



Chapter 11

Characterization of Mammalian In Vivo Enhancers Using Mouse Transgenesis and CRISPR Genome Editing

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Abstract

Embryonic morphogenesis is strictly dependent on tight spatiotemporal control of developmental gene expression, which is typically achieved through the concerted activity of multiple enhancers driving cell type-specific expression of a target gene. Mammalian genomes are organized in topologically associated domains, providing a preferred environment and framework for interactions between transcriptional enhancers and gene promoters. While epigenomic profiling and three-dimensional chromatin conformation capture have significantly increased the accuracy of identifying enhancers, assessment of subregional enhancer activities via transgenic reporter assays in mice remains the gold standard for assigning enhancer activity in vivo. Once this activity is defined, the ideal method to explore the functional necessity of a transcriptional enhancer and its contribution to target gene dosage and morphological or physiological processes is deletion of the enhancer sequence from the mouse genome. Here we present detailed protocols for efficient introduction of enhancer-reporter transgenes and CRISPR-mediated genomic deletions into the mouse genome, including a step-by-step guide for pronuclear microinjection of fertilized mouse eggs. We provide instructions for the assembly and genomic integration of enhancer-reporter cassettes that have been used for validation of thousands of putative enhancer sequences accessible through the VISTA enhancer browser, including a recently published method for robust site-directed transgenesis at the H11 safe-harbor locus. Together, these methods enable rapid and large-scale assessment of enhancer activities and sequence variants in mice, which is essential to understand mammalian genome function and genetic diseases.

Key words Gene transcription, Enhancers, *cis*-regulatory elements, Pronuclear injection, Transgenesis, Transgenic mice, CRISPR, Deletions, LacZ reporter, H11

Marco Osterwalder and Stella Tran contributed equally to this work.

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1 Introduction

Transcriptional enhancers, typically hundreds of base pairs in length, represent the predominant class of noncoding *cis*-regulatory elements in metazoan genomes, and their activities are critically required for the dynamic control of tissue-specific gene expression patterns during development and adulthood [1]. Following activation by a specific set of transcription factors (TFs), enhancers loop over to their target gene promoters and regulate gene expression independently of their position, orientation, and distance within a topologically associated domain (TAD) [2]. Putative enhancer elements are typically defined by a combination of chromatin accessibility, TF binding, coactivator signatures (e.g., CBP/p300), and histone modifications (i.e., H3K4me1 and H3K27ac), as well as three-dimensional (3D) chromatin proximity interactions [1, 3–5]. In particular, recent efforts by consortia such as ENCODE, Roadmap Epigenomics, psychENCODE, FANTOM, and the 4D Nucleome Project have yielded a wealth of regulatory annotations in both the human and mouse genomes [3, 6–9]. However, since epigenomic profiling is typically performed on heterogeneous tissue and no known molecular mark is perfectly correlated with enhancer activity, these signatures alone are often not sufficient to predict tissue- and/or cell type-specific enhancer activity *in vivo* with complete accuracy [3, 10, 11]. Therefore, experimental validation of putative enhancer elements in model organisms is essential to confirm enhancer activity and determine relevant spatial and temporal activities that regulate accurate transcription of nearby genes. Notably, the basic operational definition of an enhancer, the ability to drive transcription from a minimal promoter independently of orientation and relative position, has remained unchanged since its discovery 40 years ago [12]. Transgenic enhancer-reporter assays represent the most established method to elucidate the diverse and frequently complex enhancer activities at cellular resolution, and thousands of *in vivo* enhancers have been identified and characterized by the systematic use of *LacZ* reporter transgenes in mouse embryos [13–15] (VISTA Enhancer Browser: <https://enhancer.lbl.gov>). For example, recent studies defining the functional properties of murine and human enhancers in craniofacial development, malformation syndromes, and inter-species variation have all relied on extensive *in vivo* enhancer validation using *LacZ* reporter transgenesis [16–18]. While these approaches were based on conventional random integration transgenesis for insertion of enhancer-*LacZ* reporter cassettes, the recently developed enSERT (for enhancer insertion) methodology makes use of CRISPR/Cas9 for site-directed insertion of enhancer-reporter transgenes into the Hpp11 (H11)

intergenic safe-harbor site, thereby enabling superior efficiency and reproducibility of enhancer-reporter activities [19].

While transgenic enhancer-reporter assays validate whether a candidate enhancer element is sufficient to drive tissue-specific gene expression, the overall endogenous transcriptional contribution to target gene dosage and the resulting phenotypic impacts are not addressed by this method. Instead, deletion of the endogenous genomic enhancer sequence is essential to define the role of enhancers in organismal development and disease [20, 21]. In particular, genomic deletions of long-range craniofacial enhancers near developmental transcription factors implicated in craniofacial malformations, such as *Msx1* or *Sox9*, have revealed specific enhancer functions required to fine-tune craniofacial morphology [16, 18]. In recent years, the engineering of genomic deletions in mice via traditional homologous recombination methods has been supplanted by CRISPR/Cas9 due to substantially higher throughput and ease of use. CRISPR/Cas9-induced deletions offer a powerful way to explore in vivo enhancer functions, either individually or in combination, in complex transcriptional landscapes [22, 23].

In this chapter, we describe how to prepare versatile enhancer-reporter vectors and CRISPR mixes to introduce enhancer-reporter transgenes or genomic deletions, respectively, into the mouse genome via pronuclear microinjection (PNI) (Fig. 1). In a first step, we focus on transgenic methods, including preparation of enhancer-reporter vectors for both conventional random integration [13, 14] and site-directed (enSERT) transgenesis [19]. We further outline how to efficiently generate injection mixes for CRISPR/Cas9-mediated genomic deletions, which enable the characterization of phenotypic consequences of enhancer loss in mice throughout prenatal and postnatal development [18, 22, 23]. Subsequently, we provide a detailed protocol describing the steps for PNI of transgenic vectors and CRISPR/Cas9 deletion mixes into mouse zygotes and the generation of genome-edited mice. Finally, we outline strategies for PCR screening and selection of founder individuals for transgenic reporter and genomic deletion mouse lines and provide instructions for X-gal staining of transgenic embryos expressing the *LacZ* reporter gene. Combining targeted enSERT transgenesis and genomic deletion strategies via CRISPR/Cas9 in mice enables efficient functional dissection of transcriptional enhancer landscapes, as well as individual enhancers, to uncover the *cis*-regulatory mechanisms underlying embryonic development and human disease [19, 20, 24].

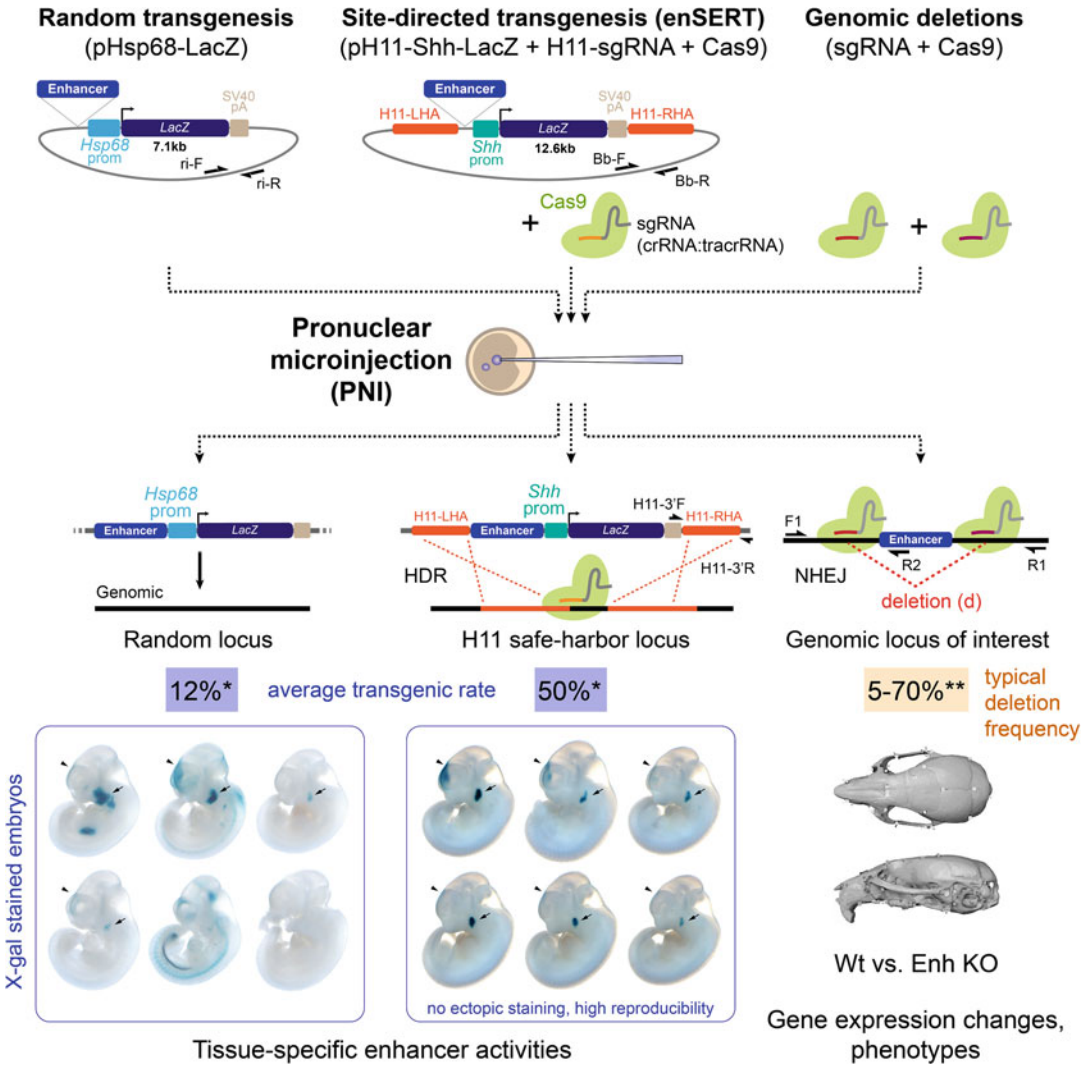


Fig. 1 Overview of PNI-based strategies for enhancer-reporter transgenesis and generation of endogenous deletions for transcriptional enhancer analysis. **Left:** Until recently, the Hsp68-LacZ reporter was used as the vector framework of choice for large-scale in vivo enhancer validation in mouse embryos [15, 16]. This vector is integrated via random transgenesis, which is enabled through injection of the linearized plasmid into the pronucleus of fertilized mouse eggs [13, 45, 46]. **Middle:** Recruitment of homology-directed repair (HDR) via CRISPR/Cas9 recently enabled the development of site-directed integration of transgenic enhancer-reporter cassettes at the intergenic and transcriptionally neutral H11 site (termed enSERT for *enhancer insertion*) [19, 32]. EnSERT is based on the pronuclear injection of a mix of circular targeting vector (pH11-Shh-LacZ), complexed H11-specific single guide (sg) RNA and Cas9 protein. EnSERT shows significantly improved transgenic rates and reproducibility when compared to random integration-mediated transgenesis, as the process is highly efficient and transgenic cassettes are not subject to position effects [19]. While the *Hsp68* promoter fragment conventionally used for random transgenesis shows rather leaky activity in the H11 locus, the *Shh* minimal promoter is suitable for validation of developmental enhancers in a broad range of embryonic tissues [19]. X-gal-stained embryos illustrate representative transgenic results following pronuclear injection of indicated reagents (using the hs2580 enhancer element) [19]. Arrows and arrowheads mark reproducible staining (enhancer activity) in the mandibular arch and forebrain, respectively. crRNA, CRISPR-RNA; tracrRNA, trans-activating crRNA. **Right:** To explore functional properties of transcriptional enhancers, genomic

