarose gel electrophoresis (setup and consumables)

- PCR cloning kit (e.g. CloneJET PCR Cloning Kit, ThermoFisher, K1231)

- Chemical competent E. coli DH5α (ThermoFisher, 18265-017)

- LB medium and LB agar plates

- Carbenicillin (Sigma, C3416)

- Plasmid DNA Miniprep kit (e.g. QIAprep Spin Miniprep Kit, Qiagen, 27104)

Protocol:

1. Prepare PCR products following section 3.1, steps 1. -3.

2. For subcloning of the PCR amplicons, set up the ligation reaction on ice:

   10 µl 2x Reaction Buffer

   1 µl purified PCR product (10-50 ng/µl)

   6 µl nuclease free water

   1 µl DNA blunting enzyme

3. Vortex briefly and spin down.

4. Incubate at 70°C for 5 min, chill on ice.

5. Add 1 µl pJET1.2/blunt cloning vector and 1 µl T4 DNA ligase to the reaction.

6. Vortex briefly and spin down.

7. Incubate at room temperature for 5 min.

8. The ligated PCR product can be stored at -20°C or immediately used for transformation in competent bacteria:

9. Thaw one vial of bacteria on ice.

10. Add 4 µl from the ligation reaction, mix by flicking and chill on ice for 20 min.

11. Incubate the tube for exactly 1 minute at 42°C in a water bath or heat block.

12. Chill tube for 2 min on ice.

13. Add 500 µl LB medium.

14. Incubate at 37°C for 1 h with agitation.

15. Streak bacteria on a LB-agar plate containing 50 µg/ml carbenicillin and incubate plate at 37°C overnight.

16. The next day, pick 5-10 single colonies from the LB-agar plate and inoculate 3 ml LB medium containing 50 µg/ml carbenicillin and incubated overnight at 37°C in a shaking incubator.

17. Isolate the plasmid DNA by using the mini-prep kit according to the manufacturer's instructions.

18. Sanger sequence the plasmid DNA with pJET1.2 forward or reverse sequencing primer.

19. Check the chromatograms of each colony allele and compare with a wildtype sequence to identify the type of mutation.

4. Anticipated results

4.1 Example 1 – Targeting of the *Cybb* gene

As an example for genotyping of founder mice by Sanger sequencing, we include results of mice derived from microinjection of cr/tracrRNA targeting the coding nucleotide C517 in exon 6 of the *Cybb* gene, recapitulating an X-CGD (X-linked chronic granulomatous disease) patient-derived mutation. The intended single nucleotide deletion should be introduced by HDR using a mutagenic oligonucleotide lacking C517. We used a primer pair for amplification of 314 bp of the target region in the *Cybb* gene. Below we show three exemplary genotypes of mutants from this gene editing experiment. Since the *Cybb* gene is located on the X chromosome, PCR products derived from male founders represent a single target allele and can directly reveal mutant sequences in comparison to the wildtype sequence. Male founder #8255 showed a 15 bp deletion in the target region generated by NHEJ (Fig. 4A). Male founder #8327 showed the intended single nucleotide deletion at C517

(Fig. 4B) and was selected for breeding to establish the mutant mouse line. Female founder #8257 showed mixed sequence peaks in the target region (Fig. 4C), indicating the presence of one or more mutant alleles which could not be resolved by simple sequence alignment. To reveal the exact sequence of the mutant alleles, the PCR product was subcloned into a plasmid vector. Among 12 sequenced subclones we found several alleles harboring deletions but none of these alleles represented the desired deletion of nucleotide C517.

- Figure 4 -

4.2 Example 2 – Targeting of the Rosa26 locus

In order to optimize the efficiency of CRISPR/Cas9-mediated gene targeting in zygotes without the efforts of embryo transfers, a PCR-based assay on mouse blastocysts can be performed. Here, after delivery of CRISPR/Cas9 reagents by microinjection or electroporation, the zygotes are grown in culture into morulas or blastocysts. Then DNA is extracted from individual embryos (see section **3.5**) and the Rosa26 target region is amplified by nested PCR followed by RFLP analysis or sequencing. We use a Rosa26 specific guide RNA which targets the XbaI site located in the first intron, defining the border between the up- and downstream regions which are commonly used as homology regions in Rosa26 targeting vectors. For assessing the efficiency of HDR we use an ssODN which introduces a BamHI restriction site and monitor knockin events by digestion of PCR products with this enzyme. Sequence deletions can be monitored by digestion of PCR products with XbaI. Unmodified wildtype PCR products are XbaI sensitive whereas sequence deletions lead to the loss of the XbaI site and the resistance of PCR products to digestion.

