An efficient i-GONAD method for creating and maintaining lethal mutant mice using an inversion balancer identified from the C3H/HeJJcl strain

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Abstract
As the efficiency of the clustered regularly interspaced short palindromic repeats/Cas system is extremely high, creation and maintenance of homozygous lethal mutants are often difficult. Here, we present an efficient in vivo electroporation method called improved genome editing via oviductal nucleic acid delivery (i-GONAD), wherein one of two alleles in the lethal gene was selectively edited in the presence of a non-targeted B6.C3H-In(6)1J inversion identified from the C3H/HeJJcl strain. This method did not require isolation, culture, transfer, or other in vitro handling of mouse embryos. The edited lethal genes were stably maintained in heterozygotes, as recombination is strongly suppressed within this inversion interval. Using this strategy, we successfully generated the first Tprkb null knockout strain with an embryonic lethal mutation and showed that B6.C3H-In(6)1J can efficiently suppress recombination. As B6.C3H-In(6)1J was tagged with a gene encoding the visible coat color marker, Mitf, the Tprkb mutation could be visually recognized. We listed the stock balancer strains currently available as public bioresources to create these lethal gene knockouts. This method will allow for more efficient experiments for further analysis of lethal mutants.

Keywords: in vivo electroporation; i-GONAD; CRISPR/Cas9; inversion balancer; lethal mutation

Introduction
Clustered regularly interspaced short palindromic repeats (CRISPR/Cas9-mediated mutagenesis has been widely used to disrupt genes in mice, rats, zebrafish, fruit flies, and nematodes (Pennisi 2013). However, lethal genes are often difficult to disrupt because both alleles are frequently disrupted simultaneously (Gurumurthy et al. 2019a). Approximately one-third of mouse genes are essential for life, and the mouse null-phenotypes for 61–62% of genes are currently unknown (Hrabe de Angelis et al. 2015; Dickinson et al. 2016). For efficient knockout studies of disease-causing and essential genes, it is crucial to establish methods that aid in the generation and analysis of lethal mutants.

A recent study reported that microinjection of the CRISPR/Cas9 system into one blastomere of two-cell embryos can be performed to efficiently generate mouse strains carrying heritable lethal mutations (Wu et al. 2019). However, this method requires specialized equipment and highly skilled personnel. To overcome this limitation, we recently introduced a recessive lethal knockout by targeting an allele in F1 hybrid mice via improved genome editing via oviductal nucleic acids delivery (i-GONAD) of Cas9 and guide RNAs (gRNAs) into mouse zygotes (Iwata et al. 2019). The i-GONAD method reported by Ohtsuka et al. (2018) employs intraoviductal instillation of genome editing components and subsequent electroporation of the oviduct and, therefore, does not require handling of preimplantation embryos. A recent study showed that compared with microinjection, electroporation results in a higher rate of embryo survival and development (Alghadban et al. 2020). However, statistical calculations indicated that even speed congenic approaches require a minimum of four backcrosses to eliminate a fully unwanted donor genome from F1 hybrid mice (Rogner and Avner 2003). Moreover, maintenance of deleterious mutations in heterozygotes from one generation to the next requires the selection of heterozygous individuals, which is labor-intensive.

In this study, we developed the i-GONAD method in which one allele was selectively edited using a B6.C3H-In(6)1J inversion identified from C3H/HeJJcl. Appropriately marked inversions were used as balancer chromosomes to maintain mutations in the corresponding chromosomal region (Zheng et al. 1999; Nishijima et al. 2003). Using this method, we generated the first Tprkb null knockout mouse with an embryonic lethal mutation that was stably maintained in heterozygotes. Finally, we listed the inversion balancer strains currently available via public bioresources to create these lethal gene knockouts using the above method.
Materials and methods

Animal strains
C57BL/6NCrSlc, C3H/HeJYokSlc (Japan SLC, Shizuoka, Japan), and C3H/HeJcl mice (CLEA Japan, Tokyo, Japan) were used in this study. The animals were maintained at a constant temperature (22 ± 2°C) and humidity (50 ± 10%), with a 12-hours light/12-hours dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee of Chubu University (Permit Numbers #2910066, #2910067 at Chubu University) and were conducted in accordance with institutional guidelines.

