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Germline transgenesis in rodents by pronuclear microinjection of *Sleeping Beauty* transposons

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We describe a protocol for high-efficiency germline transgenesis and sustained transgene expression in two important biomedical models, the mouse and the rat, using the *Sleeping Beauty* transposon system. The procedure is based on co-injection of synthetic mRNA encoding the SB100X hyperactive transposase together with circular plasmid DNA carrying a transgene construct flanked by binding sites for the transposase, into the pronuclei of fertilized oocytes. Upon translation of the transposase mRNA, enzyme-mediated excision of the transgene cassettes from the injected plasmids followed by permanent genomic insertion produces stable transgenic animals. Generation of a germline-transgenic founder animal by using this protocol takes approximately three months. Transposon-mediated transgenesis compares favorably in terms of both efficiency and reliable transgene expression to classic pronuclear microinjection, and offers comparable efficacies to lentiviral approaches, without limitations on vector design, issues of transgene silencing, and the toxicity and biosafety concerns of working with viral vectors. Transposition-mediated gene delivery can easily be implemented by any laboratory, thereby providing an attractive method to genetically modify animals for biomedical and biotechnological purposes.

INTRODUCTION

Mice and rats are the most common species used as experimental model systems for the analysis of physiological traits, to investigate the pathogenesis of human diseases and for developing new therapies. Both species offer unique advantages for use in biomedical research such as their versatility and the abundance of available resources, including mutant archives produced by the large-scale *N'*-ethyl-*N'*-nitrosourea (ENU) mutagenesis screens, the International Knockout Mouse Consortium (IKMC), the International Gene Trap Consortium (IGTC) and the European Mouse Mutant Archive (EMMA)[1-6]. A large number of inbred strains have been successfully generated for mice and rats; these genetically standardized and phenotypically characterized strains are valuable and well documented tools available for the scientific community. The availability of the full genome sequence of the mouse and the rat further strengthened their position as leading mammalian model organisms[7,8]. A comprehensive comparison with the previously decoded human genome revealed a high similarity in the genomic sequences presumably reflecting similarities in gene function between humans and rodents[9].

The Norway rat (*Rattus norvegicus*) was the first mammal domesticated for scientific research[10]. Laboratory rats are widely used in pharmacological and toxicological testing but also in basic research for modelling physiological processes of human organ systems. Its larger size and often closer similarity to human biology would seem to make the rat a better experimental tool than the mouse (*Mus musculus*). However, despite the many advantages of rats as model organisms, the laboratory mouse is currently the predominant mammalian species in biomedical research. This is mainly due to the availability of several genetic modification technologies developed and continuously advanced in mice over the past 30 years, which enabled gene trapping and the introduction of targeted mutations[11-15].

Mutagenesis is currently the most frequently used method to examine gene function and regulation, and several strategies have been developed to experimentally induce mutations into rodent germlines. Mutagenesis by homologous recombination for producing knockout and knockin animals was limited to mouse models owing to the successful isolation and culture of pluripotent

embryonic stem cells (ESCs). After more than two decades of intensive effort, pluripotent rat ESC lines have been successfully isolated and cultured[13,16]. The application of the 2i cell culture system developed for murine ESCs has further improved the derivation of rat ESCs[17,18], and enabled for the first time generating a targeted mutation in the rat genome by homologous recombination[19]. However, the use of ESCs to modify the rat genome is still very challenging. Therefore, apart from the common technologies for genetic engineering, new strategies to manipulate the mouse and the rat genome have been recently developed. New tools for targeted mutagenesis that can not only be applied in ESCs but are efficient enough for genome engineering directly in living embryos include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the CRISPR/Cas system[20-26].

Germline transgenesis in rodents

Germline transgenesis is an important procedure for the generation of heritable, loss-of-function or gain-of-function phenotypes in model species for investigations into the functions of biological pathways as well as for animal biotechnology. Since 1981, the most routinely applied method in mice has been pronuclear DNA-injection[27]. This methodology has also been adapted to rats and is performed in a very similar way[28,29]. For mice, pronuclear injection of transgenes often yields more than 20% founders among the live offspring[27,30]. In rats the reported efficiency of classical microinjection is much lower than in mice, possibly due to the significantly lower percentage of ova surviving the microinjection procedure[28,31,32]. Standard pronuclear injection of a transgene involves the introduction of a small number of identical copies of a linearized DNA molecule into a freshly fertilized oocyte. Linearization of transgene constructs is important, because the integration frequency is much higher for linearized than for circular DNA molecules[33]. However, linearized molecules recombine with each other immediately after pronuclear injection and before chromosomal integration to form concatemeres[34]. Consequently, the gene copy number per transgenic locus is unpredictable and unique for each founder line. In addition, transgenic concatemeres can persist in a nonintegrated form for several cell divisions, resulting in unequal

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transgene distribution between the blastomeres of the early embryo[35]. Therefore, a high percentage of transgenic founders produced by pronuclear injection are genetic mosaics[36]. A further limitation associated with this technology is that multicopy concatemers are prone to undergo transcriptional silencing[37], thereby compromising the production of transgenic stocks with reliable spatio-temporal patterns of transgene expression. In addition, integration of pronuclear injected transgenes often causes severe chromosomal alterations at the chromosomal integration site(s), including deletions, duplications and translocations[38-40].

Another alternative approach to producing transgenic rodents is sperm-mediated gene transfer (SMGT) combined with intracytoplasmic sperm injection (ICSI)[41-43]. In this procedure, spermatozoa are subjected to disruption of their head membranes, hence the need for the sophisticated ICSI for fertilization. The resulting frequency of founders produced by this technique is comparable to standard pronuclear injections. An improved efficiency of SMGT was demonstrated for large constructs cloned in YACs[44]. Furthermore, founders produced by SMGT are only rarely genetic mosaics[44]. However, there are major concerns regarding the impact of the procedure on the genetic material of the sperm and, consequently, on the genome of the resulting transgenic animal[45,46].

Higher transgenic rates were reported by injecting recombinant lentiviral vectors into the periviteline space of ova in various animal models, including rodents and certain livestock species[47,48]. The use of lentiviral vectors for transgenesis has been optimized in rats and resulted in 22 % average frequency of producing founders. Although this frequency certainly represents an improvement over classic pronuclear microinjection of naked DNA, it is much lower than the reported 80 % in mice[47,49]. Several problems with using lentiviral vectors remain, such as genetic mosaicism due to vector integration into the genome in later developmental stages, limitations of transgene size, vector-related toxicity, transcriptional silencing of proviral insertions by cellular mechanisms and preferential insertion into endogenous genes. Thus, methods with the capacity to yield more robust germline transmission rates coupled with predictable transgene expression

patterns, technical ease and greater safety would significantly improve transgenic studies in mammalian models.

Transgenesis with the Sleeping Beauty transposon

Similarly to retroviruses, DNA transposons integrate into the chromosomes of the host cells, a feature that forms the basis of their use for gene delivery. In nature, these elements contain a gene encoding a transposase enzyme flanked by inverted terminal repeats (ITRs) that carry binding sites for the transposase. Under laboratory conditions, transposons are applied as bi-component vector systems, in which a DNA sequence of interest can be cloned between the transposon ITRs and mobilized by providing the transposase enzyme *in trans*, as an expression plasmid or as *in vitro* synthesized mRNA (**Fig. 1A**). During transposition, the transposase excises the genetic element from its donor plasmid and integrates it into a chromosomal locus (**Fig. 1B**). Based on fossil record of transposons that were active >10 million years ago in diverse fish species, an ancient transposon was "awakened" (molecularly reconstructed) and named *Sleeping Beauty* (SB), after the Grimm brothers' fairy tale[50]. SB was the first transposon shown to be capable of efficient transposition in vertebrate cells, thereby opening new avenues for genetic engineering in animal models (reviewed in ref. 51).

The SB system combines the advantages of retroviral vectors (permanent gene insertion into recipient genomes) with those of naked DNA molecules (simple and safe production and application). Because transposition is a cut-and-paste mechanism that does not involve reverse transcription, transposon-based vectors can tolerate larger and more complex transgenes. The SB system is not strictly limited by the size of expression cassettes[52]. Indeed, inserts as large as bacterial artificial chromosomes (BAC) were recently shown to transpose with SB at reasonable efficiencies in mouse ESCs[53]. When performing transgenesis, a single copy transgene insertion, which is not disturbing endogenous gene functions is desirable. Chromosomal integration of SB transposons is nearly random at the genome level resulting in ~60 % of the SB transposon

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integrations being intergenic[54-57]. Furthermore, transposons have been shown to efficiently deliver a

wide variety of transgene cassettes (reviewed in refs. 51, 58 and 59).

Because the transposase is only transiently present in the cell, the integrated transposon is

stable (i. e., will not undergo further rounds of transposition), thereby rendering transposons easily

controllable, conditional DNA delivery tools that can be used for versatile applications, including

germline gene transfer. We have recently developed a hyperactive variant of the SB transposase,

called SB100X, by *in vitro* evolution[60]. SB100X supports efficient germline transgenesis in mice[60,61],

rats[61], rabbits[61,62] and pigs[63,64]. We optimized the SB100X-mediated protocol by carefully titrating

the relative amounts of transposase and transposon to obtain optimal rates of transgenesis to

generate founders, and extensively evaluated it for efficacy, toxicity, mosaicism, germline

transmission, insertion site preferences, transgene copy number and silencing. Genotyping of

numerous transgenic lines produced by SB-transposition demonstrated single-copy integrations of

the transposon as expected from the cut-and-paste integration reaction catalyzed by the SB-

transposase[61]. The numbers of integrations per genome were shown to be dependent on and

hence controllable by the concentration of both components of the transposon system in the

injection cocktail. The best results in terms of frequency of founder animals with mostly single

integrations were achieved by a reduction of the amount of transposon plasmid DNA[61].

One of the most important aspects of using this protocol is that no major mosaicism was

observed, and transgene expression was maintained for several generations in all species tested.

The lack of mosaicism is in sharp contrast to any other current, non-viral or viral approach for

transgenesis. This is likely due to the very nature of transgene integration: transposition results in

precise (the ends of the integrating DNA are well defined) genomic integration of monomeric

transgene units within a short timeframe following administration, thereby minimizing mosaicism.

Furthermore, unlike retroviral vectors[48,65-67], SB100X-catalyzed transgene integration does not

seem to trigger transcriptional silencing[56,61]. Therefore, the application of the *Sleeping Beauty*

transposon system described here will significantly enhance the mouse and rat genomic toolbox.

Limitations

DNA transposons, including SB, are regulated by overproduction inhibition, which means that overexpression of the transposase has a negative effect on transposition[56,68]. The practical consequence of this phenomenon is that an optimal ratio of transposon donor plasmid and transposase mRNA needs to be established. As a rule of thumb, the injection mixture should contain 5 ng/µl SB100X mRNA and 0.4 ng/µl transposon donor plasmid for an SB vector of a total size of ~6.1 kb containing a ~2.5 kb transgene cassette[60]. For larger transgenes, the concentration of the donor plasmid in the microinjection mixture has to be increased to maintain optimal molar ratios between transposon and transposase. It has been found that embryos tolerate concentrations of transposon donor plasmid up to 2 ng/µl in mice[60].

Experimental Design

The generation of transgenic rodents by SB-mediated transgenesis is achieved through microinjection of a plasmid carrying a gene of interest, cloned between the ITRs of SB, and synthetic transposase mRNA (**Fig. 1**) into the pronucleus of a fertilized oocyte, with frequencies of transgenic founders of 50-70% for both mice and rats[61]. The protocol consists of the following major steps:

Preparation of *Sleeping Beauty* transposon components for microinjection (Steps 1-26). These steps include molecular cloning of a gene of interest into SB transposon vectors and purification of the resulting plasmid construct; preparation of mRNA encoding the transposase by *in vitro* transcription, and preparation of a nucleic acid mixture consisting of the purified transposon plasmid carrying the ITR-flanked transgene and the transposase mRNA.

Transgenesis with *Sleeping Beauty* in mice (Steps 27-39) and rats (Steps 40-74). These steps include preparation of donor mice, collection of ova, microinjection of the nucleic acid mixture into ova and transfer of the microinjected ova into surrogate mothers.