- Figure 5 –

1. Sequence of the guide RNA to target the mouse Rosa26 locus

5'-ACUCCAGUCUUUCUAGAAGA-3'

2. Sequence of the Rosa26 ssODN (XbaI - underlined; BamHI – bold):

5'-
CTGGGAGAATCCCTTCCCCCTCTTCCCTCGTGATCTGCAACTCCAGTCTT<u>TCTAGA</u>**GGATCC**
AGATGGGCGGGAGTCTTCTGGGCAGGCTTAAAGGCTAACCTGGTGTGTGG-3'

3. PCR Primers for genotyping of Rosa 26:

First PCR product (341 bp)

Rosa26 fw-1: 5'-ACCTTTCTGGGAGTTCTCTGCTGCC-3'

Rosa26 rev-2: 5'-ACTCCGAGGCGGATCACAAGCA-3'

Second PCR product (196 bp)

Rosa26 fw-2: 5'-GCCTCCTGGCTTCTGAGGACCG-3'

Rosa26 rev-2: 5'-TCTGTGGGAAGTCTTGTCCCTCC-3'

The nested PCR (section **3.5**) is performed using the following conditions:

98 °C	5 min	
95 °C	20 sec	
60 °C	20 sec	35x
72 °C	20 sec	
72 °C	3 min	

- Figure 6 -

Acknowledgements

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Figure Legends

Figure 1. The CRISPR/Cas9 nuclease system

A: Cas9 and sgRNA recognize 20 nt target sequences located upstream of the invariant 3 bp NGG PAM ('protospacer adjacent motif', red letters). The double-strand break occurs 3 bp upstream of the NGG PAM sequence (red arrows). **B:** Cas9 bound to sgRNA screens DNA for sequences complementary to the first 20 nt of the sgRNA. If a target sequence is located upstream of the PAM site, each of the RuvC and HNH nuclease domains of Cas9 cut one strand of the target DNA (red arrows).

Figure 2. Gene editing in mouse zygotes using CRISPR/Cas9.

Mouse zygotes are microinjected into the (larger) male pronucleus with Cas9 protein or mRNA, sgRNA and a repair template plasmid vector or oligonucleotide (ODN). The double-strand break (DSB) in the target gene can be either repaired by NHEJ, leading to small sequence deletions and gene knockout alleles. Precise sequence modifications (Knockin alleles) are introduced by HDR with the homology regions of DNA template molecules (ODN or plasmid vector). Mice derived from microinjected zygotes (F_0 generation) represent a variety of mutant founder alleles. Mating of the F_0 founders transfers individual mutant alleles to their F_1 progeny.

Figure 3. Timing of CRISPR/Cas9 gene editing projects

A: Three days before microinjection, C57BL/6 donor females are treated with hormones and mated. At the next day, zygotes are collected, microinjected with CRISPR/Cas9 reagents, and transferred to pseudopregnant CD1 foster mothers (mated to vasectomized males)(**B**). **C:** After 7 weeks, biopsies for genotyping can be obtained from 4-week-old pups derived from the microinjected zygotes. **D:** Selected adult F_0 founder mutants can be further mated to obtain F_1 progeny (4-week-age) 11 weeks later for the second round of genotyping.

Figure 4: Sequence analysis of *Cybb* gene founder mutants.

The sequence of the wildtype *Cybb* gene (exon 6) is aligned to sequenced PCR products of individual founder mice. Nucleotide C517 is shown in a red circle, the DSB site created by Cas9 is indicated by an arrow.

Figure 5. Rosa 26 gene targeting in mouse zygotes andnested PCR assay on blastocysts. **A:** The guide RNA target site within the Rosa26 locus is indicated in red, the PAM site in orange. A BamHI site is introduced by the Rosa26 ssODN and is shown in blue. The genomic XbaI site is underlined. **B:** Overview of the PCR assay performed on the mouse blastocysts. A nested PCR is performed followed by XbaI and BamHI restriction digests.

Figure 6. RFLP analysis of Rosa26 PCR products.