2 Materials

2.1 Vector Cloning and Preparation for In Vivo Transgenic Reporter Analysis

1. Cloning vector containing minimal promoter and *LacZ* reporter gene:
 - (a) Random transgenesis vector: The pHsp68-LacZ (Hsp68-LacZ-Gibson, Addgene plasmid #170102) template vector for random integration is a Gibson cloning-compatible (KpnI digested) version of an older Gateway cloning-compatible reporter vector (Hsp68-LacZ-Gateway, Addgene plasmid #37843).
 - OR
 - (b) Site-directed transgenesis vector: The pH11-Shh-LacZ (pCR4-Shh::lacZ-H11) template vector for site-directed transgenesis via enSERT [19] can be obtained from Addgene (#139098).
2. Column-based PCR purification kit.
3. Midiprep plasmid purification kit.
4. DNA quantification device.
5. Restriction enzyme and buffer for enhancer cloning:
 - (a) Random vector: KpnI and NEBuffer 1.1.
 - (b) enSERT vector: NotI and NEBuffer 3.1.
6. Water bath or thermomixer at 37 °C and 50 °C.
7. Agarose gel electrophoresis equipment and buffers.
8. Gel extraction kit.
9. Thermocycler (for PCR).
10. Phusion high-fidelity (HF) polymerase and 5X HF buffer.
11. Vector-specific oligonucleotides with Gibson overhangs (Fig. 2):
 - (a) Random vector:
 - (Ia) pHsp68-LacZ_Gibson_Fwd (5'- CACTAAAGG GAACAAAAGCTGGTAC-3').

Fig. 1 (continued) deletions can be introduced in a highly efficient manner using a mix of Cas9 protein and CRISPR sgRNAs (or a crRNA:tracrRNA duplex) targeted to the flanking ends of the endogenous enhancer locus. CRISPR ribonucleoprotein (RNP) complexes can also be delivered efficiently via CRISPR-EZ, using electroporation of zygotes, to bypass PNI [47]. *average transgenic rate determined from [19]. **range of deletion frequencies observed for CRISPR deletion founder mice generated in [22] based on the described protocol. Primers used for transgenic screening and genotyping are indicated as arrows (sequences provided below). LHA, left homology arm; RHA, right homology arm; Enh KO, enhancer knockout (deletion). Mouse skull images are taken from [16] as an illustrative example of phenotype assessments (here: morphometric measurements of changes in skull morphology)

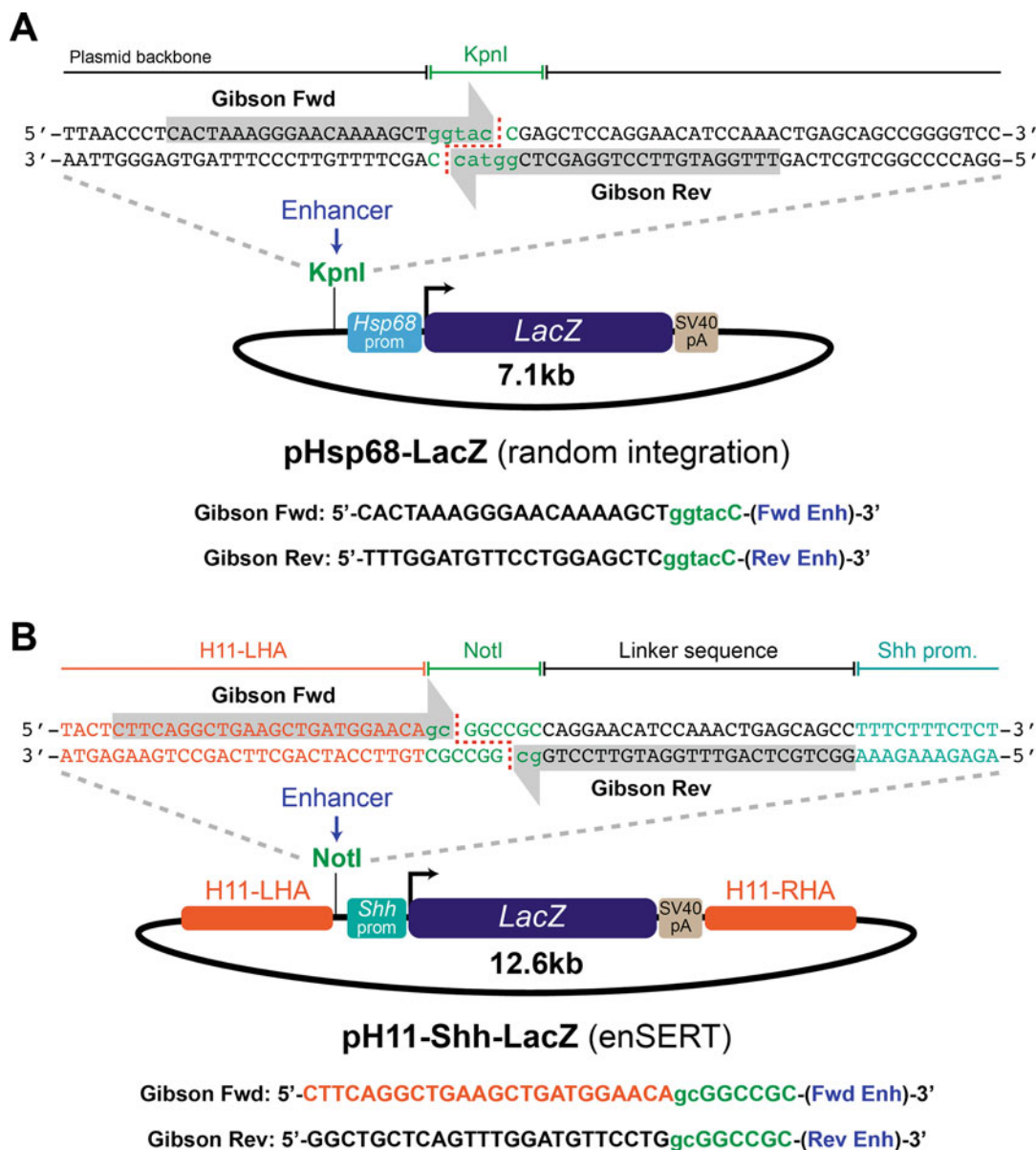


Fig. 2 Vectors used for high-throughput LacZ transgenesis. (a) The pHsp68-LacZ reporter plasmid (Addgene #170102) is a Gibson cloning-compatible version of a vector commonly used for in vivo enhancer characterization via random transgenesis (Addgene #37843). Until the development of enSERT, PNI and random genomic integration of linearized “enhancer-Hsp68-LacZ” vector was the primary method used for large-scale validation of VISTA Enhancers [13, 14, 48]. Thousands of in vivo enhancer elements with tissue-specific, embryonic activities were identified using this method, and the respective constructs are available as part of the VISTA Enhancer Browser repository (<https://enhancer.lbl.gov>) [15]. (b) The recently developed vector framework for highly efficient, site-directed transgenesis at the transcriptionally neutral H11 intergenic site [19]. This vector is also designed for Gibson cloning of the enhancer upstream of a minimal promoter and LacZ reporter. Using pronuclear injection, circular vector is injected with a mixture of Cas9/sgRNA ribonucleoprotein (RNP) complexes targeting the H11 locus. The use of H11 homology arms in the vector results in preferential transgene integration at the H11 locus through homology-directed repair (HDR). The enSERT vector is available from the Addgene plasmid repository (Plasmid #139098). Fwd, forward primer. Rev., reverse primer. LHA, left homology arm. RHA, right homology arm. Both vectors contain an ampicillin (Amp)-resistance cassette for antibiotic selection during cloning

(Ib) pHsp68-LacZ_Gibson_Rev (5'-TTTGGATGTTCCTGGAGCTCGGTAC-3').

(b) enSERT vector:

IIa) pH11-Shh-LacZ_Gibson_Fwd (5'-CTTCAGGC TGAAGCTGATGGAACAGC-3').

IIb) pH11-Shh-LacZ_Gibson_Rev (5'-GGCTGCTC AGTTTGGATGTTCTGGC-3').

12. dNTPs.
13. DNA (enhancer) template for PCR amplification (e.g., purified genomic or synthesized DNA sample, *see below*).
14. AMPure XP beads.
15. Magnetic rack for bead separation.
16. Milli-Q water (molecular biology grade).
17. Ethanol (molecular biology grade).
18. NEB 2× HiFi DNA Assembly Mix (for Gibson cloning).
19. Electroporator.
20. One Shot™ TOP10 Electrocomp™ *E. coli*.
21. Electroporation cuvettes (1 mm).
22. Orbital shaker at 37 °C.
23. SOC media.
24. LB agar plates containing ampicillin (100 µg/mL) or carbenicillin (100 µg/mL).
25. Glass plating beads.
26. LB medium containing ampicillin (200 µg/mL).
27. Miniprep kit.
28. Microinjection buffer (MI buffer): 10 mM Tris, 0.1 pH 7.5, 0.1 mM EDTA pH 8.0, prepared in ultrapure nuclease-free water, store at 4 °C.

2.2 Preparation of CRISPR Injection Mixes for Site-Directed Transgenesis

1. Alt-R® CRISPR-Cas9 H11-sgRNA, lyophilized (IDT) (5'-gctgatggaacagtaaca-3'), store resuspended 50 µL aliquots at -80 °C.
2. Alt-R® S.p. Cas9 Nuclease V3, 100 µg (e.g., IDT, Cat#1081058).
3. Microinjection buffer (MI buffer): 10 mM Tris, 0.1 pH 7.5, 0.1 mM EDTA pH 8.0, prepared in ultrapure nuclease-free water, store at 4 °C.
4. enSERT H11-targeting vector (pH11-Shh-LacZ) (Subheading 2.1).
5. Eppendorf® LoBind 1.5 mL tubes with reduced sample-to-tube binding for optimized sample recovery.

6. Centrifugal filters for 1.5 mL tubes, 0.1 µm pore size, hydrophilic PVDF.
7. Thermoblock at 95 °C.
8. Centrifuge.
9. Parafilm.

2.3 Preparation of CRISPR Injection Mixes for Genomic Deletions

1. Alt-R[®] CRISPR-Cas9 crRNA(s) (IDT) (to be designed, *see*, e.g., **Note 15**), resuspended 50 µL aliquots (50 µm) are stored at −80 °C.
2. Alt-R[®] CRISPR-Cas9 tracrRNA (IDT) (generic) (5'-AGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAA CUUGAAAAAGUGGCACCGAGUCGGUGCUUU-3').
3. Alt-R[®] S.p. Cas9 Nuclease V3, 100 µg, 10 µg/µL; Cat#1081058 (IDT).
4. Microinjection buffer (MI buffer): 10 mM Tris, 0.1 pH 7.5, 0.1 mM EDTA pH 8.0, prepared in ultrapure nuclease-free water, store at 4 °C.
5. Thermoblock at 95 °C.
6. Eppendorf[®] LoBind 1.5 mL tubes with reduced sample-to-tube binding for optimized sample recovery.
7. Centrifugal filters for 1.5 mL tubes, 0.1 µm pore size, hydrophilic PVDF.
8. Centrifuge.
9. Parafilm.