Whole-genome sequencing analysis
Raw sequencing reads of C3H/HeJ were previously sequenced by Keane et al. (2011) and deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (DRA) (https://databank.nig.ac.jp/DRASearch/, Accession: ERR008069 and ERR008070). Sequence read mapping was performed using BWA-mem software implemented in the MASER pipeline (Kinjo et al. 2018). Inversion In(6)1J breakpoints were identified as reported previously (Chen et al. 2009; Fan et al. 2014). Regions in which BreakDancer identified large inversion polymorphisms and breakpoints were visually validated using Integrative Genomics Viewer (Thorvaldsdóttir et al. 2013). Polymerase chain reaction (PCR) was performed with Ex Taq polymerase, and Sanger sequencing confirmed each breakpoint. PCR primers used to validate the inversion breakpoints are listed in Supplementary Table S1.

Test for recombination suppression
To examine whether recombination was suppressed in In(6)1J homozygous In(6)1J/C3H/HeJcl background) females were mated with C57BL/6NCrSlc males, and the F1 heterozygotes were further backcrossed for six generations to obtain C57BL/6NCrSlc mice. The single-nucleotide polymorphism (SNP) genotype of each region was determined by PCR restriction fragment length polymorphism (RFLP) analysis. We identified four SNPs (described in dbsNP) with mismatch PCR-RFLP based on the Mouse Genome Project at Wellcome Sanger Institute (https://sanger.ac.uk/sanger/Mouse_SnpViewer/rel-1505). PCR primers were used to amplify a genomic sequence containing a restriction site in C3H/HeJ mice but not in C57BL/6N mice. Following PCR amplification, the PCR products were digested for 4 hours at 37°C with 5 units of restriction enzyme and then analyzed by 1.2% agarose gel electrophoresis. The PCR primers used to validate recombination suppression are listed in Supplementary Table S1.

CRISPR solutions
Allele-specific CRISPR guide RNAs were designed using an SNP data retrieval utility, such as https://phenome.jax.org/snpp/retreivals, and cleavage efficiencies were retrieved from CHOPCHOPO (Labun et al. 2019, http://chopchop.cbu.uib.no/) (Supplementary Table S2). CRISPR RNP consists of Alt-R S.p. Cas9 Nuclease 3NLS (Integrated DNA Technologies, Coralville, IA, USA) and a custom guide RNA (cRNA): tracrRNA duplex, which includes the crRNA and a universal structural RNA (tracrRNA) (Integrated DNA Technologies). crRNA and tracrRNA were heated to 95°C for 10 minutes and slowly cooled to 25°C. This crRNA: tracrRNA duplex and the Alt-R S.p. Cas9 Nuclease 3NLS were incubated at 25°C for 10 minutes to form the RNP complex.

i-GONAD method
To synchronize the estrous cycle of female mice, 8–12-weeks-old female mice were injected intraperitoneally with 2.4 IU pregnant mare serum gonadotropin and mated with 8–24-weeks-old males 48 hours later, as previously described (Kobayashi et al. 2020). The presence of copulation plugs was confirmed the next morning via visual inspection, and plug-positive mice were subjected to i-GONAD experiments, as previously described (Ohtsuka et al. 2018; Gurumurthy et al. 2019b). To generate a lethal gene deletion, the following concentrations of CRISPR solutions were used: 540 ng/µl Alt-R S.p. Cas9 Nuclease 3NLS, 33 µM upstream and downstream crRNA/tracrRNA, and 0.05% Fast Green FCW (Wako, Osaka, Japan) marker diluted in Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA). Prior to electroporation, females were anesthetized with a mixture of medetomidine (0.75 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg). The CRISPR mixture (1 µl) was injected into the oviductal lumen upstream of the ampulla with a glass micropipette, which was made using a vertical capillary puller (NARISHIGE, Tokyo, Japan). Following injection of CRISPR solutions, the oviduct regions were grasped using tweezer electrodes (CIUY652P.5 × 4; Nega Gene, Chiba, Japan), and electroporation was performed as previously described (Kobayashi et al. 2020) using a NEPA21 (Nega Gene). The following parameters were used for electroporation: poring pulse (voltage: 40 V; pulse length: 5.0 ms; pulse interval: 50 ms; number of pulses: 3; decay rate: 10%; polarity: +); transfer pulse (voltage: 10 V; pulse length: 50 ms; pulse interval: 50 ms; number of pulses: 3; decay rate: 40%; polarity: +). Following electroporation, we placed the oviducts back in their original location and sutured the incisions. Following the operation, atipamezole hydrochloride (0.75 mg/kg) was intraperitoneally injected to reverse the effects of medetomidine.