The agarose gel image shows the RFLP analysis of Rosa26 PCR products amplified from blastocysts which were derived from zygotes electroporated with Rosa26 specific guide RNA and Cas9 protein. XbaI digestion was performed to differentiate between knockout and wildtype samples. A single 200 bp band (red stars) shows double knockout by loss of XbaI site on both alleles, two bands of 80 bp and 120 bp represent wildtype allele and three bands of 200 bp, 120 bp and 80 bp (red arrows) depict knockout on single alleles.
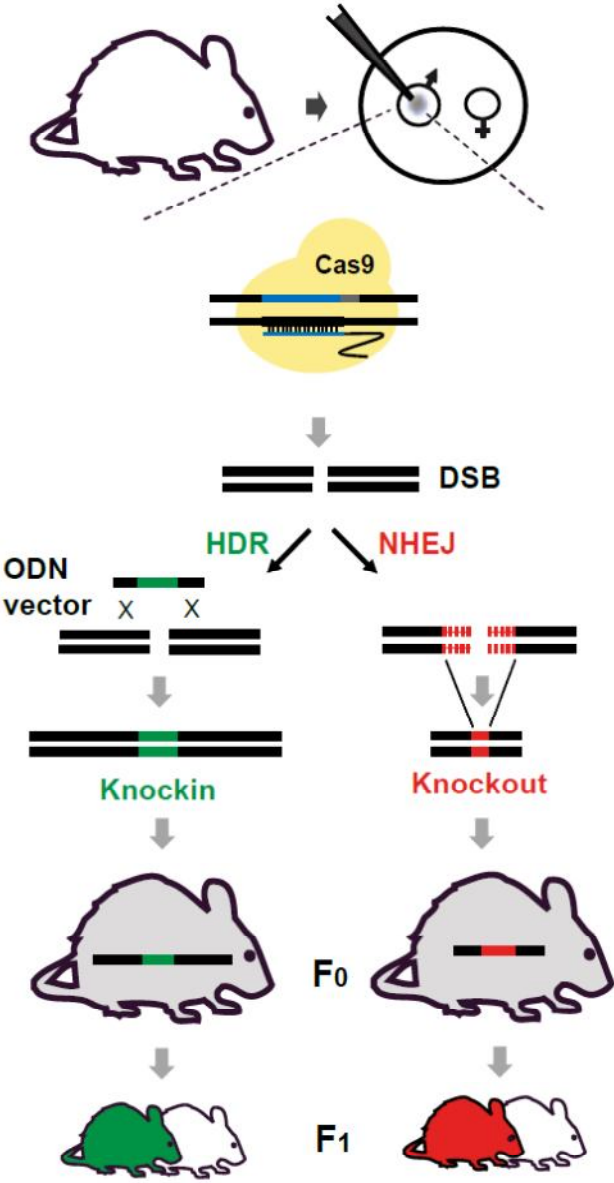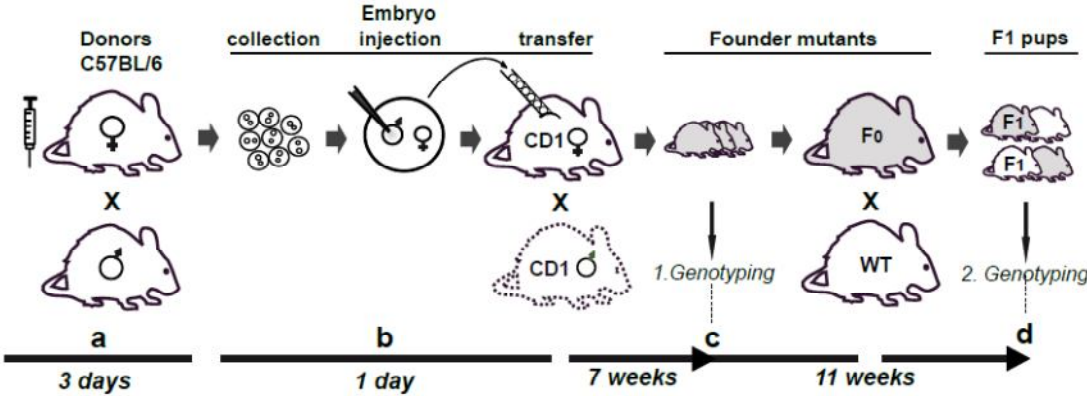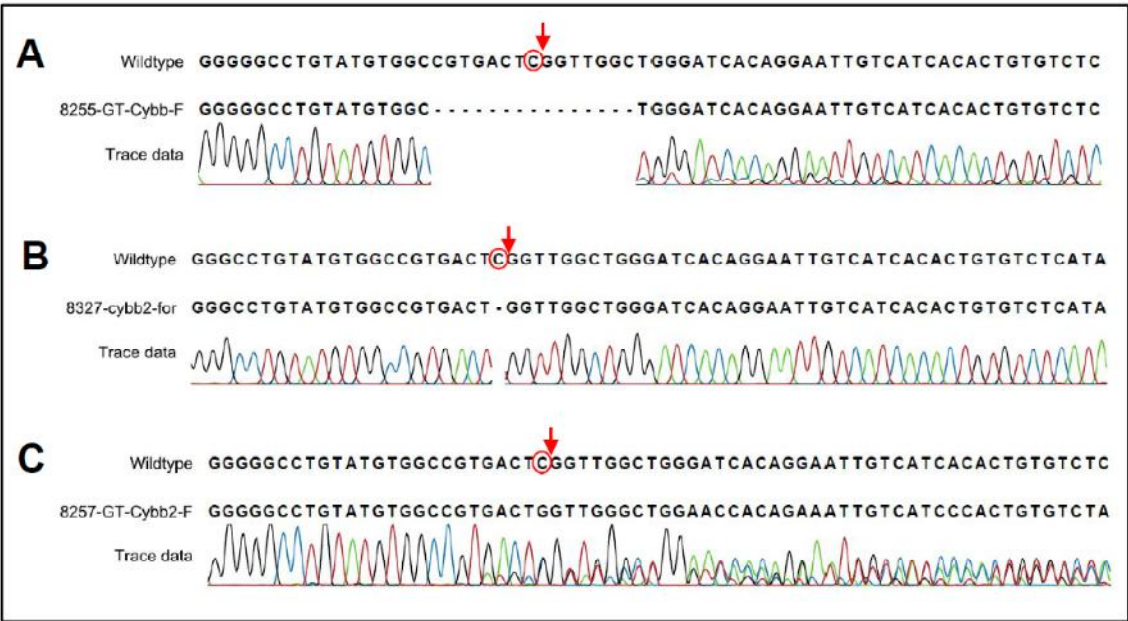