2.4 Colony Set-up for Transgenic Mouse Production, Superovulation, and Egg Collection

Mice:

1. FVB/NJ females, ordered at 7 weeks old.
2. FVB/NJ males, 8 weeks old to 8 months old.
3. CD-1 females, 26 g to 35 g.
4. BDF1 (also known as B6D2F1) males, 8 weeks to 24 months of age.

Superovulation:

5. Pregnant mare serum gonadotropin (i.e., PMSG).
6. Human chorionic gonadotropin (i.e., hCG).
7. Sterile saline solution.
8. Sterile 1 mL syringes and needles (28–31 G, length 12.7 mm).

Pulled Glass Capillaries:

9. Hand-pulled capillaries for egg handling and embryo transfer (as a component of the mouth-pipette): Borosilicate glass capillaries, thin wall without filament, OD 1 mm, ID 0.75 mm,

length 10 cm (Sutter Instruments B100-75-10) and prepared for use according to Subheading 3.4, step 6 below.

10. Bunsen burner.
11. Stereoscope with graduated eyepiece (Nikon 10×/23 with graduation in 1 mm and 100 graduations overall).
12. Diamond-tip pencil to score glass.
13. Machine-pulled glass needles with filament for zygote microinjection and holding (as components of the microinjection set-up): Borosilicate glass capillaries, thin wall with filament, OD 1 mm, ID 0.78 mm, length 10 cm (Warner Instruments, G100TF-4), and prepared for use according to **Note 36**.
14. Micropipette Puller (Sutter Instruments Flaming/Brown model P-97).
15. Microforge (Narishige MF830).

Egg Collection:

16. M2 and M16 media (Sigma).
17. Hyaluronidase.
18. Disposable underpad, 23 × 26 in.
19. Center well organ culture dish, 60 mm.
20. Square petri dish with grid, 90 × 15 mm.
21. Full size borosilicate glass capillary (Sutter Instrument B100-75-10).
22. Mouth-pipette fitted with a hand-pulled glass capillary size 8–10.
23. 70% ethanol spray.
24. One pair of toothed tissue forceps.
25. One pair of curved microdissecting serrated forceps.
26. One pair of spring scissors/iris scissors.
27. Two pairs of fine forceps (bent-curved tip).
28. Stereomicroscope/dissecting scope.
29. Incubator set to 37°C and 5% CO₂.
30. CO₂ euthanasia station.

2.5 Pronuclear Injection and Embryo Transfer

Microinjection Workstation Set-Up:

1. Inverted microscope (Nikon TE Eclipse series).
2. Manual hanging joystick micromanipulator (Narishige) or electronic micromanipulator (Eppendorf Transferrman NK2).
3. CellTram air pneumatic/air-column microinjector (Eppendorf).
4. Femtojet electronic microinjector (Eppendorf).

5. Anti-vibration microscope platform and pads.
6. Pneumatic height adjustable microscope workbench (optional).

Zygote Microinjection:

7. Machine-pulled injection needles (with filament), see above.
8. Forged holding pipet (made from a machine-pulled needle).
9. Cell culture chamber glass slide, 2-well.
10. Center-well organ culture dish, 60 mm.
11. M2 and M16 media (Sigma).
12. Incubator set to 37°C and 5% CO₂.
13. Injection mixes in microfuge tubes.
14. Centrifuge.
15. Stereomicroscope/dissecting scope.
16. Mouse eggs, collected in the morning of microinjection.
17. Mouth-pipette fitted with a hand-pulled capillary (size 8–10), as well as a full-size capillary.

Embryo Transfer:

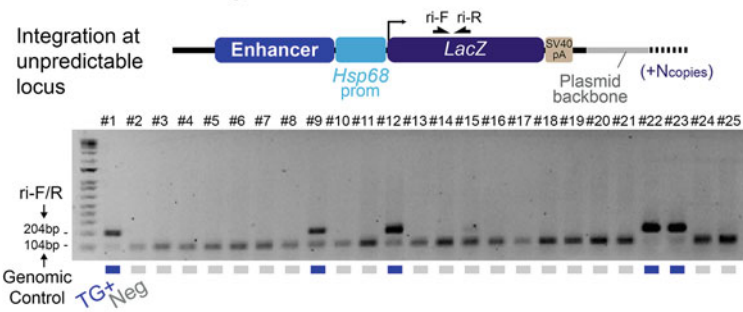
18. Nonsteroidal anti-inflammatory drug (NSAIDs) analgesic—meloxicam.
19. Saline solution.
20. 1 mL syringes with 28 G needle.
21. Inhalation anesthetic—isoflurane.
22. Veterinary-grade isoflurane vaporizer outfitted with rodent nosecones, vented induction anesthesia chamber, and gas scavenger system.
23. Oxygen supply.
24. Electric hair shaving clippers.
25. M2 medium.
26. Injected mouse zygotes in M16 medium from the incubator.
27. Pseudopregnant CD-1 females with visible copulatory plugs.
28. Dissection workbench with rubber pad.
29. Disposable sterile poly-lined drape for surgical field.
30. Two pairs of Dumont #5 titanium tip forceps 10 × 0.6 mm.
31. One pair of curved microdissecting-serrated forceps.
32. Serrefine clamp.
33. One pair of toothed tissue forceps.
34. Hemostatic forceps/Webster needle holder.

35. Chromic gut absorbable suture 5-0, tapered point.
36. Wound clip closing stapler.
37. Wound clips, 9 mm.
38. Long cotton swabs.
39. Gauze squares soaked in ethanol.
40. Antiseptic—povidone–iodine solution 10%.
41. 70% ethanol.
42. Large Kimwipes.
43. Mouth-pipette fitted with a hand-pulled capillary size 6–8.
44. Stereomicroscope/dissecting scope.
45. Animal warming chamber.

2.6 *LacZ* Transgenic Embryo Collection and X-gal Staining

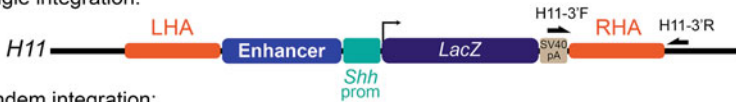
1. Cold 1× PBS buffer: 137 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), and 12 mM phosphate buffer, pH 7.4.
2. 70% ethanol spray.
3. Two-round petri dishes, 100 × 15 mm.
4. Disposable underpad, 23 × 26 in.
5. One pair of surgical scissors.
6. One pair of iris scissors.
7. Two pairs of fine forceps.
8. One pair of curved tweezers.
9. Disposable plastic transfer pipets (cut to modify the opening to accommodate embryos of various sizes).
10. 4% paraformaldehyde in 1× PBS, store at 4 °C before use.
11. CO₂ euthanasia station.
12. Embryo wash buffer: 2 mM MgCl₂, 0.02% NP-40, and 0.01% deoxycholate in 1× PBS (pH 7.3).
13. Reagents for X-gal stain, store at 4 °C:
 - (a) 200 mM potassium ferricyanide.
 - (b) 200 mM potassium ferrocyanide.
 - (c) 50 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) in dimethylformamide (DMF).
 - (d) 1 M Tris (pH 7.5).
14. Conical tubes, 15 mL and 50 mL.
15. Round-bottom microfuge tubes, 2 mL.
16. Aluminum foil and/or an opaque box.
17. 48-well flat-bottomed culture dishes for embryo storage.

A Random transgenesis

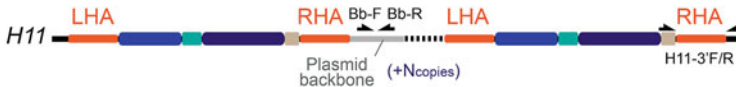


B Site-directed transgenesis (enSERT)

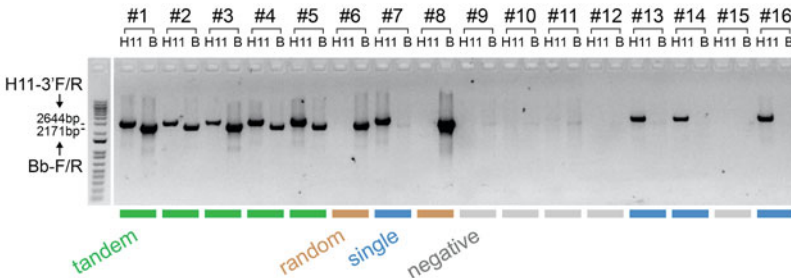
1) Single integration:



2) Tandem integration:



3) Random integration:



C Genomic deletions

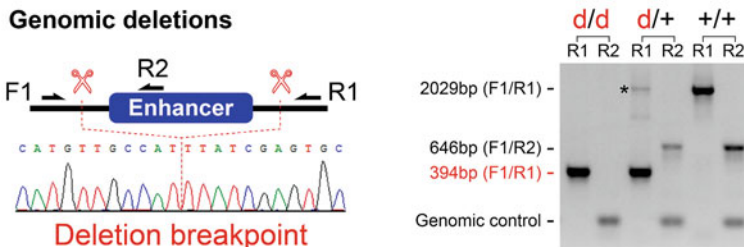


Fig. 3 Genotyping strategies for detection of *LacZ* reporter transgenes and enhancer deletions in mouse genomic DNA samples. **(a)** For random integration of linearized vectors, primers targeted to the *LacZ* coding region (ri-F/R) are utilized to identify transgenic embryos. Primers amplifying an unrelated genomic region (genomic control primers) are used in the same mix as control. Random integration of linearized DNA molecules typically leads to highly variable copy numbers [49]. TG⁺, transgene positive. Neg, transgene negative. **(b)** Routine PCR genotyping strategy to determine different transgene configurations resulting from enSERT [19]. Primer pairs targeted to the 3' junction of the right homology arm (RHA) (H11: H11-3'F/3'R) and the vector backbone (B: Bb-F/R) are utilized to indicate the following configurations: (1) insertion of a single

18. 96-well plates for sac collection.
19. Rocking platform.

2.7 Genotyping

1. DirectPCR (Tail) lysis reagent (ViaGen), store at 4 °C.
2. Proteinase K, 600 U/mL, store at 4 °C.
3. Water bath or thermomixer at 56 °C and 85 °C.
4. Thermocycler for PCR.
5. DNA polymerase (e.g., Taq).
6. (I) Primers for Hsp68-LacZ transgene detection (random integration) (Fig. 3):
 - (a) ri-F: 5'-GTCGTTTGCCGTCTGAATTT-3'.
 - (b) ri-R: 5'-CATTAAAGCGAGTGGCAACA-3'.
 - (c) ri amplicon size: 204 bp.
- (II) Genomic control (gc) primers:
 - (d) gc-F: 5'-GAGCAGTAGGGAGCAGAGGA-3'.
 - (e) gc-R: 5'-GGCAGGGGATACTCCATTTT-3'.
 - (f) gc amplicon size: 104 bp.
7. Primers for verification of site-directed integration at H11 (enSERT) (Fig. 3):
 - (a) 5' junction:
 - (b) H11-5'F: 5'-ACACTAAGGAACCCTGGCTGTG-3'.
 - (c) H11-5'R: (enhancer-specific primer).
 - (d) H11-5' amplicon size: variable.
 - (e) H11-3'F: 5'-spiepr A3B2 show [LongWord]TGCATTC-TAGTTGTGGTTTGTCCAspiepr H11-5' amplicon size:

Fig. 3 (continued) copy of the transgene at the H11 locus, (2) tandem insertion of the transgenic vector at the H11 locus, and (3) integration of the vector at random loci. Genomic control primers are run in a parallel reaction. Embryos harboring tandem integrations of the transgene at the H11 locus show superior sensitivity over single integrations (due to the presence of multiple copies), while retaining the high reproducibility of *LacZ* patterns (due to absence of position effects). Tandem integrations were observed in 52% (4256/8135) of transgenic embryos using the described enSERT strategy (single: 26%, random: 18%). Rarely, an individual embryo with a tandem integration PCR-signature may display ectopic (nonreproducible) *LacZ* activity, suggesting a combination of site-directed (single or tandem) transgenic insertion and random integration. (c) Strategy for genotyping and selection of individual enhancer deletion alleles obtained via CRISPR/Cas9 genome editing followed by nonhomologous end joining (NHEJ), e.g., used to genotype founder mice. The F1/R1 primer pair detects the wildtype and deletion allele. The F1/R2 fragment only amplifies in the presence of the wild-type allele. The F1/R1 deletion allele-specific band is gel extracted and analyzed via Sanger sequencing to verify clean deletion breakpoints (e.g., to rule out the presence of indels). Asterisk indicates the preference of PCR for amplification of only the shorter amplicon (deletion band) in samples from heterozygotes (d/+). Images in C are reused from [22] (enhancer mm1179). PCR primer IDs and related amplicon sizes (in base pairs, bp) are indicated on the left of the gel images

variable.H11-3'F: 5'- TGCATTC
TAGTTGTGGTTTGTCCA-3'.