Analysis of CRISPR/Cas9-engineered mice
To screen for CRISPR/Cas9-induced deletions, genomic DNA was isolated from the tails or ears of founder mice using lysis buffer [100 mM NaCl, 200 mM sucrose, 10 mM ethylenediaminetetraacetic acid, 300 mM Tris (pH 8.0), and 1% sodium dodecyl sulfate], and DNA was examined by PCR amplification. PCR products were cloned into the pTAC-1 vector (Biodynamics, Tokyo, Japan), and the sequences of individual clones were determined by Sanger sequencing (Eurofins Genomics, Tokyo, Japan). The PCR primers used for genotyping are listed in Supplementary Table S1.

RT-PCR
Reverse transcriptase (RT)-PCR was performed using total RNA. Total RNA was isolated from ear tissue using ISOSPIN Cell & Tissue RNA (Nippon Gene, Tokyo, Japan). Template cDNA was obtained using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). The RT-PCR products were directly analyzed by Sanger sequencing (Eurofins Genomics, Tokyo, Japan). The PCR primers used for genotyping are listed in Supplementary Table S1.

Test for the balancer chromosome
B6.C3H-In(6)1J was selected to examine whether it could balance a recessive lethal mutation. A homozygous B6.C3H-In(6)1J Mitfem1Cu male was mated with a heterozygous Tprkbem1Cu female. Following inbreeding of F1 Tprkbem1Cu/B6.C3H-In(6)1J Mitfem1Cu mice, their offspring were phenotypically distinguishable. A schematic diagram of the assay and the expected results is depicted in Figure 5, A and B. In the assay, two PCR reactions are used to genotype the Tprkb deletion. The first reaction utilizes the
external primers to specifically amplify only the Tprkb-deleted product. In the second reaction, the internal primers are used to detect the Tprkb-exon 10 fragment, which was engineered to be deleted by the genome editing. Crude DNA derived from each embryo was prepared as a PCR template according to published methods (Shea and Geijsen 2007; Martin and Cockroft 2008). The Tprkb<sup>em1Cu</sup> genotype of each individual was determined by PCR with the primers listed in Supplementary Table S1.

**Statistical analysis**
For Mendelian genotype ratios of progeny obtained from sibling mating between Tprkb<sup>em1Cu</sup>/In(6) J mice, the chi-square test was performed using Excel version 16.36 (Microsoft, Redmond, WA, USA). The threshold for statistical significance was P < 0.05.

**Data availability**
The B6.C3H-In(6) J Mitf<sup>em1Cu</sup> mice used in this study are available at the RIKEN BioResource Research Center and Center for Animal Resources. The C3H/HeJ genome assembly is available for download from the DRA search of DDBJ (https://ddbj.nig.ac.jp/DRASearch/). Supplementary material is available at figshare: https://doi.org/10.25387/g3.14677524.