Genotyping of transgenic animals (Steps 75-100). These steps include PCR-based analysis of F0 as well as F1 offspring to establish founders and germline transmission. A simple, quick PCR

test can be applied to determine the presence of integrated transposon sequences from genomic DNA samples. The PCR primers amplify sequences in the left ITR of SB; thus, this protocol can be universally applied irrespective of the gene of interest that was cloned in the SB vector. In order to assess copy numbers of integrated transposons and map the genomic integration sites, a ligation-mediated PCR procedure is applied[69]. The procedure consists of a restriction enzyme digest of the genomic DNA, ligation of an oligonucleotide adapter to the ends of the fragmented DNA, PCR amplification of a transgene/genomic DNA junction in two rounds of PCR with primers specific to the adapter and to the ITRs of the SB transposon, and sequencing of the junctions to map the insertion to the reference genome[70]. Finally, a locus-specific PCR is applied to distinguish and track the individual integrations in the F1 and later generations.

MATERIALS

Reagents

Animals

- Two different strains are required, the donor strain to produce ova and the recipient strain, which is used to provide pseudopregnant surrogate mothers. **Donors:** predominantly used inbred strains are C57BL/6 mice and F344 rats. **Surrogate mothers and sterile males:** recipients of embryos are usually outbred due to their superior fertility. Examples of preferred strains are NMRI and ICR mice at an age of 8-20 weeks and Wistar, Sprague Dawley and Long-Evans rats at an age of 11-18 weeks. **! Caution** *Animal experiments must be approved by and conducted according to institutional and national regulations.*

Molecular biology reagents

- mMessage mMachine® T7 kit (Invitrogene/Ambion, cat no. AM1344)
- Plasmid DNA preparation kit (Qiagen, cat no. 27106)
- Agarose (DNase, RNase none detected) (Sigma–Aldrich, cat no. A4718)

- Ethidium bromide 1 % solution in water (Sigma–Aldrich, cat no. E7637) **! Caution** *This is a hazardous chemical. Avoid contact with skin, eyes and airways.*

- *Cla*I restriction endonuclease (New England Biolabs, cat no. R0179S)

- *Bfa*I restriction endonuclease (New England Biolabs, cat no. R0568S)

- *Dpn*II restriction endonuclease (New England Biolabs, cat no. R0543S)

- Taq DNA polymerase, provided with PCR buffer (10 x) and $MgCl_2$ (25 mM) (New England Biolabs, cat no. M0267S)

- dNTP (10 mM) (New England Biolabs, cat no. N0447S)

- Injection buffer, EmbryoMax® (Millipore, cat no. MR-095-10 F)

- Sodium chloride (Sigma–Aldrich, cat no. S3014)

- Sodium acetate 3 M pH 5.5 (RNase-free) (Invitrogen/Ambion, cat no. AM9740)

- RNaseZap (Invitrogene/Ambion, cat no. AM9780)

- 2-propanol (ROTH, cat no. 6752.1)

- Ethanol (RNase-free) (MERCK, cat no. 108543)

- Water (RNase-free, filtered) (Sigma–Aldrich, cat no. W4502) **CRITICAL** *The use of filtered RNase-free water is recommended for solutions coming in contact with the injection material.*

- Phenol/chloroform/isoamyl alcohol, Roti®-Phenol/C/I (ROTH, cat no. A156.2) **! Caution** *This is a hazardous chemical. Avoid contact with skin, eyes and airways.*

- Chloroform/isoamyl alcohol, Roti®-C/I (ROTH, cat no. X984.2) **! Caution** *This is a hazardous chemical. Avoid contact with skin, eyes and airways.*

- Diethylpyrocarbonate (DEPC) (Sigma–Aldrich, cat no. D5758) **! Caution** *This is a hazardous chemical. Avoid contact with skin, eyes and airways.*

- 5 x TBE buffer, Nuclease-free (Sigma–Aldrich, cat no. 93306)

- TE buffer (Sigma–Aldrich, cat no. 93283)

- T4 DNA ligase, provided with ligase buffer (10x) (New England Biolabs, cat no. M0202S)

- 100-bp DNA Ladder (Thermo Scientific, cat no. SM0242)

- 100-10.000-bp DNA Ladder (Thermo Scientific, cat no. SM0331)

- QIAqick Gel Extraction kit (Qiagen, cat no. 28706)

- pGEM-T Vector Systems (Promega, cat no. A3600)

- Transposon donor plasmids are available from Addgene: pT2/BH
  (http://www.addgene.org/26556/) or pT2/HB (http://www.addgene.org/26557/).

- Transposase expression plasmids: pCMV(CAT)T7-SB100X is available from Addgene
  (http://www.addgene.org/34879/) and pcGlobin2-SB100X is available from Dr. Zsuzsanna
  Izsvak, Max Delbrück Centrum for Molecular Medicine, Berlin.

Animal work

- Equine chorionic gonadotropin/Pregnant mare's serum gonadotropin (PMSG) (Folligon®,
  Intervet, cat no. 113064 R1 or Sergon®, Bioveta a.s., reg no. 96/116/92-C)

- Human chorionic gonadotropin (hCG) (Chorulon®, cat no. 044976 or Intervetor Pregnyl®,
  N.V.Organon, reg no. 56/279/91-C/C)

- M2 medium (Sigma-Aldrich, cat no. M7167, St. Luis, USA)

- M16 medium (Sigma-Aldrich, cat no. M7292)

- G-IVF PLUS medium (Vitro Life, cat. no. 10136)

- Mineral oil (Sigma-Aldrich, cat no. M8410-500ML)

- Penicillin G (Sigma-Aldrich, cat no. P3032)

- Streptomycin sulfate salt (Sigma-Aldrich, cat no. S1277)

- Hyaluronidase (Sigma-Aldrich, cat no. H3506-100MG)

- Ketasol® Ketamine (aniMedica, cat no. 14.057.1004)

- Xylasol® Xylazine (aniMedica, cat no. 14.073.1011)

- Metacam® MeloxicamXylapan (Xylazinum 20mg/ml, Vetoquinol, cat no. 96/010/01 C)

- Narketan (Ketaminum 100 mg/ml, Vetoquinol, cat no. 96/010/01 C)

- Eye ointment (Oleovit, Fresenius Kabi cat no. V0005652/10)

- Sigmacote (Sigma-Aldrich, cat no. SL2-100ML) **! Caution** *Corrosive, flammable, harmful in contact with eyes, skin and airways.*

- Sulfuric acid (Sigma-Aldrich, cat no. 320501-1L) **! Caution** *Corrosive, harmful in contact with skin.*

- Hydrofluoric acid (Sigma-Aldrich, cat no. 339261-100ML) **! Caution** *Corrosive, harmful in contact with skin.*

Equipment

For molecular biology

- Refrigerated centrifuge capable of high speed (12000 × *g*) (Thermo Scientific, cat no. 75008162)

- Water bath, 37 °C (Thermo Scientific, cat no. 2824)

- NanoDrop® ND-2000 Spectrophotometer (Thermo Scientific, cat no. 91-ND-2000)

- Milli-Q Water Purification System (Merck Millipore, cat no. ZRXQ003T0)

- 1.5-mL tubes, free of DNase and RNase (Eppendorf, cat no. 0030123.328)

- Pipette tips, free of DNase and RNase [Eppendorf, cat no. 0030077.504 (0.1– 10 µl), cat no. 0030077.539 (2–20 µl), cat no. 0030077.555 (2–200 µl), cat no. 0030077.571 (50–1000 µl)]

- Thermal cycler capable of temperature increments for touchdown PCR (Thermo Scientific, cat no. TCA0001)

- Electrophoresis apparatus including running chamber, well combs, gel tray and power supply (BioRad, cat no. 164-5050)

- 100-ml glass flask for gel preparation (SIMAX, cat no. B1812)

Capillaries

- Capillary tubes, length 75 mm, capacity 60 µL (KAVALIER Ltd.)

- Borosilicate glass tubes without filament to produce transfer capillaries for embryo transfer (Science Products, cat no. GB150-10) **! Caution** *Sharp objects should be disposed into approved containers.*

- Commercial injection capillaries (Femtotip II, Eppendorf, cat no. 5242957000) **! Caution** *Sharp objects should be disposed into approved containers.*

- Commercial holding capillaries (VacuTip, Eppendorf, cat no. 5175108000) **! Caution** *Sharp objects should be disposed into approved containers.*

- Glass tubes to produce injection capillaries (Narishige GD-1) **! Caution** *Sharp objects should be disposed into approved containers.*

- Glass tubes to produce holding capillaries (Narishige G-1 or G-100) **! Caution** *Sharp objects should be disposed into approved containers.*

- Pipette Puller (Sutter Instrument P-97 or Narishige PC-10)

- Microforge (H. SAUR, cat no. A031102 or Narishige, cat no. MF-900)

- Glass cutter (Hilgenberg, cat no. 4018303)

Embryo production, microinjection and embryo transfer

- Petri dishes 100, 60, 30 mm, sterile (Sarstedt, cat nos. 82.1474, 82.1194, 82.1135)

- 22 µm syringe filter (Sigma-Aldrich, cat no. Z359904-50EA)

- Stereomicroscopes with large working distance (Leica MZ 75, Leica MZ 125, SZX9 Olympus)

- Blunt hypodermic flushing needle (32G Point Style 2 51mm, Hamilton)

- Vibration-damped microscope table

- Inverted microscope with DIC (Zeiss Axiovert 200 or Olympus IX70)

- Microinjector for holding capillary (CellTram Oil, Eppendorf, cat no. 5176000025)

- Microinjector for injection capillary (CellTram Vario, Eppendorf, cat no. 5176000033)

- FemtoJet injector (Eppendorf, cat no. 5247 000.013)

- Eppendorf microloader, 0.5 µL - 20 µL (Eppendorf, cat. no. 335 242 956 003)

- 2x micromanipulator (TransferMan NK2, Eppendorf, cat no. 5188000012 or NT-88, Narishige)

- Laminar hood (preferred with heated surface and with integrated transmission and reflecting microscope (IVF-Workstations, K-Systems, Kivex Biotech Ltd)

- Tubing with mouthpiece and capillary holder (Eppendorf, cat no. 5176 190.002)

- Concave ground superfrost microscope slides (VWR International, cat no. 631-9475)

- Microscope slides, 25 x 75 mm (Sigma-Aldrich, Product no. C8902-1CS)

- Cover glass, 24 x 60 mm (Sigma-Aldrich, Product no. C9056-1CS)

- Glue stick (KORES S.A.)

- *In vitro* fertilization 4-well plate (Nunc, cat no. 144444)

- Watchmaker forceps (07.60.05, Medicon or Sigma-Aldrich, cat no. F6521-1EA) **! Caution** *Sharp objects.*

- Surgical instruments **! Caution** *Sharp objects.*

- Shaving razors, stainless steel surgical prep blades (Personna, cat. no. 74-0001) **! Caution** *Sharp objects.*

- Hair clipper (Hyundai PC 550, Oster)

- Micro-dissecting scissors (Sigma-Aldrich, cat no. S3271-1EA) **! Caution** *Sharp objects.*

- Nontraumatic suture material (Chirmax, EP2.5-USP, sterile, cat no. 6319)

- Michel wound clips (Medin 7.5 x 1.75 mm, cat. no. 132 91 0391)

- Gauze sponges 7.5 x 7.5 cm (PANEP MULLTEX DIN 61630)

- Vliwasoft sterile dressing 7.5 x 7.5 150 ks (sterile) (Lohmann-Rauscher 12082)

- Nontraumatic and absorbable suture material (Vicryl 6-0, V301G, Ethicon)

- Disposable skin stapler (Precise, 3M Health Care, cat no. 78-8083-1351-0)

REAGENT SETUP

Preparation of the transposase mRNA. Synthetic mRNA encoding the SB100X transposase can be produced (Step 17) from either the pCMV(CAT)T7-SB100X or the pcGlobin2-SB100X plasmids[60] (see **Reagents**). The latter vector supports *in vitro* synthesis of SB100X mRNA containing zebrafish β-globin 5'- and 3'-UTRs and a 30-mer synthetic poly(A) sequence, from a T7 promoter[71].

DEPC treatment of water. Add 1 ml DEPC to 1 L Milli-Q water to make a 0.1 % (vol/vol) solution and shake vigorously. Continuously stir the solution for 12 hours at room temperature (~20 °C). Autoclave it for 1 h to remove any trace amount of DEPC, as residual DEPC reacts with purine residues in RNA.

NaCl solution for oligonucleotide annealing. Prepare a 500 mM NaCl stock solution in Milli-Q water, and sterilize with autoclaving or filtration. Prepare the working solution by diluting the stock 10x in sterile TE buffer. Keep frozen at -20 °C.

Quality control of synthetic mRNA on agarose gel. Clean the running chamber, gel tray, comb and flask for gel preparation with 70 % (vol/vol) ethanol and decontaminate them from RNases with RNaseZap. Rinse the reagent off with RNase-free Milli-Q water (DEPC-treated). Incubate 1 µl of *in vitro* synthesized mRNA in 10 µl injection buffer for 1 hour at 37 °C and run on an RNase-free 1 % (wt/vol) agarose gel.