(f) H11-3'R: 5'-ACCTTTGCTCTTGGGGCTTAGA-3'.

(g) H11-3' amplicon size: 2644 bp.

8. Primers for detection of enSERT vector backbone (e.g., pH11-Shh-LacZ) (Fig. 3):

(a) Bb-F: TCTGACGCTCAGTGGAAACGAAA.

(b) Bb-R: AGACTGGGCGGTTTTATGGACA.

(c) Bb amplicon size: 2171 bp.

3 Methods

3.1 Vector Cloning and Preparation for In Vivo Transgenic Reporter Analysis

1. Prepare pHsp68-LacZ (for random integration) or pH11-Shh-LacZ vector (for enSERT) for Gibson-mediated integration [25] of a putative enhancer element into the KpnI (pHsp68-LacZ) or NotI (pH11-Shh-LacZ) site located upstream of the minimal promoter (Fig. 2). Digest 10 µg of vector with 10 U of restriction enzyme (1 µL) in 100 µL of NEBuffer (NEBuffer 1.1 for KpnI, 3.1 for NotI) at 37 °C overnight.
2. Purify the linearized plasmid using a spin column-based PCR purification kit and quantify the resulting DNA. Run 250 ng of the linearized vector on a 0.8% agarose gel to verify complete linearization (*see Notes 1 and 2*).
3. Amplify the enhancer sequence by PCR using high-fidelity DNA polymerase (e.g., Phusion), in combination with primers containing vector-specific overhangs for Gibson-ligation (*see* Fig. 2 and Subheading 2.1 for sequence details). Predicted enhancer regions can be amplified from (commercially available) genomic DNA (e.g., mouse genomic DNA from Clontech, <http://www.takarabio.com>), plasmids or bacterial artificial chromosomes (BACs) containing the genomic region of interest, or from a synthesized DNA fragment (e.g., gBlocks from IDT, <http://www.idtdna.com/gblocks>) (*see Note 3*).
4. Per 20 µL reaction, the PCR conditions are as follows: 30–50 ng template genomic DNA (or 2–3 ng of plasmid, BAC or synthesized DNA template), 1 µL Fwd/Rev. Gibson primer (each with appropriate overhangs, 10 µM stock) (*see* Fig. 2), 4 µL 5× Phusion HF buffer, 0.2 µL Phusion DNA polymerase (2 U/µL), 0.4 µL of dNTPs (10 mM), and 12.9 µL of water. The thermocycler is programmed as follows:

- (a) 45'' at 98 °C.
 - (b) 28 cycles of 7'' at 98 °C, 15'' at $X^{\circ}\text{C}$ (where X is calculated from the New England Biolabs Tm Calculator, *see Note 4*), 30'' per kb at 72 °C.
 - (c) A final elongation step of 7' 30'' at 72 °C (*see Note 5*).
5. Confirm the size of the desired amplified PCR product by gel electrophoresis.
6. To prevent carryover of primers, salts, or contaminants, purify PCR product with AMPure XP beads (*see Note 6*). Depending on the size of the fragment of interest, combine PCR product and homogenous bead solution following the manufacturer's instructions regarding the proper beads:insert ratio (*see Note 7*).
7. Mix beads and PCR product by thorough pipetting, followed by incubation at room temperature (RT) for 15 min. After bead separation on the magnetic rack for 2 min, discard supernatant and add 500 μL of 70% ethanol (EtOH) to each sample. Incubate samples on the magnetic rack for 30 s at RT. Next, remove the EtOH and repeat the procedure for a total of two washes.
8. Discard EtOH and air-dry beads on the magnetic rack for up to 5 min at RT. Resuspend the beads in 20 μL of nuclease-free water by pipetting up and down ten times to elute the PCR fragments. Place samples back on the magnetic rack for 30 s to separate eluent from the beads. Transfer the eluate to a new tube and measure the concentration of purified PCR-amplified fragments.
9. For Gibson-mediated insertion of the PCR-amplified enhancer fragment into pHsp68-LacZ or pH11-Shh-LacZ, use the New England Biolabs Ligation Calculator to determine the mass of insert required at specific molar insert:vector ratios (<https://nebiocalculator.neb.com>). Here, a 3:1 insert:vector ratio is used as an example. First, add 1.5 μL of digested vector (~50 ng/ μL) to 5 μL of NEB 2 $\times$ HiFi DNA Assembly Mix. Then add calculated amounts of insert (PCR fragment) and water (*see Note 8*). Incubate the reaction mix at 50 °C for 1 h for Gibson ligation.
10. To grow and screen for ligated constructs of interest, transform competent *E. coli* cells with the Gibson reaction mix following standard procedures for bacterial electroporation. Briefly, add 20 μL of cells to each cuvette on ice (*see Notes 9 and 10*). Add 0.75 μL of Gibson ligation reaction mix to the cuvette and electroporate using a preset bacterial protocol for 1 mm cuvettes (*see Note 11*). Following electroporation, immediately add 250 μL of SOC and pour the mixture into a 1.5 mL tube.

Incubate bacteria in an orbital shaker at 250 rpm and 37 °C for 30–45 min.

11. Distribute bacteria (20–250 µL) on prewarmed LB agar plates containing ampicillin/carbenicillin for both pHsp68-LacZ and pH11-Shh-LacZ vectors. Incubate plates upside-down at 37 °C overnight to allow growth of clonal colonies.
12. *Optional:* For initial and rapid screening of vectors with inserts, bacterial colonies can be subjected to a “PCR colony screen” (*see* **Note 12**). First, pick colonies into 30 µL of LB broth in a 96-well PCR plate, which is later stored at 4 °C and used again in **step 13**. The 20 µL PCR is set up on ice as follows: 1.5 µL of picked colony in LB broth, 2 µL Fwd/Rev. Gibson cloning primers (10 µM) (used in **step 4**), 4 µL 5× Phusion HF buffer, 0.2 µL Phusion DNA polymerase, 0.4 µL of dNTPs (10 mM), and 11.9 µL water. Use the following PCR cycling conditions:
 - (a) 45'' at 98 °C.
 - (b) 25 cycles of 7'' at 98 °C, 15'' at X °C (calculated from NEB Tm Calculator), 30'' per kb at 72 °C.
 - (c) A final elongation step of 7' at 72 °C.

Analyze the PCR using agarose gel electrophoresis and select colonies based on expected insert band size.

13. Purify plasmid DNA from selected bacterial colonies subjected to growth using miniprep (post-PCR colony screen) and subsequent midiprep to generate PNI-ready samples (*see* **Note 13**). For midiprep, air-dry the final pellet for 5–10 min and resuspend DNA in 200 µL of MI buffer. Transfer the sample to a new 1.5 mL tube. Measure eluted plasmid concentration and validate sequence integrity by Sanger sequencing.
14. To confirm successful ligation, digest an aliquot of the midiprep plasmid sample with KpnI (pHsp68-LacZ) or NotI (pH11-Shh-LacZ) to release the insert and validate its size. Measure the concentration of the digested vector.
15. pH11-Shh-LacZ vectors for site-directed transgenesis are microinjected in a *circular* form in a mix with Cas9 and a single-guide (sg) RNA targeted to the H11 locus [19]. Follow Subheading 3.2 for preparation of the enSERT CRISPR injection mix.
16. pHsp68-LacZ vectors for random integration are injected in a *linearized* form and contain a unique NotI site located downstream of the SV40pA. In a 100 µL reaction, use 4–5 µg of the midiprep construct for linearization with 1 U of NotI restriction enzyme (1 µL) in 10 µL of NEB buffer 3.1 and water. Incubate overnight at 37 °C. Purify the reaction using a spin column-based PCR purification kit by following the manufacturer's instructions. Elute the plasmid in 30 µL of MI buffer,

quantify, and store the sample at -20°C . To verify full plasmid linearization, run 250 ng of linearized vector on a 0.8% agarose gel.

17. To prepare injection mixes, dilute the pHsp68-LacZ construct in MI buffer at 25 ng/ μL (final volume of 50 or 100 μL). Confirm plasmid concentration and store the PNI-ready sample at 4°C for up to 6 months. On the day of PNI, dilute the construct to 1.5 ng/ μL in MI buffer to load injection needles. Follow Subheading 3.5 for the microinjection procedure.

3.2 Preparation of CRISPR Injection Mixes for Site-Directed Transgenesis

1. This section describes the preparation of CRISPR microinjection mix (using the Alt-R CRISPR-Cas9 sgRNA system, IDT) containing donor vector DNA (e.g., pH11-Shh-LacZ) for site-directed enhancer insertion (enSERT) at H11. This protocol can also be used for site-directed engineering (knock-in) in other genomic loci of interest (*see Note 14*).
2. Resuspend lyophilized Alt-R CRISPR H11-sgRNA in MI buffer at a final concentration of 50 μM (1700 ng/ μL).
3. Prepare $2\times$ Cas9/sgRNA master mix (total volume: 15 μL). Dilute the stock of Alt-R CRISPR H11-sgRNA with MI buffer to a final concentration of 50 ng/ μL , and add Alt-R SpCas9 nuclease (stored at -20°C) at a final concentration of 20 ng/ μL . Incubate the mix at RT for 15 min to enable formation of CRISPR ribonucleoprotein (RNP) complexes.
4. Donor vector DNA (pH11-“enhancer”-Shh-LacZ vector) is typically injected in CRISPR injection mix at a final concentration of 12.5 ng/ μL . To this purpose, dilute the donor vector in MI buffer to 25 ng/ μL (total volume: 15 μL) in a 1.5 mL tube and add 15 μL of $2\times$ Cas9/sgRNA master mix.
5. To remove particles that may clog PNI needles, purify the final injection mix (30 μL) using 0.1 μm pore size centrifugal filters with subsequent centrifugation at $17,900 \times g$ for 2 min. Seal the sample tube with Parafilm to prevent evaporation and store at 4°C for up to 2 weeks until microinjection.