**Results and discussion**
**Isolation of inversion B6.C3H-In(6) J on chromosome 6 from C3H/HeJcl**
Previous studies showed that the inbred mouse strain C3H/HeJ carries an inversion In(6) J on chromosome 6 (Akeson et al. 2006; Ackert-Bicknell et al. 2007); however, the precise location of the breakpoints remains unknown. To identify In(6) J breakpoints in C3H/HeJ, we retrieved available C3H/HeJ WGS data from the DDBJ DRA (https://ddbj.nig.ac.jp/DRASearch/, Accession: ERR008069 and ERR008070) and analyzed them. The In(6) J breakpoints were predicted from alignment data using the BreakDancer tool version 1.4.5 (Chen et al. 2009; Fan et al. 2014). We then used Integrative Genomics Viewer (Thorvaldsdóttir et al. 2013) to predict candidate breakpoints. We attempted to detect two C3H/HeJ strains (C3H/HeJcl and C3H/HeJYokSlc) via PCR and Sanger sequencing (Figure 1). In the C3H/HeJcl strain, one breakpoint was in the intergenic region, whereas the other was in exon 2 of the uncharacterized gene Gm38889 (Figure 1D). In(6) J encompassed approximately 40% of the chromosome from 63 to 120.8 Mb, which was notably larger than the inversion predicted in previous studies (Akeson et al. 2006; Ackert-Bicknell et al. 2007).

Notably, the strain C3H/HeJYokSlc maintained at Japan SLC, Inc. did not carry In(6) J (Figure 1C); these mice originated from mice...
distributed by the National Institute of Infectious Diseases in 1985. ln(6)1J is expected to have occurred in the C3H/HeJ strain after the early 1970s (Akeson et al. 2006), suggesting that C3H/HeJYokSlc was derived from the C3H/HeJ strain before 1970. Following identification of the exact position of each inversion breakpoint, the B6.C3H-In(6)1J congenic strain was constructed by six generations of selective backcrossing into the C3H/HeJCl to C57BL/6N background. Previous studies demonstrated that recombination between the wild-type and chromosomal balancer lines does not occur within these inversion events (Zheng et al. 1999; Nishijima et al. 2003; Iwata et al. 2019). To examine whether B6.C3H-In(6)1J suppresses crossing over in the inversion interval, we determined the recombination frequencies on chromosome 6. We analyzed four PCR-RFLPs that lie external (dbSNP no. rs387767483 and dbSNP no. rs242839954) and internal (dbSNP no. rs244130831 and dbSNP no. rs238042460) to inversion ln(6)1J. Among all 16 meioses examined, the external region was recombined with the C57BL/6N strain (Figure 2, A, B, and E). In contrast, there was no crossover event within the inversion, indicating successful recombination suppression (Figure 2, C, D, and E).

**Generation of visible inversion strains using the i-GONAD method**

To facilitate B6.C3H-In(6)1J usage, we induced mutagenesis in the gene for which loss of function was expected to cause a visible phenotype. Mitf is a recessive gene within the ln(6)1J region. Mitf mutations show a reduction or lack of pigmentation in the coat, eye, and inner ear of the mouse (Steingrimsson et al. 2003). Thus, we performed the i-GONAD method to generate a line of B6.C3H-In(6)1J mice lacking Mitf (Figure 3A). This method can bypass the following three steps: (1) zygote isolation, (2) microinjection, and (3) zygote transfer (Takahashi et al. 2015; Ohtsuka et al. 2018; Gurumurthy et al. 2019b). We injected CRISPR/Cas9 RNPs into the oviduct lumen of a pregnant B6.C3H-In(6)1J female and electropreferred the oviduct in vivo (Figure 3B). Four founder F0 pups were white throughout their bodies, two had belly spot patterns, and one pup was black (Figure 3, C1 and C2). To determine the effect of disruption of Mitf on fertility, these mutant mice were mated to C57BL/6N mice and monitored for pregnancy. Breeding experiments revealed that the F0 mice with white spots are fertile; however, the unpigmented mice are sterile. Following backcrossing with C57BL/6N, the B6.C3H-In(6)1J Mitfem1Cu strain was generated, which eliminated the mosaicism. Heterozygotes for B6.C3H-In(6)1J Mitfem1Cu had normal coat pigmentation. In contrast, homozygous individuals had white spots throughout the body and less-pigmented eyes of normal size (Figure 3D). The RT-PCR analysis of the Mitfem1Cu/em1Cu mice tissues clearly detected a Mitf mRNA fragment shorter than that in the WT and indicated that the Mitfem1Cu mutation does not alter the open reading frame but yielded an in-frame deletion (Figure 3E). Sequence analysis of the RT-PCR products identified an in-frame deletion of 27 bp, which corresponded to Mitf amino acid residues 266–274 (Figure 3F).
Mitfem\textsuperscript{1Cu} mutation engineered into the B6.C3H-In(6)1J inversion chromosome acted as a coat color marker and enabled the inversion to be easily tracked.