TBE. DEPC cannot be used directly to treat Tris buffers. To prepare TBE, dilute the nuclease-free 5 x TBE (see **Reagents**) in DEPC-treated water.

Hormones. Dissolve lyophilized powder of PMSG and of hCG hormones for superovulation in *Aqua pro injectione* to the final concentration of 50 IU/ml. Store solutions refrigerated (4-6 °C) for no longer than 2 weeks, or as frozen aliquots at -80 °C.

Hyaluronidase solution. Dissolve hyaluronidase (lyophilized powder) in M2 medium to the concentration of 10 mg/ml (stock solution) or the working concentration of 1 mg/ml and store at -20 °C until use.

Media. Prepare M2 and M16 culture media by adding Penicillin G (60 mg/L) and Streptomycin (50 mg/L). Sterilize with a 22 μm syringe filter, aliquot and store media refrigerated (4-6 °C) for no longer than 4 weeks.

Isolation of genomic DNA of F0 animals from tail or ear biopsies. A simple and reliable protocol for DNA isolation from tissue samples is available at The Jackson Laboratory page (http://jaxmice.jax.org/support/genotyping/tail_phenol.html).

EQUIPMENT SETUP

Chambers for microinjection. The chamber is made from a microscope slide with a 15-mm hole drilled in the center of the slide glued to a cover glass that forms the bottom of the chamber. Store the chambers in a Petri dish (diameter 100 mm) and sterilize by UV irradiation before use. Alternatively, use heat sterilized concave ground microscope slides.

Injection and holding capillaries. Both injection and holding capillaries are commercially available. However, if transgenic animals are routinely generated in a laboratory, it is recommended to produce your own capillaries by using a puller and a microforge. The specific pulling conditions depend on the glass tubes and specific puller equipment used. Store the GD-1 glass tubes and pulled injection capillaries (with filament) in a way that avoids dust accumulation. The finely drawn injection capillary should have an outer diameter at the tip of about 1 μm. If the tip is sealed, push it slightly on the holding capillary to break the tip open. Alternatively, it can be opened prior to loading of the injection solution by submersing the very tip of the capillary into 20% (vol/vol) hydrofluoric acid for 8-12 seconds. Stop the reaction by washing the tip in sulfuric acid for 2-3 seconds, followed by washing in water (biotechnology performance certified) for 2-3 seconds, and then in pure ethanol for 2-3 seconds. Optionally, silicon-coat the injection capillary (Sigmacote for 5-7 seconds) and wash in water (biotechnology performance certified) for 2 to 3 seconds. Bend the tips of injection and holding capillaries (2-3 mm long) to an angle of 15° (using a microforge). Flame-

polish the opposite end of the injection- and the holding-capillaries over the flame of the burner (for smooth insertion of capillaries into the microinstrument holders).

The holding capillaries are pulled with specific pulling conditions from glass tubes without filament (G-1, G-100). Use a microforge to break the pulled glass at an outer diameter of about 70 μm and fire-polish the tip to an inner diameter of about 15-30 μm. If necessary, heat-sterilize both capillaries at 200 °C for 2 hours.

CRITICAL *The selection of optimal injection micropipettes significantly affects the speed of DNA injection, survival rate of injected ova, and degree of control of pronuclear injection.*

Hand-operated manipulation and transfer capillaries. Hand-pull transfer and manipulation capillaries from borosilicate glass tubes heated over the flame of a burner. Use a glass cutter to score the pulled segment at an internal diameter of about 300 μm (manipulation capillary) and 100 μm (transfer capillary) (diameters are measured using the scale of a stereo microscope), then break and fire-polish the end. This must be done very quickly in order to avoid sealing the tip. If necessary, sterilize capillaries by heat or UV irradiation before use. Transfer capillaries can be used mouth-controlled or hand-controlled. For the latter, connect the prepared capillary with rubber tubing (length 1.5-2 cm, closed with a knot on one end) fitting to the diameter of pipette barrels.

CRITICAL *Selection of optimal transfer capillaries enables fast, precise, and effective manipulation of embryos without any damage or loss throughout the entire procedure.*

PROCEDURE

CRITICAL At each step of the protocol use RNase-free plasticware and wear gloves while handling reagents and samples to prevent RNase contamination.

Preparation of RNase-free transposon (ITR-flanked transgene) donor plasmid ⬜TIMING 2-4 h

    1 Clone your gene of interest between the ITRs of an SB transposon donor plasmid by standard molecular cloning procedures.

2 Transfer at least 5 µg of transposon donor plasmid in 400 µl TE buffer into a 1.5 ml tube, and add 400 µl phenol/chloroform/isoamyl alcohol to the tube. **! Caution** This is a hazardous chemical. Avoid contact with skin, eyes and airways.

3 Vortex the tube for 15 s and leave it on the bench for 2 min. Repeat this step 3 times to completely inactivate residual RNase.

4 Centrifuge at 12000 × *g* for 5 min at room temperature.

5 Transfer the top layer to a new, RNase-free 1.5 ml tube and add 400 µl chloroform/isoamyl alcohol. **! Caution** This is a hazardous chemical. Avoid contact with skin, eyes and airways.

6 Vortex for 15 s and centrifuge at 12000 × *g* for 5 min at room temperature.

7 Transfer the aqueous top layer, containing the DNA, to a new, RNase-free 1.5 ml tube, add 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol, and precipitate the DNA for 30 min at -20 °C.

8 Spin down at 12000 × *g* for 15 min at 4 °C and discard the supernatant.

9 Wash the pellet in cold 70 % (vol/vol) ethanol (RNase-free) by keeping the ethanol on the pellet for 10 min, centrifuge at 12000 × *g* for 1 min at 4 °C, and discard the supernatant. Repeat this step to completely remove any residual chemicals that may not be tolerated by the embryos.

CRITICAL STEP DEPC is not tolerated by the embryos and RNase-free solutions that come in contact with the injected material should be purified by filtration. Therefore, use the filtered, RNase-free water for the preparation of 70 % ethanol.

10 Air-dry the pellet for 5-10 minutes and resuspend it in 100 µl EmbryoMax® injection buffer.

11 Measure the concentration of the plasmid DNA using a NanoDrop® spectrophotometer.

12 Make a 50 ng/µl dilution of the plasmid in EmbryoMax® injection buffer. This solution can be used later for the preparation of the final injection mixture (Steps 25-26).

Pause Point The plasmid stock and its 50 ng/µl dilution can be stored (preferably at -80 °C) until use for up to 2 years.

13 Thaw the plasmid DNA immediately before the preparation of the microinjection mixture (Step 25).

Preparation of the transposase mRNA ☐TIMING 10-14h

14 Linearize at least 2 µg of the pcGlobin2-SB100X plasmid with *Cla*I digestion. 1 µg of linearized plasmid will be necessary for one round of mRNA synthesis. Check complete linearization on a 1 % (wt/vol) agarose gel.

CRITICAL STEP Supercoiled plasmid DNA runs faster, whereas open circular plasmid runs slower on agarose gel than the linear form. Following full digestion, bands corresponding to the open circular and supercoiled forms of the plasmid should be no longer visible on the gel. Consequently, linearized plasmid DNA produces a single band corresponding to the size of the plasmid when compared to a linear DNA ladder size marker.

15 Prepare the digested plasmid RNase-free by phenol/chloroform extraction as described in Steps 2-10. In Steps 2 and 5, set the volumes of the digested plasmid DNA, the phenol/chloroform/isoamyl alcohol and the chloroform/isoamyl alcohol to 100 µl each. This will decrease the liquid volume during precipitation and improve DNA recovery.

16 Measure the concentration of the linearized plasmid DNA using a NanoDrop® spectrophotometer.

17 Synthesize the mRNA using the mMessage mMachine® T7 kit following the manufacturer's instructions.

18 After mRNA synthesis, perform the Turbo DNase treatment and phenol/chloroform extraction suggested in the kit manual, with the modification that after the isopropanol precipitation following the phenol/chloroform extraction, wash the pellet twice in cold 70 % (vol/vol) ethanol. Use filtered, RNase-free water for the preparation of 70 % ethanol. Air-dry the pellet for 5-10 minutes.

19 Resuspend the mRNA in 20 µl filtered, RNase-free water.

20 Measure the concentration of the *in vitro* synthesized mRNA using a NanoDrop®

spectrophotometer. The typical yield is around 1 µg/µl.

21 Prepare a 1 % (wt/vol) agarose gel using nuclease-free TBE buffer, Milli-Q water treated with

DEPC and agarose powder. **! Caution** *DEPC is a hazardous chemical. Avoid contact with*

skin, eyes and airways.

22 Load 1 µl of the *in vitro* synthesized mRNA (Step 19) in RNA loading buffer (supplied with the

mMessage mMachine® T7 kit) and a DNA size marker and run the gel. The SB100X mRNA

prepared using the T7 promoter on the *Cla*I-digested pcGlobin2-SB100X runs on a normal

agarose gel between 700-800 bp (**Fig. 2,** lane 1). Typically, the *in vitro* synthesized mRNA

runs as one band on a non-denaturing gel (**Fig. 2,** lane 1). Alternatively, two bands may be

visible (**Fig. 2,** lane 2) due to the formation of secondary structures.

? TROUBLESHOOTING

23 Prepare 50 ng/µl dilution of the mRNA (Step 19) in EmbryoMax® injection buffer for later use.

CRITICAL STEP It is advisable to test new batches of the EmbryoMax® injection buffer for

accidental presence of RNase (see **Reagent Setup**) as the manufacturer does not guarantee that it

is RNase-free. **Fig. 2,** lane 2 shows the result of an injection buffer test without visible signs of

mRNA degradation.

Pause Point The *in vitro* synthesized mRNA and its 50 ng/µl dilutions can be stored at -80 °C until

use.

24 Thaw the 50 ng/µl mRNA solution immediately before the preparation of the microinjection

mixture.

CRITICAL STEP Repeated thawing and freezing of mRNA stocks may cause mRNA degradation.

Preparation of the microinjection mixture ☐TIMING 1h

CRITICAL In order to prepare the final microinjection mixture, specific amounts of SB100X mRNA

and transposon donor plasmid have to be mixed to reach their optimal final concentrations. For

both mice and rats, the best working injection mixture contains 0.4 ng/µl transposon donor plasmid and 5 ng/µl SB100X mRNA[60].

25 Prepare 2X concentration solutions: 0.8 ng/µl of transposon donor plasmid (from the stock solution prepared in Step 12) and 10 ng/µl of transposase mRNA (from the stock solution prepared in EmbryoMax® injection buffer in Step 23) in EmbryoMax® injection buffer.

26 Mix the 2X concentration solutions at 1:1 ratio to create the final microinjection mixture. Prepare 5-10 µl aliquots of the final microinjection mixture.

Pause Point Aliquots of the microinjection mixture may be stored at -80 °C for a couple of months.

Mouse transgenesis: Preparation of the ovum donors ☐**TIMING** 3 days

CRITICAL Mouse transgenesis is described in Steps 27-39.

CRITICAL The staff of a transgenic lab and animal facility need to have substantial experience in reproductive biology, methods of assisted reproduction, cell culture techniques, mouse genetics and molecular biology.

CRITICAL The animals need to be maintained and treated under conditions which are suitable in terms of the hygienic quality. The use of healthy animals in SPF (specific pathogen free) or SOPF (specific opportunistic pathogen free) conditions will assure the best results regarding efficacy of the method and allows for the possibility of unrestricted distribution of the resulting founders to other facilities.

CRITICAL The donor strain has to be selected according to the appropriate genetic background for the transgene and the ability for efficient superovulation. Inbred strains are preferred, because they have a defined genetic background. However, they are generally less efficient in all crucial aspects of production of ova, microinjection and embryonic development than outbred or hybrid animals.

27 Apply hormonal treatment to increase the number of ova per donor. Superovulate mice by intraperitoneal (i.p.) application of 5 IU PMSG and 46-48 h later (directly before mating) with 5 IU hCG between 9 a.m. and 10 a.m. (**Fig. 3**).

28 Pair mice for mating. Put females into the male's cage for mating and not vice versa. Putting

males in a new cage may induce exploration behavior and delay the start of copulation. The

optimal dosage of both hormones can vary depending on the mouse strain used and the age

of the animals.

CRITICAL STEP The most appropriate age of donors of different genetic backgrounds needs to be

empirically determined. For example, F1-hybrid and BALB/c females are used as donors as young

adults at about 7 weeks of age. In contrast, the superovulation of C57BL/6 females is most efficient

as juveniles during their prepubescent stage of development.