3.3 Preparation of CRISPR Injection Mixes for Genomic Deletions

1. The Alt-R CRISPR-Cas9 crRNA:tracrRNA duplex system (IDT) is used to efficiently prepare CRISPR-RNP complexes targeting genomic locations upstream and downstream of the region to be deleted (*see Fig. 1 and Note 15*). The 20 bp crRNA spacer sequence complementary to the target DNA sequence (protospacer) is designed using published online tools, such as CHOPCHOP (<https://chopchop.cbu.uib.no>) [26] or CRISPOR (<http://crispor.tefor.net>) [27] using mouse mm10 (C57BL/6) reference genome and an NGG (SpCas9) protospacer adjacent motif (PAM) (*see Notes 16 and 17*).

2. The customized crRNAs, along with tracrRNA, are ordered in lyophilized form. CrRNAs (36 nt, 10 nmol) are then resuspended in 200 μL MI buffer, and tracrRNA (67 nt, 100 nmol) is resuspended in 2 mL MI buffer to obtain equimolar concentrations (50 μM); 612.9 ng/ μL and 1140 ng/ μL final stock concentrations, respectively.
3. Assemble tracrRNA with custom crRNA and Cas9 into an active CRISPR–RNP complex. First, in a LoBind tube, combine crRNAs and tracrRNA (50 μM stocks) in MI buffer at a final volume of 15 μL (50 ng/ μL final RNA concentration). Incubate the mix at 95 °C for 5 min and let it cool down to RT. Then, add Alt-R SpCas9 enzyme (stock 10 $\mu\text{g}/\mu\text{L}$, IDT) for a final concentration of 20 ng/ μL and incubate the sample at RT for 15 min for assembly of the RNP complex.
4. Filter (0.1 μm) the 30 μL RNP mix and centrifuge at $17,900 \times g$ for 2 min. The sealed mix can be stored at 4 °C for at least 2 weeks until microinjection.

3.4 Colony Set-up for Transgenic Mouse Production, Superovulation, and Egg Collection

1. For efficient production of genetically modified mice, four distinct mouse colonies are needed (*see Note 18*): female FVB mice (egg donors) to produce eggs for microinjection (*see Note 19*), stud male FVB mice (sperm donors) to mate with the egg donors (*see Note 20*), female CD-1 mice serving as pseudopregnant recipients for embryo transfer (*see Note 21*), and vasectomized (sterile) BDF1 male mice to produce pseudopregnant CD-1 females (*see Notes 22 and 23*).
2. For production of fertilized FVB-strain eggs, ~8-week-old FVB females are superovulated with PMSG and hCG hormones (*see Note 24*). Resuspend PMSG and hCG (lyophilized powder) in sterile saline solution and store in small volume stock aliquots of 2000 IU/mL at $-80\text{ }^{\circ}\text{C}$.
3. On day 1 (at 11 am), freshly dilute PMSG stock hormone to 50 IU/mL in saline solution, and intraperitoneally inject 100 μL (5 IU) per FVB female, into the lower quadrant of the abdomen (*see sequence of events, see Fig. 4*).
4. On day 3 (at 11 am), 46–48 h post-PMSG injection, freshly dilute hCG stock solution to 50 IU/mL, and intraperitoneally inject 100 μL (5 IU) into each FVB female that received PMSG on day 1. After hCG administration, mate the superovulated female by placing it in the cage of a singly housed stud FVB male (1:1 matings only) in order to produce fertilized eggs for PNI by natural mating (*see Note 25*).
5. On day 3, to produce pseudopregnant recipients for injected zygote transfer the next day (day 4), perform estrous cycle assessment on the females in the CD-1 colony. Examine the vaginal area of each female by lifting it by the tail as its front legs

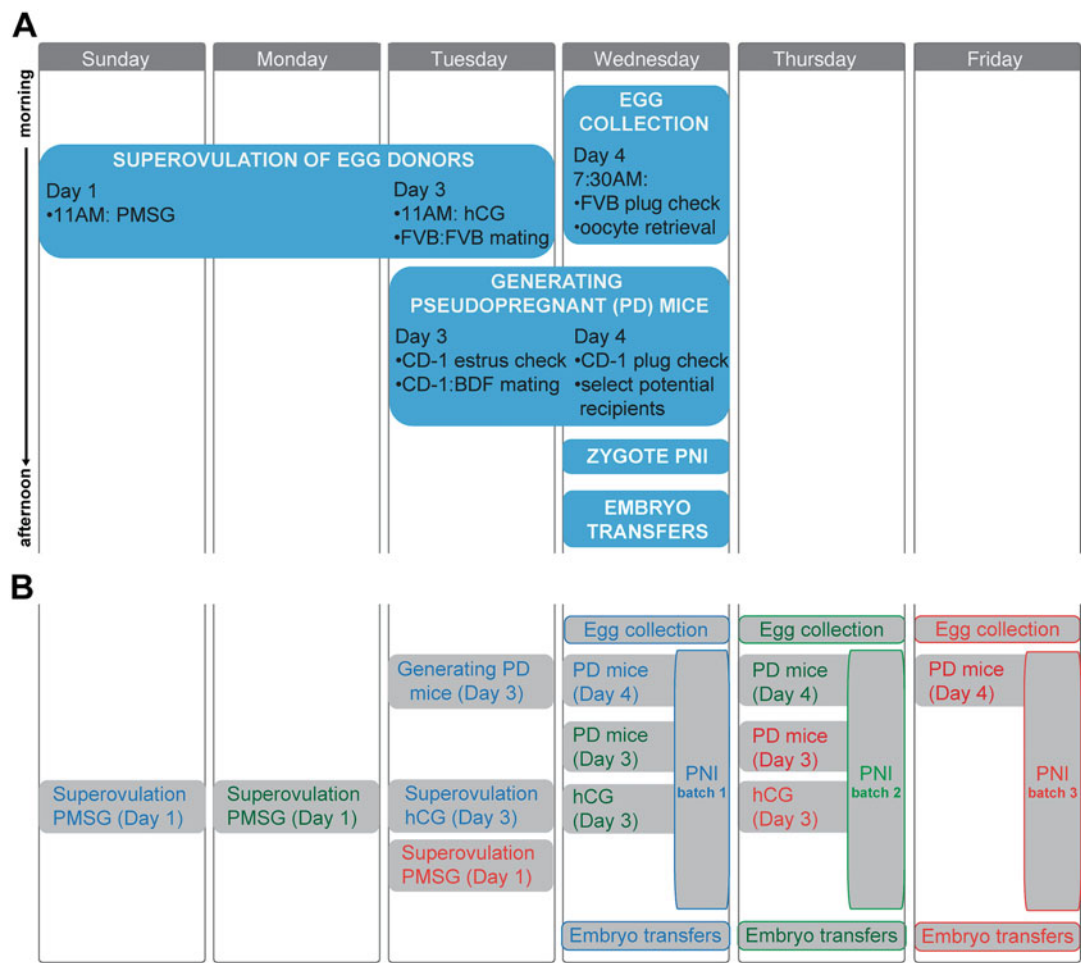


Fig. 4 Sequence of events for transgenic and mutant mouse production. **(a)** For each day of zygote pronuclear microinjection (PNI), a total of four days is required to complete all the mouse tasks leading up to and including the generation of recipients containing genome-edited embryos. Blue boxes indicate the four-day schedule for a single batch of experiments, which may be interleaved with additional sets. **(b)** Sample weekly schedule for three interleaved batches of experiments, each consisting of four consecutive days of PNI and embryo transfers (batch 1: blue, batch 2: green, batch 3: red). Typically, for logistics purposes, PNI is carried out by trained microinjectionists, whereas the remaining morning tasks (egg collection, superovulation, and production of pseudopregnant recipients) are performed by other staff members. *PMSG* pregnant mare serum gonadotropin, *hCG* human chorionic gonadotropin, *PD* pseudopregnant

hang down into the cage or a lower surface. Females deemed in estrus by visual assessment of the vaginal opening (gaping, reddish-pink tissue, swollen pronounced folds [28, 29]) are selected to be mated with the sterile BDF1 males (1 male:2 females in a single cage) (see **Notes 26** and **27**). On the morning of day 4 (PNI day), mated CD-1 mice are checked for the presence of a vaginal plug. Plugged females are physiologically

receptive to embryo transfer and serve as pseudopregnant recipients (*see Note 28*).

6. Prior to day 4, prepare hand-pulled glass capillaries as they are a component of the mouth-pipette used to handle oocytes (*see Note 29*). Different sizes of glass capillaries (no filament) are pulled over a Bunsen burner. Hold the capillary horizontally with both hands over the flame to soften the glass and withdraw it as quickly as possible while pulling both ends sharply to produce a long tube. Snap the pulled capillary in half and cut it down into two 10–15 cm tapered tubes. Score the glass with a diamond-tip pencil for a neat straight break. Using a graduated eyepiece and 40× magnification, measure the opening (narrow end) of the capillary to select for two sizes (6–8 and 8–10, *see Note 30*). Quickly wave the cut capillary through the flame to fire polish the edges of the opening. The final product should be about 8 cm in length.
7. On day 4 (around 7:30 am), separate the superovulated FVB female mice from the cages with males and check each one for a vaginal plug (*see Note 31*). Euthanize all superovulated females for egg collection. Next, quickly dissect the ovaries and attached oviducts from the abdominal cavity with scissors and transfer each oviduct into a drop of M2 medium on a first square petri dish.
8. Ovulated eggs, surrounded by cumulus cells and also called cumulus–oocyte complexes (COCs), are found in the ampulla of the oviduct. After successful ovulation, the ampulla should be visibly swollen with ridges in the epithelium. Using a stereomicroscope, dissect the COCs from the oviducts by snipping the ampulla with spring iris scissors and teasing the COC mass out with fine forceps.
9. On a second square petri dish, use the mouth-pipette (*see Note 32*) to transfer the COCs into a single 200 µL drop of hyaluronidase enzyme in order to digest the cumulus cells away from the oocytes [30]. Allow the COCs to incubate in the hyaluronidase for a few min (<5) until the cumulus cells shed off and oocytes are clean of cellular debris (*see Note 33*).
10. Using a size 8–10 capillary on the mouth-pipette, gently swirl the oocytes and proceed several washes by moving the oocytes through a minimum of six sequential drops of clean M2 medium to rinse off the hyaluronidase and cellular debris. Count the oocytes and incubate them in an organ culture dish containing 500 µL of M16 medium at 37 °C and 5% CO₂ until they will be used for microinjection (*see Note 34*).