**Generation of a lethal allele on a chromosome balanced with B6.C3H-In(6)1J using the i-GONAD method**

To produce F0 mice carrying the embryonic lethal mutation, we designed a method wherein one of two alleles of the gene was selectively edited by i-GONAD-mediated mutagenesis in the presence of a non-targeted B6.C3H-In(6)1J in heterozygotes. We attempted to disrupt a potentially essential gene, Tprkb (encoding the Tp53rk binding protein), which was expected to result in lethal phenotypes based on a previous study (Braun et al. 2017); however, this has not been accurately determined. To induce a large deletion and complete knockout of Tprkb, we cut two sites using two gRNAs that selectively target one of the C57BL/6N (B6) alleles (Figure 4A). We electroporated the genome editing CRISPR/Cas9 mixture into the oviducts of three B6 females that mated with B6.C3H-In(6)1J Mitfem\textsuperscript{1Cu} males, and seven B6 females that mated with B6 males were used as controls. Control B6/B6 strains had only three pups born through cesarean section, suggesting that most embryos died owing to the deletion of both Tprkb gene alleles. One of the three pups had a deletion mutation, but we could not obtain a surviving founder F0 (Figure 4C). In contrast, in B6/B6.C3H-In(6)1J Mitfem\textsuperscript{1Cu} hybrid strains, we obtained six F0 pups via cesarean section and found that two had large deletions in the target locus, resulting in one viable F0 mouse (Figure 4, B and C). Similarly, B6.C3H-In(6)1J females that mated with B6 males successfully obtained a viable F0 mouse with Tprkb deletion (Figure 4, B and C). This approach used fewer animals than are required by conventional methods, which is beneficial in terms of animal welfare. Unlike in our previous study performed using F1 hybrid mice (Iwata et al. 2019), the new method avoids the need for a few generations of backcrossing to achieve genetic homogeneity. Thus, the strategy in which one allele in an essential gene is selectively targeted by i-GONAD-mediated gene editing enables researchers to efficiently generate a strain carrying the lethal allele.

The i-GONAD method reported by Ohtsuka et al. (2018) was confirmed to have comparable efficiency to microinjection. Hence, our method in this study would be as effective as the standard pronuclear injection methods.

**Confirmation of B6.C3H-In(6)1J inversion to balance a lethal mutation**

We examined whether B6.C3H-In(6)1J could balance a recessive lethal mutation within the inversion interval, as described in Figure 5A. A homozygous B6.C3H-In(6)1J Mitfem\textsuperscript{1Cu} male was mated with a heterozygous female carrying a Tprkb\textsuperscript{em1Cu} mutation, and the F1 trans-heterozygotes were further intercrossed. As mentioned previously, B6.C3H-In(6)1J Mitfem\textsuperscript{1Cu} contains a coat
color marker, which allows animal carriers to be easily identified. Following inbreeding of F1 mice, the balanced strain $Tprkb^{bmi1Cu}/B6.C3H-In(6)1J$ segregated into two phenotypes: black progeny inherited the heterozygous $Tprkb^{bmi1Cu}$ mutation, whereas white spot progeny were genotyped as wild-type (Figure 5, B–D).