? TROUBLESHOOTING

Isolation of ova ▢TIMING 1 hour

29 Equilibrate M16 medium in the incubator (at 37 °C, 5% CO_2) for about 12 h before use. Pre-

warm M2 medium, mineral oil, glass slides and diluted hyaluronidase solution on a warming

plate (37 °C) before use. Pre-warm injection anesthetics, analgesics and eye ointment to

room temperature.

30 In the morning of the day intended for embryo transfer to the recipient mice, harvest the ova

from dissected oviducts of donor mice by flushing with M2 medium about 23 h after hCG

application and mating (**Fig. 3A**). At this time the ova are still in or partly released from the

cumulus complex and both pronuclei are clearly visible.

? TROUBLESHOOTING

31 Remove the remaining sticky cumulus cells of the flushed ova by incubation in the 1 mg/ml

hyaluronidase solution for 3-5 min in a Petri dish. Examine the size of the pronuclei under the

microscope, and store the ova in M2 medium on a warming plate until the pronuclei are of the

appropriate size for injection (about 15 μm in diameter) (Step 34). The optimal time window

for pronucleus injection lasts about 3 h from when the male pronucleus reaches 15 μm in

diameter to shortly before the disappearance of the fully-developed pronuclei. The diameter

of the pronucleus can be estimated by comparing it to the diameter of the ova (which is about 80 μm). We recommend implementation of all steps of ova preparation in a laminar flow bench with a 37 °C heated surface.

CRITICAL STEP Dissect the oviduct and flush the ova in cumulus complexes immediately after sacrificing the donor female. Avoid changes of osmolarity due to evaporation of the medium during culture or injection. The resulting shrinking of the ova will complicate microinjection.

Microinjection of ova □**TIMING** 2-3 hours

CRITICAL RNA is very unstable and sensitive to ribonucleases. Use gloves during all steps of handling the injection cocktail. All materials including pipette tips, injection buffer, reaction tube, DNA preparations and injection capillaries have to be clean and free of ribonucleases.

32 Place about 30 ova into the northern part of a small drop of M2 medium in the injection chamber (see **Equipment Setup**) covered by pre-warmed mineral oil.

33 Keep the transposon DNA/transposase mRNA-injection mixture from Step 26 at 4 °C during usage. Fill the injection capillary with approx. 1 μl injection mixture using a microloader or by inverting the capillary into the injection buffer and allowing it to fill-up by capillary action. Ensure that the tip of the injection capillary is open.

CRITICAL STEP Ova are not adherent to the surface of the slide and therefore need to be fixed by a holding capillary for injection.

34 By using an inverted microscope and micromanipulator, select the paternal pronucleus based on its larger size, and fix the ovum with the holding capillary in such a way that the pronucleus is located near the horizontal axis of the ovum (**Fig. 4**). Insert the injection capillary into the pronucleus and inject until the pronucleus visibly swells (about 1-2 pl). The diameter of the pronucleus will increase from about 15 μm to 20 μm). Since the plasma membrane of a mouse ovum is extremely elastic, penetration usually does not occur until the tip of the injection capillary is inside the pronucleus.

? TROUBLESHOOTING

35 Move each ovum after successful microinjection into the southern part of the oil-covered M2 drop until all ova are treated.

CRITICAL STEP Further details about pronuclear injection can be found in refs. 72 and 73.

? TROUBLESHOOTING

Preparation of surrogate mothers □TIMING 1-3 days

36 Mate surrogate females monogamously or bigamously to vasectomized, sterile males of the same outbred stock as the females (alternatively, mutant strains expressing male infertility and asthenozoospermia can be used if their mating behavior is proved to be normal and if they produce a vaginal plug) in the afternoon of the day before microinjection (**Fig. 3A**), to produce recipients for the injected embryos on the next day.

CRITICAL STEP Avoid pairing mice too early (e.g. in the morning) because any female mouse that had undergone estrus on the previous night could immediately mate and may lose the vaginal plug by the next morning. Since the presence of a vaginal plug is a marker for a successful mating, this could result in undetected pseudopregnant females. Calculate the number of matings necessary to provide the required number of surrogate mothers. The estrus cycle of mice is about 4 days and therefore 25 % of mated females are expected to have a vaginal plug. To increase the frequency of plugged females, they can be stimulated 48 h before mating by exposure to male pheromones (Whitten effect)[74]. This can be done with dirty bedding from the stud male's cage.

Embryo transfer □TIMING 2 hours

37 Identify pseudopregnant recipients by vaginal plug control on the next morning after mating. Since the microinjection is performed in the morning, injected ova can be re-transplanted in the afternoon of the same day (**Fig. 3A**). If not enough surrogate mothers are available, the remaining ova can be cultured overnight in equilibrated M16 medium in an incubator and re-transplanted the next morning as 2-cell stage embryos into 0.5 dpc pseudopregnant females. Use the instruments shown in **Fig. 5A** for embryo transfer.

38 Anesthetize the pseudopregnant female with 10 mg/100 g body weight of Ketamine (Ketasol) and 0.4 mg/100 g body weight of Xylazine (Xylasol) (**Fig. 5B**). Other anesthesia protocols are possible depending on the availability of appropriate equipment (inhalation anesthesia) or local regulations[75].

CRITICAL STEP Perform embryo transfer (following step) only after the animal has reached surgical tolerance under general anesthesia. Surgical tolerance is tested by the withdrawal reflex after pinching the hind foot. In order to avoid hypothermia, place the animals on a warming plate during surgery and until recovery from the anesthesia. Cover the eyes with eye ointment to protect them from drying.

CRITICAL STEP Pseudopregnancy can be confirmed by the swollen ampulla.

39 Transfer microinjected ova or two-cell stage embryos via the infundibulum into the oviduct as shown and described in **Fig. 5C-K**. Although there is no transmigration of mouse embryos from one uterine horn into the other, we recommend unilateral embryo transfer into the right oviduct. Transfer 20-30 injected embryos per female: too few offspring can result in bad brood care and infanticide by the mother. Further details about embryo transfer in mice can be found in refs. 73, 76 and 77.

CRITICAL STEP In order to avoid pain after surgery, implement an appropriate analgesic treatment for example by subcutaneous injection of 0.05 mg meloxicam/100 g body weight.

CRITICAL STEP Avoid exposing surrogate mothers to pheromones of an unfamiliar male for at least the first 4 days after embryo transfer. Exposure would have a drastic impact on the female's reproductive state, and may result in termination of the early pregnancy and return of the estrus cyclicity (Bruce effect)[78].

? TROUBLESHOOTING

Rat transgenesis: Preparation of vasectomized males □**TIMING** 14 days

CRITICAL Rat transgenesis is described in Steps 40-74.

40 Anaesthetize male rats from outbred Long Evans stock (80 to 90 days old) with i.p. injections of ketamine (80 mg/kg body weight) and xylazine (4 mg/kg body weight).

41 Shave lower abdomen with clippers and sterilize the shaved region with 70 % (vol/vol) ethanol. With a pair of curved forceps and scissors, make a cut across the lower abdomen (the incision in the midline wall should be about 1 cm wide).

42 Using blunt forceps and scissors to separate the *vas deferens* from the surrounding tissue, gently grasp the *vas deferens* with the forceps, lift it out of the incision, and cut off the section in the loop of the *vas deferens*.

43 Return the *vas deferens* gently back inside the abdominal cavity, and repeat the procedure on the other side. Sew the incision in the body wall with separate stitches and close up the skin with three Michel clips.

44 Keep rats in a warm place, supervise and monitor them until they are recovered from anesthesia. Allow males to recover for two weeks before using them for inducing pseudo-pregnant females.

Superovulation of female donors ☐**TIMING** 3 days

45 For stimulating of ovum donors for microinjections, select a group of 6-8 female rats (40 to 90 days old) and inject them by an i.p. injection of 20 IU of PMSG (SERGON) in 0.4 ml of diluted gonadotrophin (see **Reagent Setup**) between 9 a.m. and 10 a.m. (**Fig. 3B**).

46 For ovulation and fertilization, inject 25 IU hCG i.p. (Pregnyl) in 0.5 ml of diluted gonadotrophin) (see **Reagent Setup**) 48-49 hours after SERGON injection (**Fig. 3B**), transfer the injected rats individually into cages with a fertile male and mate them overnight.

47 On the next morning, between 9 a.m. and 10 a.m., check rats for vaginal plugs; mark positive rats on the tail and place them into clean cages.

CRITICAL STEP The day of plug observation is Day 1 of pregnancy.

Recovery and manipulation of fertilized ova ☐**TIMING** 1 hour per 8 superovulated donors

48 Sacrifice the superovulated (donor) females with detected vaginal plugs (Step 47) by cervical dislocation on Day 1 of pregnancy, between 2 p.m. to 4 p.m. (**Fig. 3B).** Sterilize their abdomen with 70 % (vol/vol) ethanol and open the abdominal cavity with scissors.

49 Hold the proximal part (about 1 mm) of the uterine horn with forceps and dissect the oviduct with the help of fine scissors. Wash the oviduct in 300-400 µL of M2 medium (warmed to room temperature) and transfer it into a 100 µL drop of M2 medium placed on the lid of a 60 mm sterile tissue culture Petri dish (each oviduct in its individual drop of M2 medium, place 4 oviducts separately on one lid).

50 Place two 200 µL drops with hyaluronidase solution besides each drop with oviducts and transfer the Petri dish lid with oviducts under a dissecting microscope. Fix the ampulla portion of the oviduct with fine forceps and tear it open with second fine forceps.

51 Release cumulus complexes with ova, transfer them (with a wide manipulation capillary) into the drop with hyaluronidase and incubate cumulus complexes in this drop for 3-5 minutes (incubate all cumulus complexes from one donor in one drop with hyaluronidase).

52 With the help of gentle pipetting (with a narrow manipulation capillary) free ova from the cumulus cells, wash them in fresh M2 medium and transfer all fertilized ova into 5 µL drops of M2 medium placed on the bottom of the injection chamber.

53 Place the microinjection chamber with ova under the inverted microscope (Olympus IX70, equipped with DIC optic, 10x eyepieces, 5x, 20x and 40x objectives). Record the number of fertilized ova, showing two pronuclei and a 2nd polar body.

54 Place the injection chamber with ova under the stereomicroscope, transfer a group of ova collected from a single donor from the M2 medium into a drop of CO_2-preincubated G-IVF PLUS medium at room temperature, wash them by repeated pipetting in this drop, and transfer them into a well (containing 300 µL of CO_2-preincubated G-IVF PLUS medium) of a 4-well plate.

55 Keep the ova in the incubator in 6% CO_2 at 37 ^{o}C for 1 to 4 hours until microinjection. Microinjection is done between 4 p.m. and 8 p.m. on Day 1.

Pronuclear injection ☐TIMING 1-3 hours per 100-200 ova

56 Place a 5 µl drop of M2 medium in the center of the microinjection chamber, and cover the drop with mineral oil.

57 For DNA microinjection, transfer a group of 25-30 fertilized ova from the 4-well plate into a 300 µL drop of M2 medium, placed on the lid of a 60 mm Petri dish, wash them and place them in the M2 medium drop in the microinjection chamber.

58 Mount the chamber with ova under the inverted microscope equipped for microinjection (Olympus IX70 with DIC-optic, Narishige micromanipulators, FemtoJet injector and CellTram Air for operation of the holding microcapillary).

59 Fill the injection capillary with 1 µL of injection mixture (Step 26) with the help of a microloader.

60 Install holding and injection capillaries into basic position close to each other in the very center of the drop of M2 medium situated in the center of the chamber and with all ova in the lower half of the drop (carry out the installation at magnification 10x eyepieces and 5x objective).

61 Fix a selected fertilized ovum showing distinct pronuclei and a 2nd polar body to the holding capillary under the DIC-optic (magnification 10x eyepieces and 20x objective), and microinject the ovum (**Fig. 6A**) so that the volume of the injected pronucleus visibly increases. Microinjection is done between 4 p.m and 8 p.m.; inject 100-200 ova per session (**Fig. 3B**).

CRITICAL STEP Injection capillaries are changed whenever the process of microinjection is out of order (clogged or broken tips, increased number of lysed ova, remnants of cytoplasm or pronuclear membranes on the surface of microinjection capillaries).

62 Collect all injected ova in the upper half of the working field (**Fig. 6A**), while the rest (unfertilized ova and all abnormal and degenerated ova) is left in the lower half of the field.

? TROUBLESHOOTING

In vitro culture of injected ova ▢TIMING 15-20 hours

63 Transfer the injection chamber with injected ova under the stereomicroscope and collect all

surviving injected ova with a manipulation capillary.