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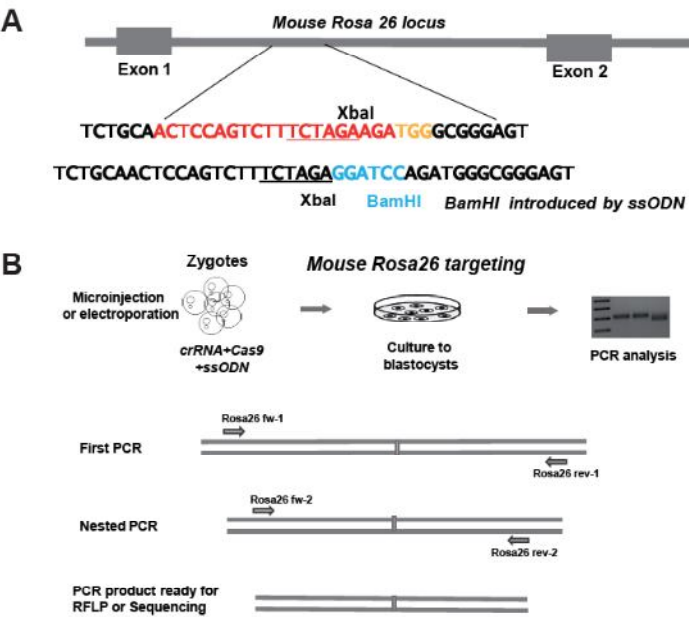
Figure 2

Figure 3

Figure 4

Figure 5

A

Mouse Rosa 26 locus

Exon 1   Exon 2

XbaI
TCTGCAACTCCAGTCTTTCTAGAAGATGGGCGGGAGT

TCTGCAACTCCAGTCTTTCTAGAGGATCCAGATGGGCGGGAGT

XbaI   BamHI   *BamHI introduced by ssODN*

B

Zygotes   *Mouse Rosa26 targeting*

Microinjection
or electroporation

*crRNA+Cas9
+ssODN*

Culture to
blastocysts

PCR analysis

First PCR

Rosa26 fw-1

Rosa26 rev-1

Nested PCR

Rosa26 fw-2

Rosa26 rev-2

PCR product ready for
RFLP or Sequencing

Figure 6