We also confirmed all breakpoints of the inversion $In(6)1J$ (Supplementary Figure S1). As homozygous $Tprkb^{bmi1Cu}$ mutants were not observed in live-born progeny, we collected embryos at embryonic days 14.5 (E14.5) and E7.5 and analyzed them. However, no $Tprkb^{bmi1Cu}$ homozygous embryo was found (Figure 5, E–G). These non-Mendelian ratios suggest that the $TprkB$ null mutation results in the death of homozygotes at the developmental stage earlier than E7.5 (Figure 5G). Previous studies showed that F0 embryos with CRISPR/Cas9 knockout of $Tprkb$ exhibited primary microcephaly (Braun et al. 2017); however, most embryos injected with gRNA and Cas9 mRNA are genetically mosaic (Yen et al. 2014; Oliver et al. 2015). Thus, these phenotypes may be attributed to a combination of mutations. Therefore, $B6.C3H-In(6)1J$ makes it easier for researchers to maintain lethal mutations and more efficient experiments in which lethal mutants can be analyzed.

**Conclusions**

The $B6.C3H-In(6)1J$ strains generated in this study will be deposited as frozen sperm at the RIKEN BioResource Research Center and Center for Animal Resources. Table 1 lists the stock balancer strains currently available via the public bioresource community. These balancers are genetically identical to the inbred strain, except for the inverted region and its surrounding region.

In this study, the allele-specific i-GONAD method in $B6.C3H-In(6)1J$ mice allowed us to efficiently generate recessive lethal strains without ex vivo handling of embryos. The edited lethal genes were stably maintained in heterozygotes, as recombination did not occur within this inversion interval. Such heritable lethal mutations are common in many human inherited disorders (Hrabe de Angelis et al. 2015; Dickinson et al. 2016; Meehan et al. 2017), and our method using mice with $B6.C3H-In(6)1J$ will be a useful tool for disrupting and analyzing disease-causing essential genes. In addition, $B6.C3H-In(6)1J$ are applicable in $\text{N}$-ethyl-$\text{N}$-nitrosourea mutagenesis screens (Supplementary Figure S2), as the balancers shown in Table 1 were previously used (Kile et al. 2003; Boles et al. 2009). Our strategy provides an easier method by which researchers can create lethal mutations and analyze the mechanisms of action of genes.

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S.I. designed and performed the experiments and drafted the manuscript. T.S. and M.N. performed the animal experiments. T.I. supervised the studies and corrected the manuscript. All authors have read and approved the final manuscript.

### Table 1

| Strains (Repository/Stock#) | Chr. | Covering | Genetic background | Phenotypes | References |
|-----------------------------|------|----------|--------------------|------------|------------|
| In[D4Mit117; D4Mit281]1Brd (MMRRC/031767-UCD) | 4    | 96742762–130172215 bp | 129S7 FVB | Light brown coat | Nishijima et al. 2003 |
| In[D4Mit281; D4Mit51]2Brd (MMRRC/031768-UCD) | 4    | 130172113–155046016 bp | 129S7 FVB | Light brown coat | Nishijima et al. 2003 |
| In(4)56Rk (JAX/001379) | 4    | 4A1–4E2 | DBA/2J | Retinal degeneration | Roderick et al. 1997 |
| In(6)1J Mitf^m1Cu | 6    | 63000846–120827193 bp | C3H/HeJ | White spots | Current study Zheng et al. 1999 |
| In(11)Tpr^m1Cu | 11   | 69580359–103817957 bp | C57BL/6J | Normal | Roderick 1983 |
| In(15)21Rk/J (JAX/000920) | 15   | 15A1–15E | DBA/2J | Embryonic lethal | Nishijima et al. 2003 |