64 Transfer and wash the ova in a drop of CO_2-preincubated G-IVF PLUS medium at room

temperature, and place them into a well (containing 300 µL of CO_2-preincubated G-IVF PLUS

medium) of a 4-well plate.

65 Insert the 4-well plates into the incubator in 6% CO_2 at 37 ^{o}C and keep the injected ova there

for 15 to 20 hours.

Preparation of pseudopregnant recipients (surrogate mothers) ▢TIMING 1 day

66 Use sexually mature Wistar female rats (90-120 days old) as foster mothers. On the day of

microinjection (Step 61), check 10 to 40 female rats for the stage of the estrus cycle by

vaginal flushing (between 1 p.m. and 2 p.m., **Fig. 3B**), and place those showing strong sign of

proestrus (mass of nucleated epithelial cells typical for proestrus) into the cages together with

two vasectomized Long-Evans males overnight.

67 On the next morning, between 9 a.m. and 10 a.m., use only the rats showing vaginal plugs as

foster mothers. Use them for embryo transfer on the same day as when vaginal plugs are

detected (between 10 a.m. and 11 a.m., **Fig. 3B**).

? TROUBLESHOOTING

Embryo transfer into recipient foster mothers ▢TIMING 1 hour per 8 recipient foster mothers

CRITICAL Embryo transfer of injected 2-cell embryos is done between 9 a.m. to 10 a.m. in order to

avoid 2-cell block. To reduce the surgery trauma only unilateral transfer into the oviduct is made.

68 Anesthetize recipient foster mother by i.p. injection of Narketan (150 mg/kg) and Xylapan (2

mg/kg), shave the abdomen (left flank, 4 x 2 cm), sterilize with 70 % (vol/vol) ethanol and

open the abdominal cavity (cut into skin and body wall, 1-2 cm).

29

69 Pull out the abdominal fat with the ovary, oviduct and proximal part of uterine horn and fix them on a strip (3 x 2 cm) of woven wound dressing soaked in saline so that the *infundibulum* is clearly visible.

70 Place the stereomicroscope in the laminar flow box, make a hole in the ovarian *bursa* (with the help of two pairs of fine forceps and avoiding bleeding) and fix the *infundibulum* on a fine strip of paper (2 mm x 5 mm) cut off from the paper towel.

71 Take the 4-well plate with injected embryos from the incubator (Step 65), transfer it under the stereomicroscope placed inside the laminar flow box, and select 2-cell embryos for the transfer into recipient foster mother.

? TROUBLESHOOTING

72 Aspirate 2-cell embryos into the manipulation capillary with narrow opening; transfer 10 to 14 embryos to one recipient foster mother, in a minimal volume of G-IVF PLUS medium, with each 2-cell embryo placed between small air bubbles.

73 Insert the manipulation capillary with 2-cell embryos into the *infundibulum* (close to the first bend of the oviduct), and gently expel the medium with 2-cell embryos into the oviduct.

74 Gently place abdominal fat with ovary, oviduct and uterine horn back into the abdominal cavity, close up the abdominal wall with a suture and the skin with wound clips. Place the foster mother in a cage and keep her in a warm place for 1-2 hours until she wakes up and is mobile.

CRITICAL STEP Avoid exposing surrogate mothers to pheromones of an unfamiliar male for at least the first 4 days after embryo transfer. Exposure would have a drastic impact on the female's reproductive state, and may result in termination of the early pregnancy and return of the estrus cycle (Bruce effect)[79].

? TROUBLESHOOTING

Genotyping of transgenic animals by confirming transposon insertions by PCR ☐**TIMING**

2.5 hours

CRITICAL If the transposon includes a visibly expressed (fluorescent) marker, early embryos and F0

offspring can be identified by fluorescence emission (**Fig. 4C** and **Fig. 6B-D**). However, transgene

integration and germline transmission must be confirmed by DNA analysis.

75 Isolate genomic DNA of F0 animals from tissue samples (see **Reagent Setup**), and set up a

PCR reaction in a 25 µl volume containing the following components:

Component	Amount per reaction	Final
PCR buffer (10x)	2.5 µl	1x
MgCl$_2$ (25 mM)	1.5 µl	1.5 mM
dNTP (10 mM)	0.5 µl	0.2 mM
Primer SB short (10 pmol/µl) (**Table 1**)	0.8 µl	8 pmol
Primer Tbal rev (10 pmol/µl) (**Table 1**)	0.8 µl	8 pmol
Genomic DNA	1 µl	~200 ng
Taq DNA polymerase	0.4 µl	2 U
H$_2$O	17.5 µl	-

76 Run the PCR reaction using the following conditions:

Cycle number	Denature	Anneal	Extend	Hold
1	94 °C, 5 min			
2–31	94 °C, 1 min	55 °C, 30 s	72 °C, 30 s	
32			72 °C, 7 min	
33				4 °C

77 Run a 5-µl aliquot of the PCR product on a 1 % (wt/vol) agarose gel. A PCR product of 201

bp in length indicates the presence of genomically integrated SB transposons. An example

result is shown in **Fig. 7A**.

Identification of individual transgene integrations by ligation-mediated PCR (LMPCR) ☐

TIMING 1-2 weeks

78 Digest 1 µg of genomic DNA isolated from F0 animals with *Bfa*I, and in a separate reaction 1

µg with *Dpn*II (**Fig. 7B**), in 50 µl final volumes. Include a negative control sample (genomic

DNA isolated from a non-transgenic animal) as well. Follow the instructions of the enzyme

supplier. To reach complete digestion, incubate the reaction for 3 hours at 37 $^\circ$C.

CRITICAL STEP Always use high quality genomic DNA as template for PCR. Good quality

genomic DNA runs on an agarose gel as a dominant, high molecular weight band (**Fig. 7C**).

79 Add 50 µl phenol/chloroform/isoamyl alcohol to each tube.

80 Vortex for 10 s and leave on the bench for 2 min. Repeat this step 3 times.

81 Centrifuge the samples at 12000 × *g* for 5 min at room temperature.

82 Transfer the top layer (~50 µl) to a new 1.5 mL tube and add 5 µl (1/10 volume) of sodium

acetate and 125 µl (2.5 volumes) of ethanol, shake well and let the digested DNA precipitate

for 30 min at -20 °C.

83 Spin down at 12000 × *g* for 15 min at 4 °C, discard the supernatant.

CRITICAL STEP The pellet is barely visible. To avoid loss of DNA, remove the liquid using a 200 µl

pipette tip by touching only the wall of the tube that faced the inner side of the rotor.

84 Wash the pellet in cold 70 % (vol/vol) ethanol. Keep the ethanol on the pellet for 10 min.

85 Spin down at 12000 × g for 15 min at 4 °C, discard the supernatant.

CRITICAL STEP The pellet is barely visible. To avoid loss of DNA remove the liquid using a 200 µl

pipette tip by touching only the wall of the tube that faced the inner side of the rotor.

86 Air-dry the pellet for 5-10 minutes and resuspend it in 20 µl sterile Milli-Q water.

87 Measure the concentration of the digested DNA using a NanoDrop® spectrophotometer. The

typical yield is between 30-50 ng/µl.

88 To check digestion run 200 ng of each sample on a 1 % (wt/vol) agarose gel. The digested

samples should run as a smear centered between 0.5-1 kb in size (**Fig. 7D**).

Pause Point The digested genomic DNA samples can be stored at -20 °C for up to 1 year.

89 Prepare the double-stranded linkers by mixing the Linker(+) oligo with the Linker(-)BfaI or with the Linker(-)DpnII oligo (**Table 1**) in separate tubes at a final concentration of 10 pmol/µl each in 100 µl TE buffer containing 50 mM NaCl.

90 Place the tubes containing the oligonucleotide solutions into a boiling water bath for 2 min, switch off heating and leave the tubes in the bath overnight to allow a slow cool down and hybridization of the two single-stranded oligonucleotides to form the double-stranded linker.

Pause Point The annealed double-stranded oligonucleotides can be stored at -20 °C for up to 1 year.

91 Ligate the *Bfa*I linkers and the *Dpn*II linkers to the corresponding *Bfa*I- and *Dpn*II-digested genomic DNA samples, respectively (**Fig. 7B**). Set up the ligation reaction containing the components below, and incubate overnight at 16 °C.

Component	Amount per reaction	Final
Ligase buffer (10x)	5 µl	1x
*Bfa*I- or *Dpn*II-digested genomic DNA	X µl	150 ng
Annealed *Bfa*I or *Dpn*II linker (10 pmol/µl)	2 µl	20 pmol
T4 DNA Ligase	3 µl	18 U
H_2O	X µl	to final volume of 50 µl

92 Set up the 1st PCR in a 50 µl reaction volume containing the components below:

Component	Amount per reaction	Final
PCR buffer (10x)	5 µl	1x
$MgCl_2$ (25 mM)	3 µl	1.5 mM
dNTP (10 mM)	1 µl	0.2 mM
Linker Primer (10 pmol/µl) (**Table 1**)	1 µl	10 pmol
Primer Tbal rev3s (10 pmol/µl) (**Table 1**)	1 µl	10 pmol
Ligated DNA (Step 91)	2 µl	-
Taq DNA polymerase	0.5 µl	2.5 U
H_2O	36.5 µl	-

93 Run the 1st PCR reaction using the following conditions:

Cycle number	Denature	Anneal	Extend	Hold
1	96 °C, 2 min			
2–11	92 °C, 40 s	60 °C -1 °C/cycle, 40 s	72 °C, 2 min	
12-36	92 °C, 40 s	50 °C, 40 s	72 °C, 1 min	
37			72 °C, 10 min	
38				4 °C

94 Set up the 2nd PCR in a 50 µl reaction volume containing the components below:

Component	Amount per reaction	Final
PCR buffer (10 x)	5 µl	1x
$MgCl_2$ (25 mM)	3 µl	1.5 mM
dNTP (10 mM)	1 µl	0.2 mM
Nested Primer (10 pmol/µl) (**Table 1**)	1 µl	10 pmol
Primer Tbal (10 pmol/µl) (**Table 1**)	1 µl	10 pmol
100x diluted 1st PCR sample	1 µl	-
Taq DNA polymerase	0.5 µl	2.5 U
H_2O	37.5 µl	-

95 Run the 2nd PCR reaction using the following conditions:

Cycle number	Denature	Anneal	Extend	Hold
1	96 °C, 2 min			
2–7	92 °C, 40 s	66 °C -1 °C/cycle, 40 s	72 °C, 1 min	
8-21	92 °C, 40 s	59 °C, 40 s	72 °C, 1 min	
22			72 °C, 10 min	
23				4 °C

96 Run a 10-µl aliquot of the PCR product on a 1 % (wt/vol) agarose gel. An example result is

shown in **Fig. 7E**. Each band represents a transposon (transgene) genomic integration.

? TROUBLESHOOTING

97 If strong, distinct bands are visible, isolate them from the gel using the QIAquick Gel

Extraction Kit according to the manufacturer's instructions, and sequence them. Multiple

bands often represent multiple integrations and lower intensity bands may represent mosaic

integrations - all of which need to be isolated from the gel, subcloned and sequenced.

98 Map the insertion sites by a BLAT or BLAST search of the DNA sequence directly flanking

the transposon, at the UCSC Genome Bioinformatics website (http://genome.ucsc.edu/cgi-

bin/hgBlat) or at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). One should be able

to identify the TA target dinucleotides immediately flanking the ITR in the genomic sequence,

and the *Bfa*I and/or *Dpn*II recognition sites where the LMPCR linkers were ligated. The PCR

amplifications applied in parallel on the *Bfa*I- and *Dpn*II-digested DNA methods help the user

to recover all integrations.

Tracking individual transgene integrations by locus-specific PCR ▢**TIMING** 1 week

99 Design primers matching the integration loci mapped in the founder mice (step 98). Avoid

designing primers that would bind to repetitive elements and thus amplify non-specific PCR

products. The BLAT search at the UCSC Genome Bioinformatics website directly provides a

RepeatMasker annotation of the genomic loci where the SB transposons have integrated.

When using BLAST at the NCBI website select "map viewer" for a given BLAST hit, then

select "maps & options" and choose "repeats" to see the RepeatMasker annotation. After

identification of a genomic region free of repetitive sequences in the neighborhood of the SB

ITR, design the locus-specific primer so that its T_m is between 55-60 ^{o}C and its length is

between 20-25 nt long. Run a BLAT or BLAST search with the new primer sequences to

make sure that they do not bind to other genomic locations. In addition, general rules for PCR

primer design can be found for example at

http://www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html.