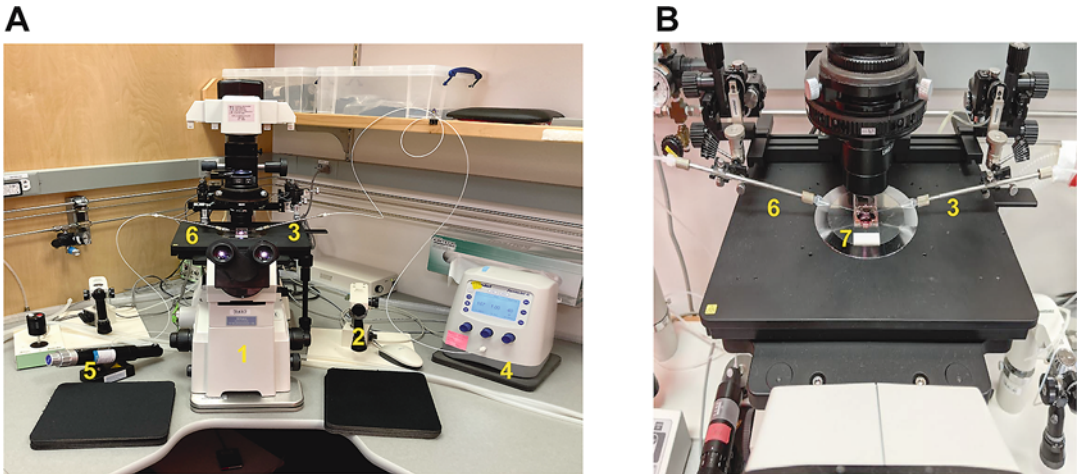


Fig. 5 Microinjection workstation. (a) Inverted microscope (1) equipped with right-hand side manual micro-manipulator (2) connected to the injection needle holder (3) and controlled by the electronic microinjector (4). Left-hand side pneumatic microinjector (5) connected to the holding pipet holder (6). (b) View of the microscope stage with chamber slide (7) containing M2 medium

3.5 Pronuclear Injection and Embryo Transfer

1. Following oocyte collection, PNI of an injection mix of choice (*see* Fig. 1) is scheduled between 8 am and 12 pm (*see* Note 35). A functional microinjection workstation is described in Note 36 and Fig. 5a.
2. Load the injection needles (*see* Note 37) by placing the blunt end into the tube containing the injection mix with vector and/or CRISPR components (Subheadings 3.1–3.3). Suspend the loaded needles pointy end down to allow air bubbles to move up and out of the sample to be injected. Air bubbles will prevent the injection mixes from flowing adequately during PNI.
3. Fit a forged glass holding pipet into the left instrument holder, which is connected to the manually-controlled pneumatic unit (CellTram). Insert a loaded injection needle into the right instrument holder, which is connected to the electronic injector (FemtoJet). Position both instrument holders at a slight angle so that the capillaries reach the drop of M2 on the chamber slide (Fig. 5b).
4. Only healthy looking fertilized eggs (zygotes), which will subsequently develop into mouse embryos, are selected for PNI. Examine the incubated culture dishes for fertilized eggs under the microdissection scope; selection is based on visual detection of two pronuclei and an overall normal oocyte morphology. Discard nonfertilized eggs, as well as trinucleates. Until injection, selected zygotes are held in a drop of RT M2 medium on the injection chamber slide (*see* Note 38).

5. PNI: Position and focus the target pronucleus on the same plane as the injection needle ($\sim 180^\circ$ to the holding pipet). The zygote can be repositioned by easing suction from the holding pipet and gently tapping the egg with the injection needle. As the zygote is secured by the holding pipet, pierce the egg zona with the loaded injection needle in a swift and gentle motion to minimize mechanical stress to the injected zygotes. Successful PNI of the injection mix should produce slight swelling of the pronucleus (*see* **Notes 39** and **40**).
6. Using the mouth-pipette, move the injected zygotes to an organ culture dish containing equilibrated M16 medium and culture in the incubator until embryo transfer. Discard any lysed zygotes (*see* **Note 41**).
7. For random transgenesis experiments, four recipients (up to 100 injected and transferred embryos) often yield enough data for analysis. For enSERT experiments, two recipients are sufficient in many cases due to the improved transgenesis rate (*see* **Note 42**). For CRISPR genomic deletion projects, a pilot study to test several sgRNA designs by injecting them into zygotes and then culturing these to the blastocyst stage for genotyping/sequencing purposes can aid in narrowing down the optimal targeting strategy. Based on those results, 4–8 recipients can then be produced by microinjection and embryo transfer to generate offspring that can be screened for F0 founders with the intended mutation.
8. Next, later during day 4, transplant 20–25 injected zygotes into each pseudopregnant recipient by performing bilateral embryo transfer surgery. All surgery reagents are sterile, and instruments are autoclaved for aseptic survival surgery (*see* **Note 43**).
9. A veterinary-grade vaporizer for isoflurane is used to anesthetize the recipient mouse for the entire duration of the embryo transfer procedure. Induce initial anesthesia in a vented chamber, and keep the mouse deeply anesthetized by maintaining its snout in a nose cone circuit connected to the vaporizer at all times. Monitor anesthesia levels, body temperature, and breathing of the mouse during the procedure.
10. Inject the anesthetized mouse with freshly diluted meloxicam (10 mg/kg in saline solution) subcutaneously. Shave two surgical sites on the dorsal flanks of the female using electrical clippers.
11. On the dissection workbench, place the mouse on its side on a 4" $\times$ 4" piece of gauze. Prepare the shaved surgical site with swabs of 70% ethanol and povidone–iodine solution. The mouse must be fully anesthetized with no reaction to a toe pinch.

12. Using a size 6–8 capillary fitted on the mouth-pipette, examine the injected zygotes from the incubator. Discard any lysed eggs. Next, move 20–25 healthy zygotes to a fresh dish of M2 medium for handling outside of the incubator. Load the transfer capillary in the following manner: small volume of M2 followed by two air bubbles, then by 10–13 embryos in a minimal volume of medium. (*see* **Note 44**).
13. To expose the oviduct of the recipient mouse, lift the shaved skin using toothed forceps and make a small incision (0.5–1 cm) perpendicular to the dorsal midline by using the iris scissors. Next, locate the muscle wall area below the last rib and tent it away from the peritoneal cavity. Cut a very small incision (<0.5 cm) into the muscle wall (*see* **Note 45**). Insert the tips of the iris scissors into the cut and use them to stretch the incision. To minimize contamination, a small piece of sterile drape with an opening to access the incision is placed over the surgical area.
14. Using the serrated curved forceps, locate the white fat pad surrounding the ovary and gently pull it out of the abdominal cavity to rest on the sterile drape. Clamp a serrefine clip to the fat pad to orient it to lay towards the spine of the mouse; this secures the ovarian oviduct outside of the body cavity.
15. Embryo transfer: Move the mouse from the dissection workbench to the stereomicroscope by lifting the piece of gauze it rests on; keep its snout in the isoflurane nosecone. Using both pairs of fine point (sharp) forceps, tear the ovarian bursa open and tuck the edges under the ovary while avoiding the blood vessels (*see* **Note 46**). The end opening to the oviduct (infundibulum) is located near the ovary; confirm by gently poking the tip of the forceps into the end of the swollen indentation of the infundibulum.
16. While looking through the stereoscope eyepieces, stabilize the ovarian tissue with fine forceps while inserting the very end of the preloaded transfer capillary into the infundibulum. Blow into the mouth-pipette to transfer the content of the glass capillary to the oviduct until the air bubbles have entered the ampulla. Embryos should have transferred with the medium preceding the air bubbles.
17. Closing the incision: Move the mouse back to the dissection workbench and unclamp the serrafine clip. Remove the small drape and lift the muscle wall away from the body cavity. Using a wick or forceps, gently push the ovarian tissue back into the peritoneal cavity. Using the toothed forceps, tent the muscle wall up away from the internal organs and add a single-layer buried stitch (instrument tie surgical knot) to close the tissue by aligning the edges. Using your fingers, align and gently

pinch the outer epidermal layers up making sure that no subdermal layer such as the muscle tissue will be accidentally stapled. Apply a metal wound clip to the outer epidermal layer to close the incision.

18. The embryo transfer surgical procedure (from **step 3**) is repeated on the other oviduct of the recipient to transfer the remaining embryos.
19. When finished, place the mouse on a heating pad or warming chamber for recovery from the anesthetic. Monitor postsurgery recipients closely. Postoperative care includes monitoring body condition and incision site healing. If the mouse loses a significant amount of blood during the surgery, administer warm saline solution by subcutaneous injection (*see Note 47*).

3.6 LacZ Transgenic Embryo Collection and X-Gal Staining

1. The day following embryo transfer of injected zygotes is defined as embryonic day 1.5 (E1.5). For embryo collection at the desired embryonic day and time point (e.g., E11.5 embryos should be dissected before noon on day E11.5), place the euthanized pregnant recipient mouse on an absorbent pad and spray the abdomen with 70% ethanol.
2. Using surgical scissors, cut the skin to expose the abdominal cavity. Lift the peritoneum with forceps and make another incision to reveal the inner organs. Push aside the intestinal components and locate the uterine horns, which harbor the embryos. Carefully dissect and separate the uterine horns from the mesometrial tissue.
3. Using fine forceps, transfer the right and left uterine horns containing the embryos into a petri dish filled with cold 1× PBS. Using fine scissors, carefully slit open the uterine muscle wall along the “string of embryos.”
4. Gently detach each embryo enveloped in its visceral yolk sac with curved tweezers. Remove and discard the placenta and Reichert’s membrane. Transfer the embryos in the yolk sac into a new dish with clean 1× PBS using a wide-bore disposable plastic pipet (*see Note 48*).
5. Rip open the yolk sac so that the fixing solution can penetrate the embryonic tissue. Leave the sac attached to the embryo for future genotyping purposes.
6. Using a plastic disposable pipet cut short to generate a wider bore, move the embryos to an appropriate container for fixing and staining (typically a 15 mL conical tube) (*see Note 49*).
7. For fixation, incubate the embryos in 4% paraformaldehyde (PFA) on a rocking platform at RT. The duration of fixation depends on the embryonic stage (e.g., 30 min for embryos at E11.5) (*see Note 50*).

8. Remove the PFA from the samples and safely dispose of it according to safety regulations. Embryos are washed immediately in embryo wash buffer on a rocking platform three times for 10–30 min.
9. Prepare the X-gal staining solution by mixing the following reagents in the listed order (from top to bottom). For 50 mL of X-gal stain (*see* **Note 51**):
 - (a) 46.2 mL of embryo wash buffer.
 - (b) 1.0 mL of 200 mM potassium ferricyanide.
 - (c) 1.0 mL of 200 mM potassium ferrocyanide.
 - (d) 1.0 mL of 1 M Tris (pH 7.5).
 - (e) 0.8 mL of 50 mg/mL 5-Bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-gal) in DMF.
10. Discard the liquid from the last wash. Fully submerge the embryos in a minimal volume of X-gal staining solution to ensure submersion for the entire duration of incubation on the rocking platform. Wrap the tube in aluminum foil as X-gal is light sensitive. Place tube with embryos in staining solution on the rocking platform for overnight incubation at RT (*see* **Note 52**).
11. The following morning, remove the X-gal solution and safely dispose it in the appropriate waste container. Immediately add 1 $\times$ PBS and wash embryos three times with 1 $\times$ PBS for 10–30 min on a rocking platform.
12. Embryo sorting: Transfer the embryos to a petri dish of cold 1 $\times$ PBS. Using a stereomicroscope, examine each embryo in detail from all angles for the presence of blue staining (oxidized X-gal product) in embryonic cells.
13. Collect the yolk sac or a piece of embryonic tissue for DNA extraction and subsequent PCR genotyping.
14. For long-term storage, submerge selected embryos in 4% PFA in round-bottom tubes or multiwell plates (add thermaseal film to prevent evaporation).

3.7 Genotyping

1. Collect embryonic (yolk sac) or mouse (ear punch tissue) biopsies in 1.5 mL tubes or multiwell plates. These tissue samples can be stored at -20°C until used.
2. A time- and cost-efficient protocol for crude DNA extraction is used, and tissue lysis master mix is prepared for the desired number of samples (n) according to the following formula: $1.1n \times [75 \mu\text{L DirectPCR lysis (tail) reagent} + 0.67 \mu\text{L Proteinase K}]$ (*see* **Note 53**).