**Figure 5** Chromosomal inversion B6.C3H-In(6)1J can balance a lethal mutation. (A) Overview of animal crossing schemes used to test whether B6.C3H-In(6)1J could balance a Tprk^b mutation. Sibling matings between Tprk^b^In(6)1J mice generate three classes of F2 mice, In(6)1J/In(6)1J, Tprk^b^/In(6)1J, and Tprk^b^/Tprk^b^, which can be distinguished by the presence of the Mitf mutation on the balancer chromosome. (B) Schematic representation of the PCR primer positions for detecting the Tprk^b^ genotype. The internal primers are designed to amplify only the Tprk^b^ product. The internal primers are only able to bind the wild-type product. The expected results of the PCR amplifications are shown for the three potential genotypes at the right of the figure. (C) Appearance of In(6)1J/In(6)1J and Tprk^b^/In(6)1J pups at 14 days of age. In(6)1J/In(6)1J mice had a white spot phenotype. Tprk^b^/In(6)1J mice were phenotypically normal. (D) Genotyping of seven pups was performed to confirm Tprk^b^ genotypes. (E) Appearance of In(6)1J/In(6)1J and Tprk^b^/In(6)1J fetuses at E14.5. The In(6)1J/In(6)1J fetus had less-pigmented eyes, indicated by the red arrowhead. (F) Genotyping of six fetuses was performed to confirm Tprk^b^ genotypes. (G) Summary of In(6)1J/In(6)1J, Tprk^b^/In(6)1J, and Tprk^b^/Tprk^b^ offspring proportions. Asterisks indicate a significant difference, as determined using the chi-square test (*P* < 0.05, **P** < 0.01).
Conflicts of interest

None declared.

Literature cited

Ackert-Bicknell CL, Salisbury JL, Horowitz M, DeMambro VE, Horton LG, et al. 2007. A chromosomal inversion within a quantitative trait locus has a major effect on adipogenesis and osteoblastogenesis. Ann N Y Acad Sci. 1116:291–305.

Akeson EC, Donahue LR, Beamer WG, Shultz KL, Ackert-Bicknell C, et al. 2006. Chromosomal inversion discovered in C3H/HeJ mice. Genomics. 87:311–313.

Alghadban S, Bouchareb A, Hinch R, Hernandez-Pliego P, Biggs D, et al. 2020. Electroporation and genetic supply of Cas9 increase the generation efficiency of CRISPR/Cas9 knock-in alleles in C57BL/6J mouse zygotes. Sci Rep. 10:12.

Boles MK, Wilkinson BM, Maxwell A, Lai L, Mills AA, et al. 2017. Mutations in KEOPS-complex genes cause nephrotic syndrome with primary microcephaly. Nat Genet. 49:1529–1538.

Chen K, Wallis JW, McLellan MD, Larson DE, Kalicki JM, et al. 2009. BreakDancer: an algorithm for high-resolution mapping of genomic structural variation. Nat Methods. 6:677–681.

Dickinson ME, Flenniken AM, Ji X, Teboul L, Wong MD, et al. 2016. High-throughput discovery of novel developmental phenotypes. Nature. 537:508–514.

Fan X, Abbott TE, Larson D, Chen K. 2014. BreakDancer: identification of genomic structural variation from paired-end read mapping. Curr Protoc Bioinformatics. 45:15.6.1–15.6.11.

Gurumurthy CB, O’Brien AR, Quadros RM, Adams J, Alcaide P, et al. 2019a. Reproducibility of CRISPR-Cas9 methods for generation of conditional mouse alleles: a multi-center evaluation. Genome Biol. 20:

Gurumurthy CB, Sato M, Nakamura A, Inui M, Kawanou N, et al. 2019b. Creation of CRISPR-based germine-genome-engineered mice without ex vivo handling of zygotes by i-GONAD. Nat Protoc. 14:2452–2482.

Hrabé de Angelis M, Nicholson G, Selloum M, White JK, Morgan H, et al.; EUMODIC Consortium. 2015. Analysis of mammalian gene function through broad-based phenotypic screens across a consortium of mouse clinics. Nat Genet. 47:969–978.

Iwata S, Nakadai H, Fukushi D, Jose M, Nagahara M, et al. 2019. Simple and large-scale chromosomal engineering of mouse zygotes via in vitro and in vivo electroporation. Sci Rep. 9:8.

Keane TM, Goodstadt L, Danecek P, White MA, Wong K, et al. 2011. Mouse genomic variation and its effect on phenotypes and gene regulation. Nature. 477:289–294.