100 Perfom the locus-specific PCR with the primer designed in Step 99 and primer Tbal (**Table

1**) to trace specific transgene integrations by the presence or absence of an amplified

product. To maximize specificity of primer annealing to the genomic target, the use of touchdown PCR is recommended consisting of 5-10 touchdown cycles stepwise decreasing the annealing temperature by 1 ^{o}C per cycle down to the final annealing temperature, at about 2 °C below the T_m of the lower T_m primer, and 25 additional standard cycles. **Fig. 8** shows an example of locus-specific PCR test of a rat founder and its F1 descendants.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

Table 2| Troubleshooting table.

Step	Problem	Possible reason	Possible solution
Step 22	Smear is detected in the size range lower than 800 bp.	RNase contamination occurred during or after *in vitro* mRNA synthesis.	Change solutions and maintain RNase-free conditions.
	More than two bands are detected.	Different length products of the *in vitro* mRNA synthesis	Consult the troubleshooting instructions of the mMessage mMachine® T7 kit.
		The mRNA runs aberrantly due to the formations of secondary structures	
Steps 27, 30	Small numbers of ova or unfertilized oocytes despite superovulation of the donors.	Hormone treatment failed or is suboptimal.	Prepare fresh hormone solutions and store up to 4 weeks at -20°C, if necessary up to 6 months at -80 °C until use.
		Age of animals is suboptimal.	Use females and males of appropriate age as described.
			Use experienced males. However, males of the inbred strain C57BL/6 are often bad breeders if their age exceeds 6 months.
		External disturbances to the mice during preparation of ova.	Avoid cage changing or other activities during superovulation and mating of donors.
Step 34	No visible swelling of the injected pronucleus.	Injection capillaries clogged.	Remove remaining particles from the injection mixture by centrifuging for 10 min at 16.1 x *g* at 4 ^{o}C, and carefully transfer

| | | | the upper part of the injection solution to a fresh 1.5 mL tube (Steps 25-26).

Pull new, clean injection capillaries. |
|---|---|---|---|
| Steps 35, 62 | High rate (over 20 %) of lysed ova immediately or shortly after DNA microinjection. | Shrinking due to suboptimal culture conditions during microinjection, excessive injection volume, or poor quality of injection capillaries. | Optimize culture conditions during the injection procedure, reduce the injection pressure to enable better control of injection volume, or change the injection capillaries. |
| Steps 39, 74 | Surrogate mothers do not become pregnant. | Surrogate mothers were stimulated with vasectomized males in an inappropriate phase of the estrus cycle. | The presence of a vaginal plug does not ensure psuedopregnancy. Therefore, pseudopregnancy must be confirmed by inspection of the ampulla, the ovaries and ovarian bursa (*corpus hemorragicum* and blood vessels, respectively) during embryo transfer. Recipients can also be pre-selected by vaginal cytology to select females in proestrus for mating. |
| | | Induction of a pregnancy block by exposure to the pheremones of a foreign male (Bruce effect). | Avoid exposing recipients to foreign male phermones during early pregnancy. |
| | | Failure/inefficient establishing of surrogate mothers. | Confirm pseudopregnancy of plugged surrogate mothers by the swollen ampulla.

Avoid exposure of surrogate mothers to olfactory cues of foreign males. |
| Steps 39, 74 | Small litters. | Residual harmful chemicals remaining in the microinjection mixture. | Carefully washing the precipitated plasmid DNA and mRNA with 70 % ethanol (Steps 9 and 18) is important for the removal of residual phenol/chloroform.

DEPC is not tolerated by the ova. Consequently, RNase-free solutions that come in contact with the injected material should be purified by filtration.

Prepare new batches of culture media. Test media and oil for toxicity by overnight culture with ova. |
| Step 65 | High rate (over 20 %) of uncleaved ova after *in vitro* culturing of injected | Suboptimal *in vitro* culture conditions, suboptimal injection procedure, and damage of ova during manipulation *in vitro*. | Lowering the amount of DNA/mRNA mix down to 50 % of the original concentration in the injection solution (Step 26) might |

	ova.		remarkably increase the number of 2-cell embryos suitable for transfer into the foster-mothers.
Steps 77, 96	Low frequency of transgenic founders per litter.	Integrity of transposase mRNA compromised	Always use RNase-free laboratory plastic and glassware and wear gloves while handling reagents and samples to prevent RNase contamination. Do not re-use injection mixture after microinjection due to the increased risk of RNase contamination.
		Larger transgenes may cause a drop of transgenesis rates.	Increasing the amount of transposon (transgene) donor plasmid in the final injection mixture (preferably not over 2 ng/µl; Step 26) may help to increase the efficiency in case of larger transgenes.
		Transgene detection problems, e. g., because the genomic DNA template used in the PCR tests is degraded.	Always use high-quality genomic DNA for PCR, as defined in Step 78. Include a positive control (DNA from an established transgenic animal) in the PCR tests.

TIMING

Praparation of *Sleeping Beauty* transposon components for microinjection

Steps 1-13, Preparation of RNase-free transposon (ITR-flanked transgene) donor plasmid: 2-4 h

Steps 14-24, Preparation of the transposase mRNA: 10-14 h

Steps 25-26, Preparation of the microinjection mixture: 1 h

Transgenesis with *Sleeping Beauty* in mice

Step 27-28, Preparation of the ovum donors: 3 days

Steps 29-31, Isolation of ova: 1 hour

Steps 32-35, Microinjection of ova: 2-3 hours

Step 36, Preparation of surrogate mothers: 1-3 days

Steps 37-39, Embryo transfer: 2 hours

Transgenesis with *Sleeping Beauty* in rats

Steps 40-44, Preparation of vasectomized males: 14 days

Steps 45-47, Superovulation of female donors: 3 days

Steps 48-55, Recovery and manipulation of ova: 1 hour per 8 superovulated donors

Steps 56-62, Pronuclear injection: 1-3 hours per 100-200 ova

Steps 63-65, *In vitro* culture of injected ova: 15-20 hours

Steps 66-67, Preparation of pseudopregnant recipients (surrogate mothers): 1 day

Steps 68-74, Embryo transfer into recipient foster mothers: 1 hour per 8 recipient foster mothers

Genotyping of transgenic animals

Steps 75-77, Confirming transposon insertions by PCR: 2.5 hours

Steps 78-98, Identification of individual transgene integration events by LMPCR: 1-2 weeks

Steps 99-100, Tracking individual transgene integrations by locus-specific PCR: 1 week

ANTICIPATED RESULTS

The frequency of transgenic founders using SB transposon-mediated transgenesis is expected to be in the range of 50-70 % of live births in mice and 14-65 % in rats (**Fig. 7**). We found that for efficient transposition of a transposon of about 3 kb in length, using the optimized concentration of 0.4 ng/µl of plasmid and 5 ng/µl of SB100X mRNA will result in a high frequency of founders with single integration sites. Depending on the size of the transposon it may be necessary to modify these concentrations. The number of independent integration sites per genome (**Fig. 8**) can be more efficiently controlled by modifying the concentration of injected transposon donor plasmid than by modifying the concentration of transposase mRNA[61]. Lowering the concentration of transposon plasmid should lead to a decrease in multiple integrations. Transposon insertions are expected to be randomly distributed in the genome, with most transgenes located in intergenic regions. Nearly all founders produced by SB100X transposition are expected to transmit the transgene to the next generation. Although some differences in transgene expression levels and patterns between the founder lines are expected, there is no significant transgene silencing in the F0 and F1 generations.

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Competing financial interests statement

The authors declare that they have no competing financial interests.

Author contributions statement

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[CE: references 62 and 64 are sister protocols and will be published by us along with this protocol.]