3. For digestion, add 75 μL of tissue lysis master mix per sample. If multiwell plates are used, seal plates with thermaseal cover to prevent cross-well contamination.
4. Following centrifugation at $17,900 \times g$ for 10 s, incubate samples on a thermoshaker at 300 rpm for 4–12 h at 56 °C.
5. Incubate samples at 85 °C for 50 min to inactivate Proteinase K. Place samples at 4 °C for short-term storage until used for PCR or at –20 °C for later use (*see Note 54*).
6. Centrifuge the crude DNA lysate samples at $1,600 \times g$ for 15 min. Dilute 10 μL of the supernatant in 100 μL of millipore H_2O in a separate tube as working solution for PCR.
7. Genotyping PCR: Select respective PCR primers for detection of either *LacZ* transgenes or genomic deletions (*see Subheading 2* for primer sequences). Per 20 μL reaction, the PCR components are as follows: 1–3 μL of diluted DNA lysate, 2 μL 10 $\times$ PCR buffer, 0.4 μL of dNTPs (10 mM), 0.4 μL Fwd/Rev. primer (10 μM stock), and 0.2 μL (1 U) Taq DNA polymerase, completed with water. The thermocycler is set up as follows:
 - (a) 30'' at 95 °C.
 - (b) 30–35 cycles of 30'' at 95 °C, 30'' at 45–68 °C (calculated from NEB T_m Calculator), 1' per kb at 72 °C.
 - (c) A final elongation step of 5' at 72 °C.
8. Founder individuals encoding transgene insertions or CRISPR-engineered deletions are selected based on the PCR strategies outlined in Fig. 3. For genomic deletions, the deletion breakpoints are determined using Sanger sequencing from PCR-amplified fragments (*see Fig. 3*).

4 Notes

1. Confirm enzyme digestion by assessing linearized and uncut (control) samples of the vector by electrophoresis.
2. If the undigested form of the vector is still detected by electrophoresis separation after overnight enzyme digestion, isolate the linearized form of the vector from the agarose gel and purify it by using a gel extraction kit.
3. DNA synthesis enables testing of mutated enhancer variants [19] or enhancers from species for which DNA samples may not be readily available [31].
4. The NEB T_m Calculator (<http://tmcalculator.neb.com>) is used to determine the annealing temperature of “enhancer”-specific forward and reverse primer sequences for PCR; and the average value is used.

5. If antibiotic resistance cannot be used to select for the target vector, eliminate the template vector from the final PCR product by digesting with DpnI enzyme (1 h at 37 °C followed by inactivation for 20 min at 80 °C). This will result in DpnI cleavage of methylated DNA from plasmids prepared from *E. coli* dam⁺ strain, such as the One Shot TOP10 Electrocomp cells, but will not affect the unmethylated PCR-amplified DNA.
6. Purification of PCR product prior to ligation is important in order to remove impurities, salts, and primer dimers. Consult the manufacturer's manual to determine the appropriate beads: desired product ratio in order to purify the fragments of interest (according to size). Bead purification is recommended for efficient Gibson ligation, especially when using batch cloning or cloning of large fragments. Alternatively, PCR-amplified DNA fragments can be purified using a spin column-based PCR purification kit (if no vector contaminants are present) or a gel extraction kit. The latter may yield traces of gel particles which can reduce the efficiency of the Gibson ligation mix.
7. For fragments smaller than 800 bp, adjust the beads:insert ratio [0.65×–1.8×] based on desired PCR product size to purify.
8. For batch cloning of PCR products or synthesized fragments (e.g., gBlocks from IDT), use an insert:vector ratio of 15:1 or 60:1, respectively. For these ligation reactions, use of 10 µL of NEB 2× HiFi DNA Assembly Mix mixed with 1.5 µL of vector.
9. One 50 µL vial of TOP10 *E. coli* cells can be diluted 1:1 in 10% glycerol and be used for up to five reactions of 20 µL cells/glycerol each.
10. Competent cells are sensitive to shearing and temperature fluctuations. Use a P1000 tip to handle and expel the cells directly in the center of the cuvette in order to avoid pipetting any cells onto the interior walls. Do not prechill the cuvette, as moisture/condensation buildup on the walls of the container will interfere with electroporation.
11. Here, a BioRad Gene Pulser Xcell electroporator is used with the following settings: 4—Preset, 1—Bacterial, 1–0.1 cm gap. Wipe off any ice/water around the cuvette, and gently tap the cuvette on the table to remove air bubbles before putting it into the electroporator. Close the electroporator lid and press pulse (duration approx. 2 s). If the cuvette sparks or an error “ARC DETECTED” is shown on the display, the electroporation was possibly unsuccessful and may need to be repeated.
12. For efficient screening of batch-cloned colonies, the Equi-Phi29 DNA polymerase kit (Thermo Fisher) can be used in a 96-well plate format with the following “DNA master mix” per

reaction/colony: 0.0625 μL 10 $\times$ reaction buffer for EquiPhi29, 0.125 μL exo-resistant random primers, 0.3125 μL ddH₂O. The “amplification master mix” contains the following reagents per reaction/colony: 0.25 μL 10 $\times$ reaction buffer for EquiPhi29, 0.25 μL 10 mM dNTPs, 0.025 μL DTT, 0.125 μL EquiPhi29 DNA polymerase, and 1.225 μL H₂O. Following completion of the reaction on a thermocycler (45 °C for 180 min, 65 °C for 10 min, hold on 4 °C), add 10 μL of H₂O to each PCR product and use 0.5 μL for validation by Sanger sequencing.

13. Generally, midiprep samples display higher purity for PNI than miniprep samples and result in higher embryo yield from injected zygotes.
14. The procedure described enables highly efficient transgenic knock-in at the neutral H11 locus [19, 32]. In an analogous manner, this protocol can be used to prepare and inject CRISPR-Cas9 mixes for engineering of any mouse genomic locus of interest, by modifying/exchanging the donor plasmid and the sgRNA sequence. For example, an analogous version of this protocol has been used to replace the endogenous *Shh* ZRS limb enhancer sequence with a range of species-specific variants [31].
15. Alternatively, single-guide RNAs can be utilized, either ordered commercially (for time-saving purposes) or synthesized in the lab, e.g., using a cloning-free, oligo-based method [33].
16. For genomic deletions, a sequence interval of interest on each the 5' and 3' side of the region to be deleted is screened for a crRNA spacer sequence with optimal specificity/efficiency. CRISPOR is a particularly versatile web tool integrating data from eight SpCas9 off-target studies with widely used algorithms to predict off-target sites and to support the selection of specific and efficient sgRNAs in more than 150 genomes [27, 34].
17. As FVB mouse strain zygotes are used for PNI of CRISPR reagents, the integrity of the selected gRNA target sequence (including PAM) has to be verified in FVB genomic sequence. This validation can be conducted by using either the ENSEMBL platform (<http://www.ensembl.org>) (BLAT, Genetic variation, Resequencing, FVBNJ) or the FVB UCSC genome browser track (<http://hgdownload.soe.ucsc.edu/hubs/mouseStrains/hubIndex.html>).
18. For large-scale generation of transgenic mouse embryos [14, 19, 35], a team of three experienced microinjectionists can inject up to 500–600 zygotes daily and generate an average of 16 recipients of embryo transfers a day. A different team of

support staff usually handles the other mouse tasks on PNI days.

19. The choice of mouse strain to use as egg (and sperm) donors will determine the genetic background of the transgenic and mutant mice. The FVB/N inbred strain is advantageous for microinjection, as fertilized FVB eggs exhibit large prominent pronuclei and good resistance to lysis [36, 37]. The average litter size of FVB mice ($n = 9.5$) provides an additional advantage in accelerating the establishment of subsequent stable mouse lines (e.g., when compared to the smaller litter sizes of the more commonly used C57BL/6J strain) [36]. A potential drawback of using the FVB strain is the homozygous *Pde6b*^{rd1} allele, which results in early onset of retinal degeneration. Therefore, the FVB genetic background would be a poor choice for studies focusing on behavioral phenotyping based on visual cues. FVB mice are also often reported to be refractory to hormone stimulation [38–40] but 20–25 eggs per superovulated female can be routinely obtained in our facility with notable seasonal variation. Although females of the optimal age and weight for maximal egg yield are not always available commercially, it is generally more convenient and cost-effective to regularly purchase females of breeding age instead of maintaining an in-house breeding colony if the egg donor strain is available from research animal suppliers.
20. Stud FVB males are individually housed to avoid fighting and injury. Ideally, mating should be spaced a few days apart to maximize sperm count and generation of fertilized eggs. Thus, if the transgenic operation aims to obtain zygotes from 28 females per day for three consecutive days of PNI a week, this will require a colony of 84 fertile FVB males that will need to be replaced every 6–8 months. A cage card record should be kept for each male (date of plug check with female with presence (+) and absence (–) of a plug) in order to flag underperforming males based on copulation plug checks.
21. Oviducts from CD-1 females have large ampullae, which facilitate embryo transfer surgeries. CD-1 females are also known for their ability to sustain large litters with good nurturing maternal instinct. In addition, the CD-1 strain is an affordable option (usually less than half the cost of an FVB female) and a widely available outbred model. CD-1 females are ordered to be between 26 and 28 g, and heavier mice (>35 g) are culled from the colony because they become unsuitable for oviduct transfer due to the large fat pads surrounding the ovaries.
22. Vasectomized mice can be commercially purchased, or males can be rendered sterile by performing the vasectomy procedure (the vas deferens are cut and tied) in-house. Before using a

vasectomized male in a mating experiment, it is best practice to test for sterility by mating it with isolated females to monitor for pregnancy in plugged females. Using BDF1 strain males (dark coat color) in combination with CD-1 females allows for visual detection of vasectomy failure: if the resulting embryos/mice show dark eyes instead of red (albino) eyes, it suggests that they are not of the FVB strain background from the transferred zygotes. A colony of sterile males can be mated on sequential days to establish pseudopregnant females. Consequently, a smaller BDF1 colony of 40–60 sterile males is sufficient to mate with multiple cohorts of up to 80–120 CD-1 females in estrus per week (1 male:2 females).

23. Male mouse colonies should be replaced as staggered cohorts to ensure performance continuity since new males need 1–2 matings to reach peak breeding condition after puberty at ~8 weeks of age. Four sequential negative mating events (lack of copulation plug in the female) indicate poor male mating performance; therefore, the male should be replaced by a younger individual. To ensure peak reproductive performance in the mouse colony, stud FVB males should be replaced at 6–8 months old, whereas vasectomized BDF1 males can be used until 18–24 months of age.
24. Superovulation increases egg production and reduces animal use, labor, and costs. It is recommended to allow 5–7 days of acclimatization in the animal facility if mice are purchased from an external source. Females are prepped for superovulation by adding soiled bedding from FVB males to their cages to induce synchronized estrous cycling. This will result in most of the females being in estrus approximately 3 days later via the Whitten effect [41], which makes them more receptive to mating and optimizes oocyte yield [42].
25. To maximize the number of fertilized oocytes recovered from superovulated and mated females (on day 4), it is critical that the stud males exhibit good plugging performance and a high sperm count. As males will mate with females at about the midpoint of the dark period, zygotes should be at E0.5 on the morning of the scheduled microinjection.
26. The murine estrous cycle is 4–5 days, which means up to 25% of the randomly cycling female colony to be in estrus at any time. Thus, in order to find 25 females in estrus, one may have to examine up to 100 females. Trained animal technicians usually mate 80 females in estrus with 40 sterile males to obtain at least 20–30 pseudopregnant (plugged) potential recipients the following day. A colony of naturally cycling CD-1 females is kept in order to maintain a steady-state stock from which ~75 pseudopregnant (plugged) females can be produced per week

(25/day) for embryo transfer. Unplugged and unused pseudopregnant females can be integrated back into the CD-1 colony.