Kile BT, Hentges KE, Clark AT, Nakamura H, Salinger AP, et al. 2003. Functional genetic analysis of mouse chromosome 11. Nature. 425:81–86.

Kinjo S, Monma N, Misu S, Kitamura N, Imoto J, et al. 2018. Maser: one-stop platform for NGS big data from analysis to visualization. Database. 2018:bay027.

Kobayashi Y, Aoshima T, Ito R, Shinmura R, Ohtsuka M, et al. 2020. Modification of i-GONAD suitable for production of genome-edited C57BL/6 inbred mouse strain. Cells. 9:957.

Labun K, Montague TG, Krause M, Torres Cleuren YN, Tjeleides H, et al. 2019. CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. Nucleic Acids Res. 47:W171–W174.

Martin P, Cockroft DL. 2008. Culture of postimplantation mouse embryos. Methods Mol Biol. 461:7–22.

Meehan TF, Conte N, West DB, Jacobsen JO, Mason J, et al.; International Mouse Phenotyping Consortium. 2017. Disease model discovery from 3,328 gene knockouts by The International Mouse Phenotyping Consortium. Nat Genet. 49:1231–1238.

Meng X, Zhao L, Lai C, Liu J, Weng X, et al. 2015. High-throughput discovery of novel developmental phenotypes. Nat Genet. 47:969–978.

Meng X, Zhao L, Lai C, Liu J, Weng X, et al. 2015. High-throughput discovery of novel developmental phenotypes. Nat Genet. 47:969–978.

Morgan H, Selloum M, White JK, Morgan H, et al.; EUMODIC Consortium. 2015. Analysis of mammalian gene function through broad-based phenotypic screens across a consortium of mouse clinics. Nat Genet. 47:969–978.

Oliver D, Yuan S, McSwiggin H, Yan W. 2015. Pervasive genotypic mosaicism in founder mice derived from genome editing through pronuclear injection. PLoS One. 10:e0129457.

Pennisi E. 2013. The CRISPR craze. Science. 341:833–836.

Roderick TH. 1983. Using inversions to detect and study recessive lethals and detrimental in mice. In: FJ de Serres, editor. Utilization of Mammalian Specific-Locus Studies in Hazard Evaluation and Estimation of Genetic Risk. New York, NY: W. Sheridan Plenum Press. p. 135–167.

Roderick TH, Chang B, Hawes NL, Heckenlevy JR. 1997. A new dominant retinal degeneration (Rd4) associated with a chromosomal inversion in the mouse. Genomics. 42:393–396.

Rogner UC, Avner P. 2003. Congenic mice: cutting tools for complex immune disorders. Nat Rev Immunol. 3:243–252.

Shea K, Geijsen N. 2007. Dissection of 6.5 dpc mouse embryos. JoVE. 2:160.

Steingrümsson E, Arnheiter H, Hallsson JH, Lamoreux ML, Copeland NG, et al. 2003. Interallelic complementation at the mouse Mitf locus. Genetics. 163:267–276.

Takahashi G, Gurumurthy CB, Wada K, Miura H, Sato M, et al. 2015. GONAD: genome-editing via oviductal nucleic acids delivery system: a novel microinjection independent genome engineering method in mice. Sci Rep. 5:11406.

Thorvaldóttir H, Robinson JT, Mesirop J. 2013. Integrative genomics viewer (IGV): high-performance genomics data visualization and exploration. Brief Bioinformatics. 14:178–192.

Wu Y, Zhang J, Peng B, Tian D, Zhang D, et al. 2019. Generating viable mice with heritable embryonically lethal mutations using the CRISPR-Cas9 system in two-cell embryos. Nat Commun. 10:1–13.

Yen ST, Zhang M, Deng JM, Usman SJ, Smith CN, et al. 2014. Somatic mosaicism and allele complexity induced by CRISPR/Cas9 RNA injections in mouse zygotes. Dev Biol. 393:3–9.

Zhang B, Sage M, Cai WW, Thompson DM, Tavsanli BC, et al. 1999. Engineering a mouse balancer chromosome. Nat Genet. 22:375–378.