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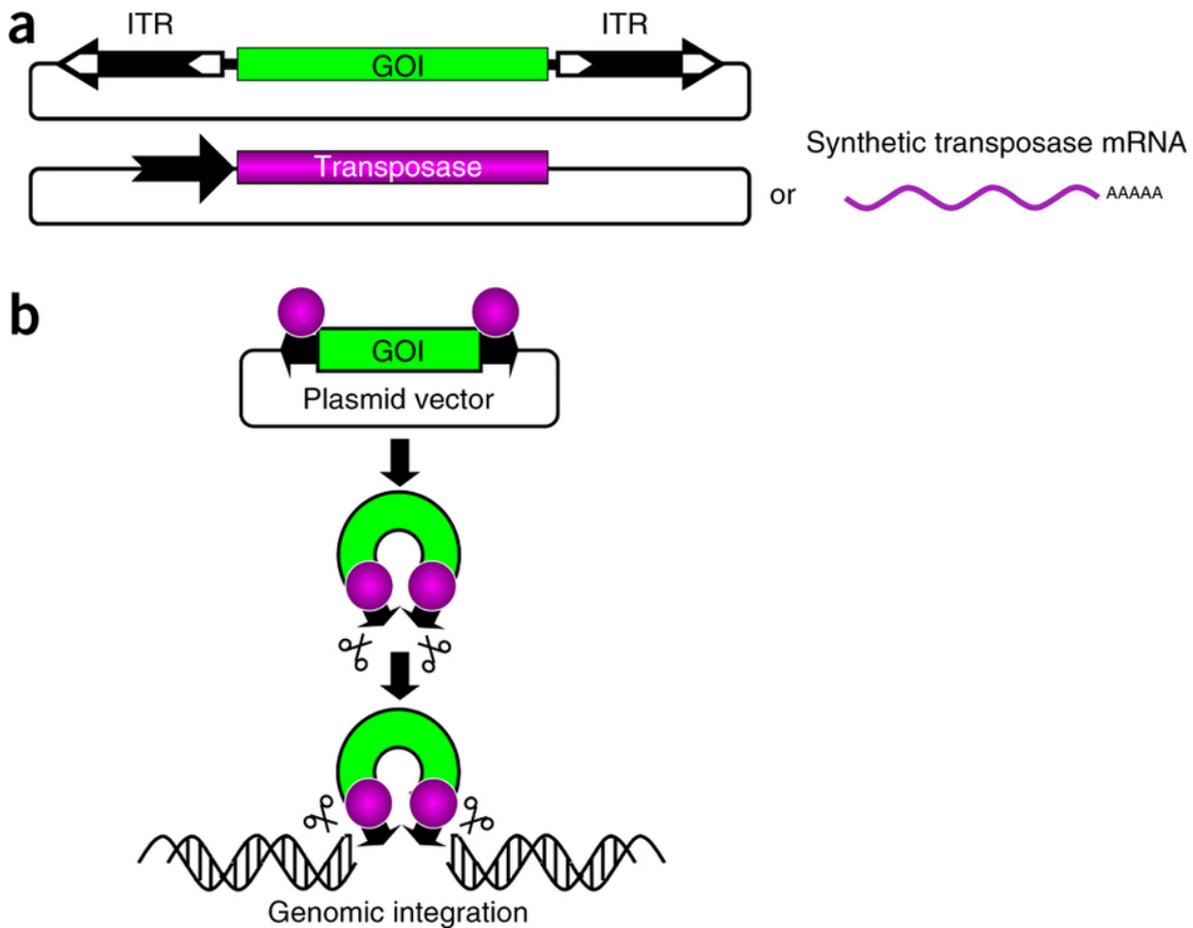
Figure 1. Application of *Sleeping Beauty* transposons for gene delivery. (**A**) A bi-component transposon system for delivering transgenes in plasmids. One component contains a gene of interest (GOI) cloned between the transposon inverted terminal repeats (ITR, black arrows) encoded by a plasmid. The other component is either a transposase expression plasmid, or synthetic mRNA encoding the transposase. (**B**) The transposon carrying a GOI is excised from the donor plasmid and is integrated at a chromosomal site by the transposase (purple spheres).
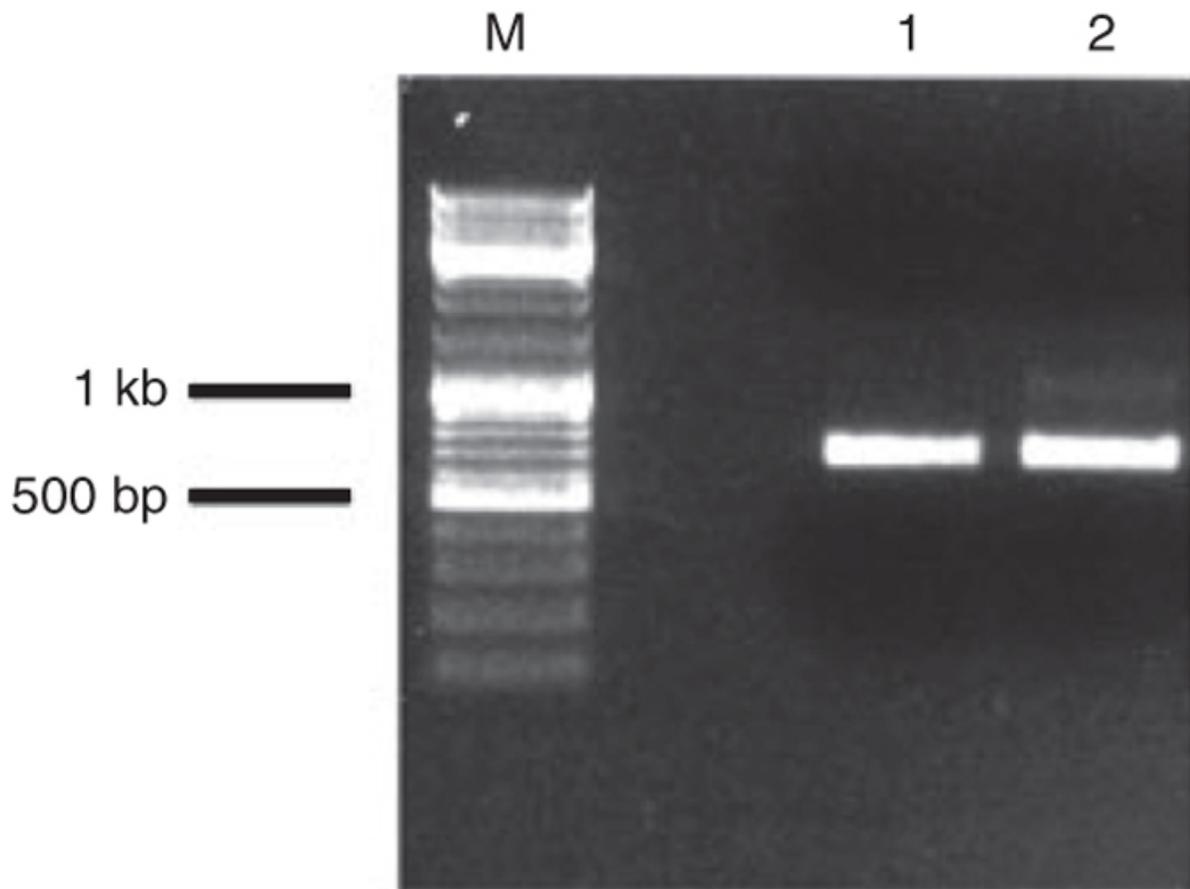
Figure 2. Result of *in vitro* mRNA synthesis and test of the microinjection buffer. mRNA quality can be determined by standard RNase-free agarose electrophoresis and ethidium bromide staining. Samples are run on a 1 % (wt/vol) RNase-free non-denaturing agarose gel. Lanes: M) DNA size marker; 1) 1 µl of *in vitro* synthesized mRNA; 2) 1 µl of *in vitro* synthesized mRNA incubated (1 h, 37 °C) in 10 µl EmbryoMax® injection buffer to detect the potential presence of RNase in the buffer (reproduced with permission from ref. [80]). Shown is a result without visible signs of mRNA degradation.
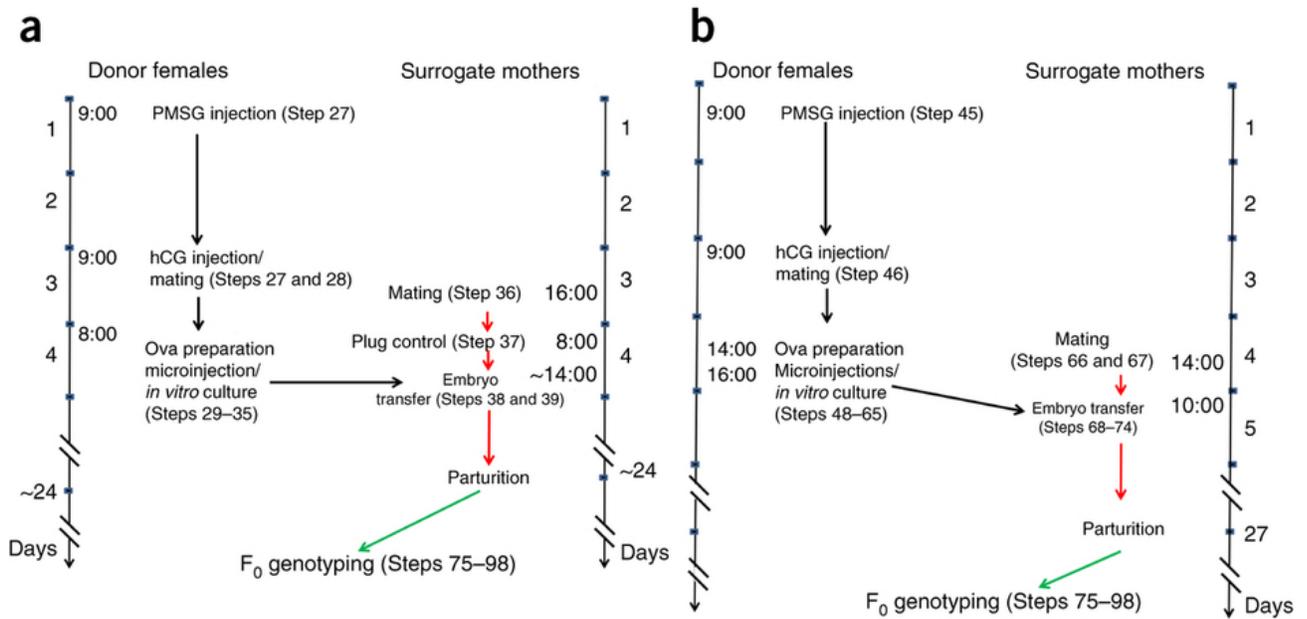
Figure 3. Timelines for animal manipulations. Timing (in days) of the important protocol steps for mice (**A**) and rats (**B**) starting from the preparation of ova donors and ending with parturition and genotyping of the F0-offspring.
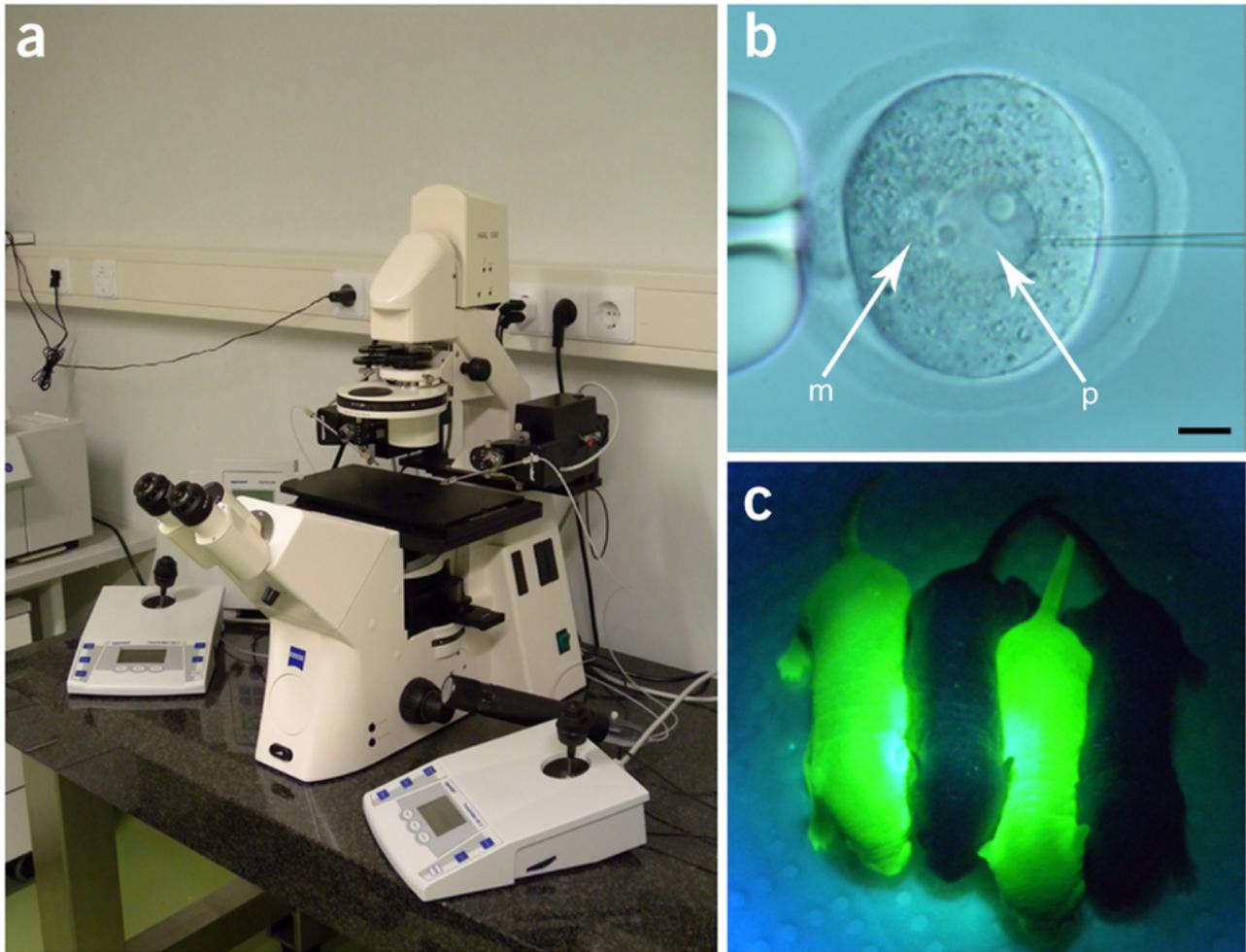
Figure 4. Overview of mouse transgenesis. (A) Inverted microscope equipped with Normarski optic (DCI), microinjectors for holding, and injection capillaries and micromanipulators on a vibration-damped microscope table. (**B**) Co-injection of the ITR-flanked transgene plasmid and transposase mRNA into the paternal pronucleus (p) of a mouse ovum fixed by a holding capillary. The maternal pronucleus (m) is visible but out of focus in this picture. (**C**) Detecting successful transposition and transgenesis among F0 offspring, expressing the *Venus* fluorescent reporter gene.
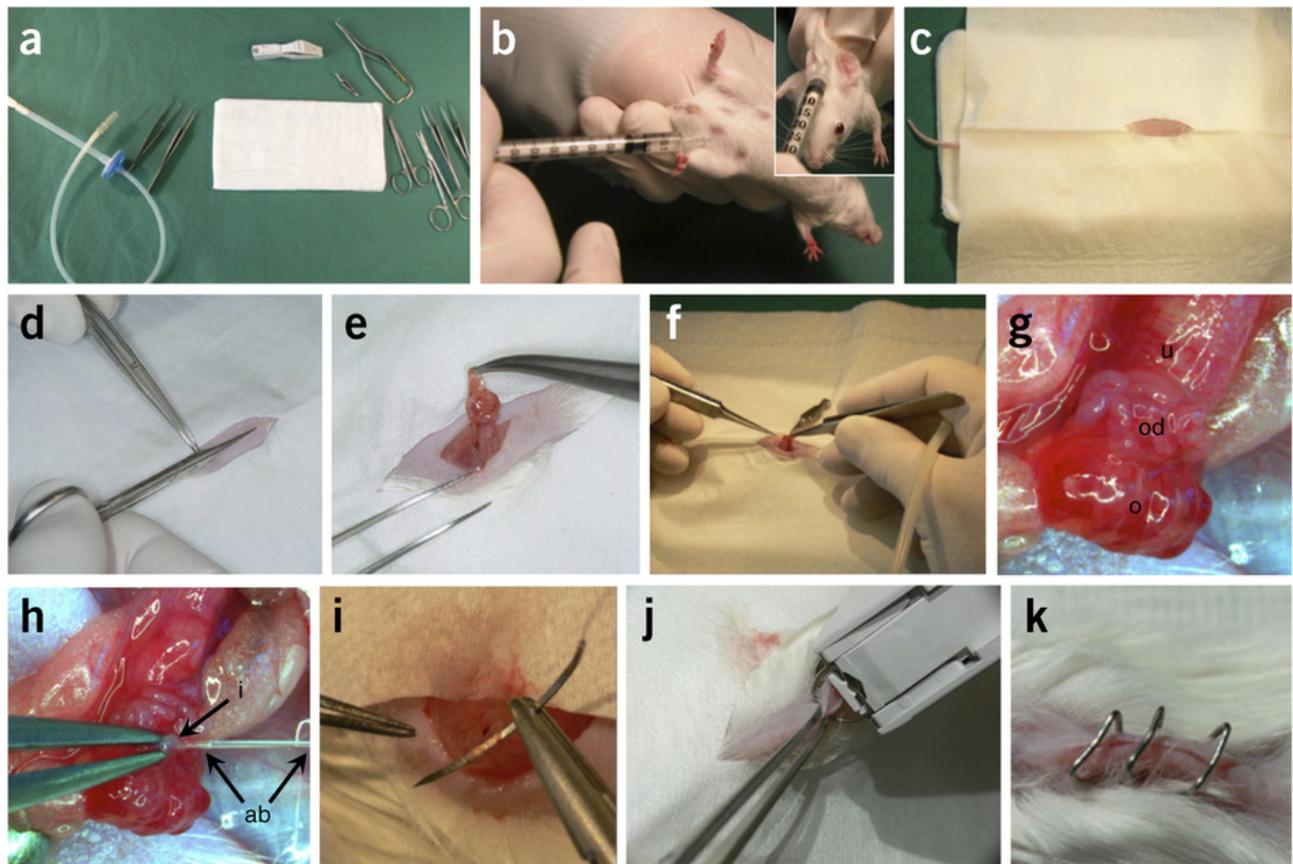
Figure 5. Equipment and steps for mouse transgenesis. (**A**) Specific equipment for the embryo transfer (2 watchmaker forceps, 2 curved dissecting forceps, sharp and blunt scissors, bulldog clamp, needle holder with absorbable atraumatic sutures, skin stapler, tubing with holder for the transfer capillary). (**B**) Intraperitonial injection of anesthetics to a pseudopregnant surrogate mother. Before she awakens from anesthesia, the mouse is treated for analgesia using a subcutaneous injection of Meloxicam (small picture). (**C**) The partially shaved and disinfected mouse in deep anesthesia prepared for surgery. (**D**) Incision of the skin about 1.5 cm-long along the dorsal midline below the last rib allows for access to both sides of the reproductive organs. Alternatively, separate incisions can be made on each flank. (**E**) Incision of the abdominal wall close to the visible ovary allows for the gentle extraction of the ipsilateral ovary, oviduct and top of the uterine horn. (**F**) The fat pad on the ovary is used to fix the female genital tract using a bulldog clamp. To reach the *infundibulum*, the *bursa* (a thin transparent connective tissue membrane that covers the ovaries) is carefully opened with two watchmaker forceps without injuring blood vessels. (**G**) Embryo transfer