27. Unplanned adverse events can arise during embryo transfer surgery, so having more pseudopregnant candidates on hand is desirable. In our laboratory, we microinject and transfer the zygotes the same day, so the number of recipients needed must always be overestimated on day 3 to ensure enough pseudopregnant females are available for embryo transfer on day 4.
28. Unused pseudopregnant CD-1 females should begin to cycle again 10 days after plugging so they can be labeled as such and reintegrated into the randomly cycling colony. Unplugged mice are returned to the colony or remated (if assessed as still in estrus) if more pseudopregnant females are needed the following day.
29. Mouth-pipetting is still the prevalent technique for oocyte handling in embryology laboratories where the super fine control of air pressure powered by one's lungs (suction) allows for precise egg and embryo handling. A typical mouth-pipette is assembled from an aspirator mouthpiece, elastic soft tubing and rubber stop, and a pulled glass capillary at the very end; our prototype has a 10 μ M filter in the tubing, as well as a spacer, to prevent any accidental inhalation of liquid (*see* Fig. 6).

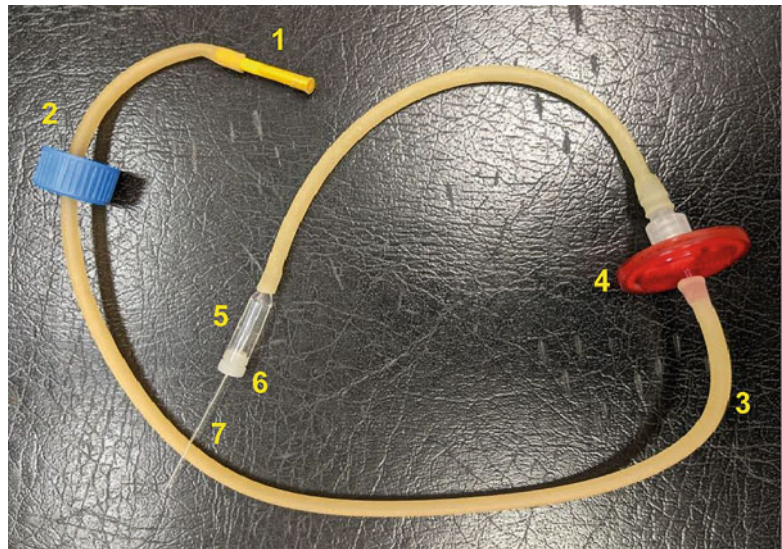


Fig. 6 Mouth-pipette and its components. Aspirator mouthpiece (1), cap with a drilled hole (2) which allows for the mouthpiece to be stored in a 15 mL conical tube for hygiene purposes, soft elastic tubing (3), 10 μ M filter (4), spacer (5), rubber stopper (6) fitted with a pulled glass capillary (7)

30. Hand-pulled capillaries with a smaller opening (outside diameter measured to be 6–8 graduations at 40 $\times$ or size 6–8) are used for embryo transfers, whereas larger ones are useful for oocyte handling (outside diameter measured to be 8–10 graduations at 40 $\times$ or size 8–10). Generally, a capillary with a lumen diameter of 0.2–0.3 mm is desirable [30].
31. Although both plugged and unplugged superovulated FVB females are euthanized for egg collection, a plugging record is kept for each stud FVB mouse to flag underperforming males. It is recommended to euthanize the egg donor females in batches in order to minimize the time between euthanasia and dissection for egg collection. Since up to 1000 eggs can be collected from 28 donors, it may be more time-efficient to split the collection procedure between 2 and 3 technicians and assign groups to the collection culture dishes (eggs from plugged vs. unplugged females) to facilitate visual detection of fertilized eggs later.
32. COCs should be handled with a full-size unaltered glass capillary (no filament).
33. Timing of hyaluronidase digestion should be as short as possible (3–5 min depending on the batch of enzyme) to avoid overdigestion of oocytes, which can lead to soft eggs and increased lysis during microinjection. COC underdigestion will leave sticky cumulus cells on the oocytes, which may clog the injection needles and disrupt PNI.
34. Organ culture dishes (without mineral oil overlay) are used for oocyte culture as a replacement for microdrop cultures. No issues have been observed with evaporation if egg collection, microinjection, and embryo transfer are completed within 12 h. If necessary (e.g., lack of pseudopregnant recipients), injected zygotes can be cultured in KSOM^{AA} medium with oil overlay overnight and be transferred the following day when the embryo develops to the two-cell stage. If zygotes are to be cultured to the blastocyst stage, be aware of the cleavage arrest at the two-cell stage and use the appropriate culture medium to support the metabolic requirements for embryonic development.
35. Timing of PNIs depends on the superovulation schedule: the optimal window for DNA integration and cell division, as well as egg turgidity, is between 8 am and 12 pm (day 4) for an experimental schedule based on hCG administration 20 h prior to egg collection from the FVB donors. PNI performed later than 12 pm may yield no integration, and the eggs begin to soften, exacerbating lysis. Under ideal conditions, FVB colonies will exhibit a 50–75% fertilization rate. Mouse strain, age, and weight of the female mice, as well as superovulation

conditions (dose, timing), must be optimized empirically for each facility. Fertilized eggs can still be identified up to 3 h after oocyte collection, as initially pronuclei of zygotes with slightly delayed development might be difficult to detect visually due to smaller size.

36. The microinjection station consists of an inverted microscope with two micromanipulators connected to each side. One micromanipulator controls the forged holding pipet that stabilizes the zygote and is connected to a manual pneumatic/air-column microinjector for zygote stabilization and handling under the microscope. The other micromanipulator controls the injection capillary needle containing the mixes of transgenic vectors; this is connected to an electronic microinjector that has ultrafine pressure features. The microscope and accessories should be located on suitable antivibration platforms or an air table to ensure stability and damper interfering vibrations that can exacerbate egg lysis during microinjection. Zygote microinjection is a task that involves repeated movements and microscopy work for prolonged periods of time within a restrained space, so it is highly recommended to consult an ergonomics expert to prevent discomfort and injury related to this work. The microinjection station is usually located next to a dissection scope that is used for egg collection, zygote identification, and counting, as well as embryo handling.
37. To produce PNI needles and holding pipets, commercially available glass capillaries (with filament) are shaped into needles with sharp-tapered ends using a needle puller device (Flaming/Brown Micropipette Puller). Fresh needles are pulled on a daily basis for microinjection; they are fragile and should be kept very clean. Unused needles are saved to be cut and polished to a tapered smooth opening on a microforge; these forged capillaries become holding pipets that are used to secure the zygote in position during PNI on the chamber slide.
38. Cell culture chamber slides (with the growth chamber removed) are used as an injection chamber to keep zygotes on the microscope stage for injection. A drawn blue line across the bottom of the slide will serve as a visual marker; noninjected eggs are deposited below the line and already injected eggs are placed atop the blue line. Zygotes are sensitive to temperature fluctuations and therefore their time outside of the incubator should be kept to a minimum.
39. The injection needle is loaded with injection mix (containing plasmid and/or CRISPR reagents) by capillary action from the filament. 50 μL of injection mix should be sufficient for >10 needle loadings. Microinjection pressure from the Femtojet determines the amount of injection mix injected into the

pronucleus and should be optimized to yield good transgenesis results with minimal egg lysis.

40. Some injectionists prefer to inject on a constant-flow/back pressure function. Flow rate should be inversely proportional to the opening of the injection needle (smaller opening = higher flow rate). If the microinjection needle is dirty with cellular debris or clogged by precipitates from the injection mix, the needle has to be replaced by a new one. As the nucleolus is very sticky, it is recommended to position it (as well as the polar bodies) away from the path of the injection needle. A dirty needle will damage the zygote and cause lysis.
41. Up to ~10% lysis rate is acceptable for a trained microinjectionist, but this can vary depending on the purity and nature of the injection mix as well as the microinjection conditions (e.g., timing, quality of harvested zygotes, vibrations).
42. enSERT mixes have a highly efficient transgenesis rate of ~50%, but we have observed that they generate fewer embryos per recipient when compared to random transgenesis mixes (average of $n = 5$ vs. $n = 8$ –10, respectively).
43. It is best practice to get extensive hands-on training to perfect this survival surgery technique, which should be approved by your institutional animal care and user committee (IACUC). Isoflurane is a commonly used anesthetic in animal surgeries and may be toxic to humans. Safety measures and engineering controls such as using a gas scavenger and veterinary-grade vaporizer are recommended to limit exposure to the user.
44. Carefully prop the mouth-pipette on a clean Kimwipe ensuring that the tip of glass capillary containing the embryos remains untouched (*see* Fig. 6).
45. Always lift and tent the tissue away from the body cavity during incisions, suturing, and wound clip application. If any internal organs are accidentally compromised during surgery, humane euthanasia of the mouse is recommended as it can cause post-surgical sepsis.
46. Avoid touching the ovary with any of the sharp instruments as it can injure the tissue. It will also cause bleeding that can obscure your field of vision and clog your transfer capillary. It is possible to retrieve the precious embryos from a clogged capillary by carefully breaking the tip of it into a spare dish of media. Absorbent wicks can help to prop the ovarian tissue or absorb bodily fluids. To make wicks: tightly twist a long strip of Kimwipe moistened with 70% ethanol, cut into ~2 cm sections and autoclave in a microfuge tube to make sterile.
47. Hypothermia is a risk during rodent anesthesia and surgery. It results in depressed physiological functions including

respiration and cardiac activity and may lead to death [43, 44]. Consider using a thermal support device (e.g., circulating warm water blanket) under the mouse during survival surgery.

48. Disposable plastic transfer pipets can be cut to produce different sized openings to handle embryos. The finer tip transfer pipets are useful to suction liquid away from the embryos.
49. Make sure that there is enough space in the plasticware container to allow for sufficient rocking movement to fix and stain the embryos correctly. In our experience, a 2 mL round-bottom tube can be used to hold up to ten embryos at E11.5. Place the dissected embryos on ice while finishing the collection procedure.
50. To achieve full tissue paraformaldehyde penetration, fixing time depends on the size of the tissue and age of the mouse embryos. For embryos at E9.5, fixing time is 20 min and can go up to 60 min for embryos at E14.5 or older.
51. X-gal is prepared from reagents stored at 4 °C. It should therefore be prepared well in advance so it can equilibrate to RT on the rocking platform before addition to the embryos. Reagents and final staining solution must be protected from light. Anecdotally, X-gal can precipitate and form crystals that cling to the embryo, which is problematic regarding the image quality. Letting the cold staining reagents warm up to RT seems to alleviate this issue.
52. If the protocol (sorting) cannot be continued on the following day, it is possible to perform the X-gal staining step at 4 °C and leave it rocking for up to 3 days, checking the staining intensity and quality regularly. Resume the protocol with washing and sorting steps.
53. This formula will produce an extra 10% volume of master digestion mix to account for pipetting error. Depending on tissue sample size, the digestion volume can be scaled up to 200 µL per sample.
54. As a simple and rapid protocol for crude DNA extraction is used here, samples stored at 4 °C should be genotyped within a week, as there is a risk for degradation of genomic DNA from fixed tissue. Alternatively, commercially available DNA extraction kits can be utilized for time-efficient extraction yielding stable genomic DNA for long-term storage.

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