is performed with a stereo microscope with about 25x magnification (o = ovary, od = oviduct, u = uterus). (**H**) Injected zygotes are transferred with a mouth controlled glass capillary directly via the *infundibulum* (i) into the oviduct. The inserted capillary is fixed with watchmaker forceps. To ensure correct transfer into the ampulla of the oviduct, the embryos in M2 medium are located between two air bubbles in the glass capillary (ab) which can be followed during the procedure. (**I**) After the transfer, the female genital tract is gently placed back into the abdominal cavity and the incision is closed with an abdominal suture. (**J**) The cut in the skin is carefully closed with suture clips. (**K**) Suture clips do not need to be removed because they are either rejected after wound healing or do not disturb the surrogate mother until weaning of the offspring.
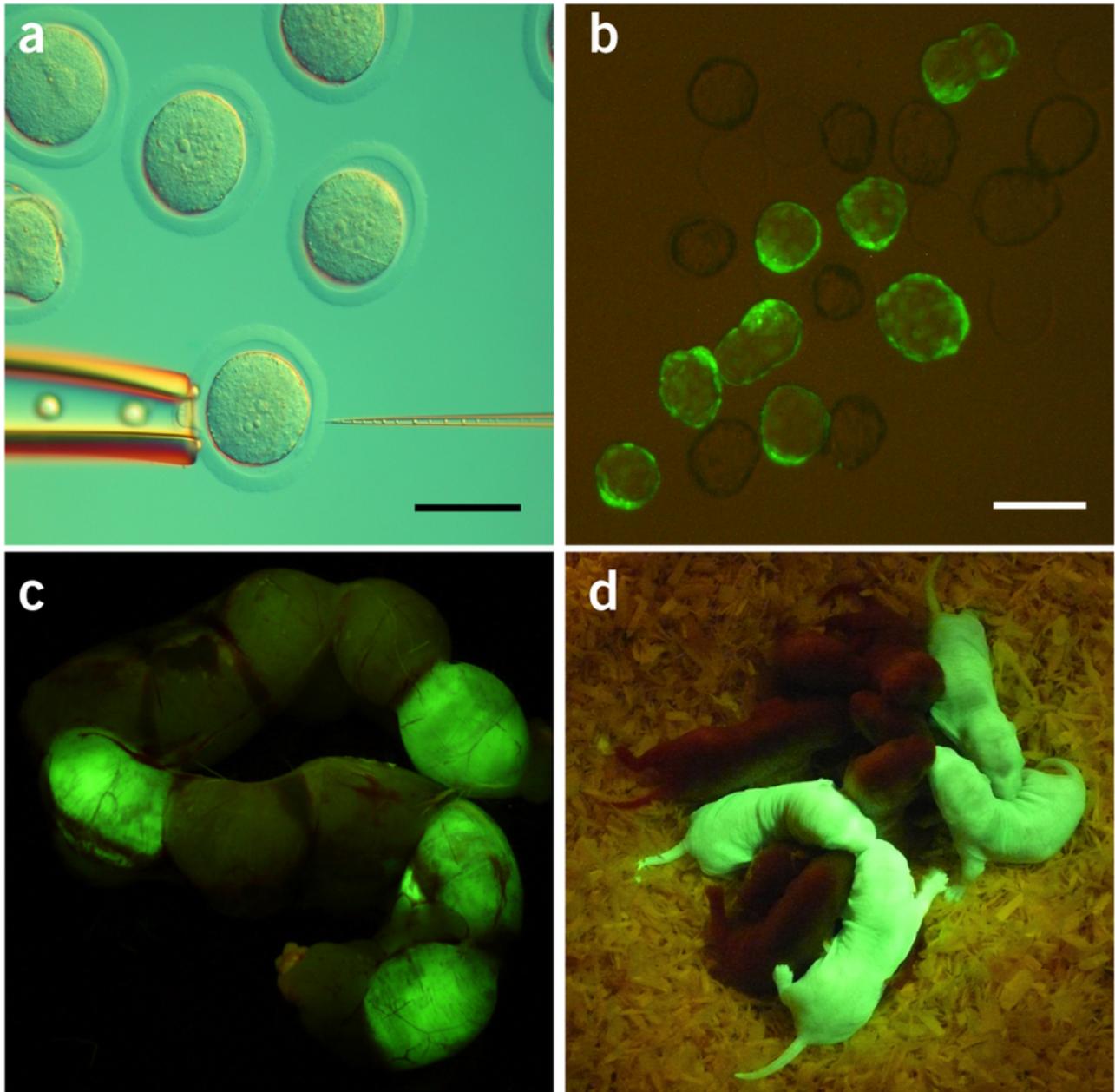
Figure 6. Derivation of transgenic rats with a *Sleeping Beauty* transposon vector encoding the *Venus* fluorescent protein. (**A**) Pronuclear microinjection of an ovum with the help of holding (left) and injection (right) capillaries; ova already injected are shown in the upper part; (**B**) Blastocysts at day 5 express the *Venus* transgene; (**C**) 14-day old fetuses expressing the *Venus* transgene; (**D**) 2-day old transgenic rats and their nontransgenic sibs.
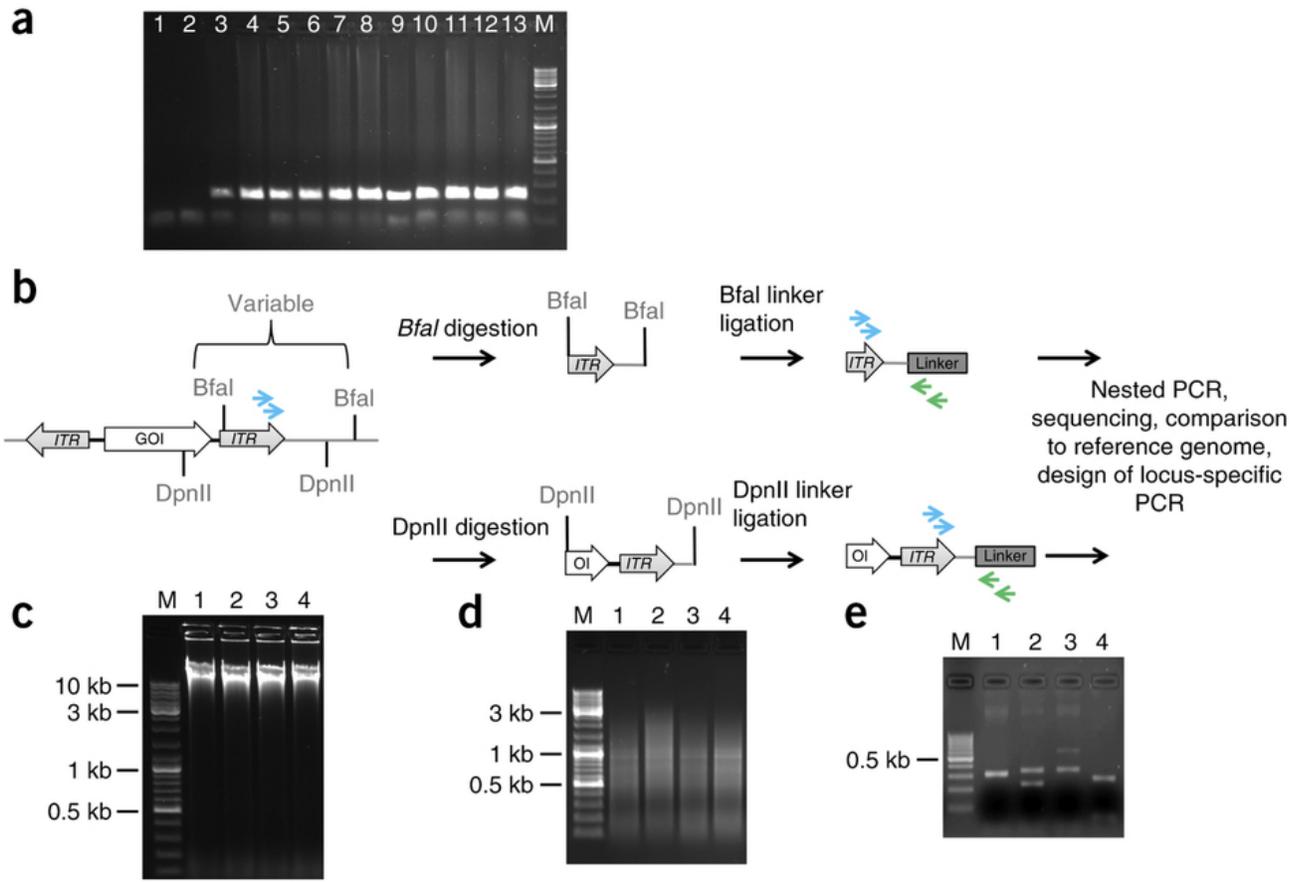
Figure 7. Identification of transgene integration by PCR. (**A**) Identification of integrated transposon sequences from genomic DNA samples by PCR with primers that amplify the left ITR of SB. Lanes: 1) H₂O; 2) mouse genomic DNA, negative control; 3) mouse genomic DNA, positive control; 4) transgenic founder #1; 5-9) F1 offspring of transgenic founder #1; 10) transgenic founder #2; 11-13) F1 offspring of transgenic founder #2; M) 100-bp molecular size marker. (**B**) Ouline of the LMPCR procedure. Digestion of genomic DNA with the frequently cutting restriction enzymes *Bfa*I and *Dpn*II and ligation of linkers with a known sequence allows for specific LMPCR amplification of transposon/genomic DNA junctions using primers specific to the transposon ITR (blue arrows) and the linkers (green arrows). Linker-to-linker amplifications are blocked by the 3' amino modifications on Linker(-) oligos (**Table 1**)[69]. Amplification products are then sequenced and compared to the reference genome. GOI, gene of interest; ITR, inverted terminal repeat. (**C**) Agarose gel with genomic DNA samples. Lanes 1-4) genomic DNA samples of rat founders, 500 ng each; M) DNA size marker. (**D**) Agarose gel with genomic DNA

samples digested with *Bfa*I restriction endonuclease. Lanes 1-4) *Bfa*I-digested DNA samples of rat founders, 200 ng each; M) DNA size marker. (**E**) Agarose gel with LMPCR products. Lanes 1-4) result of nested PCR following *Bfa*I linker ligation; M) DNA size marker.
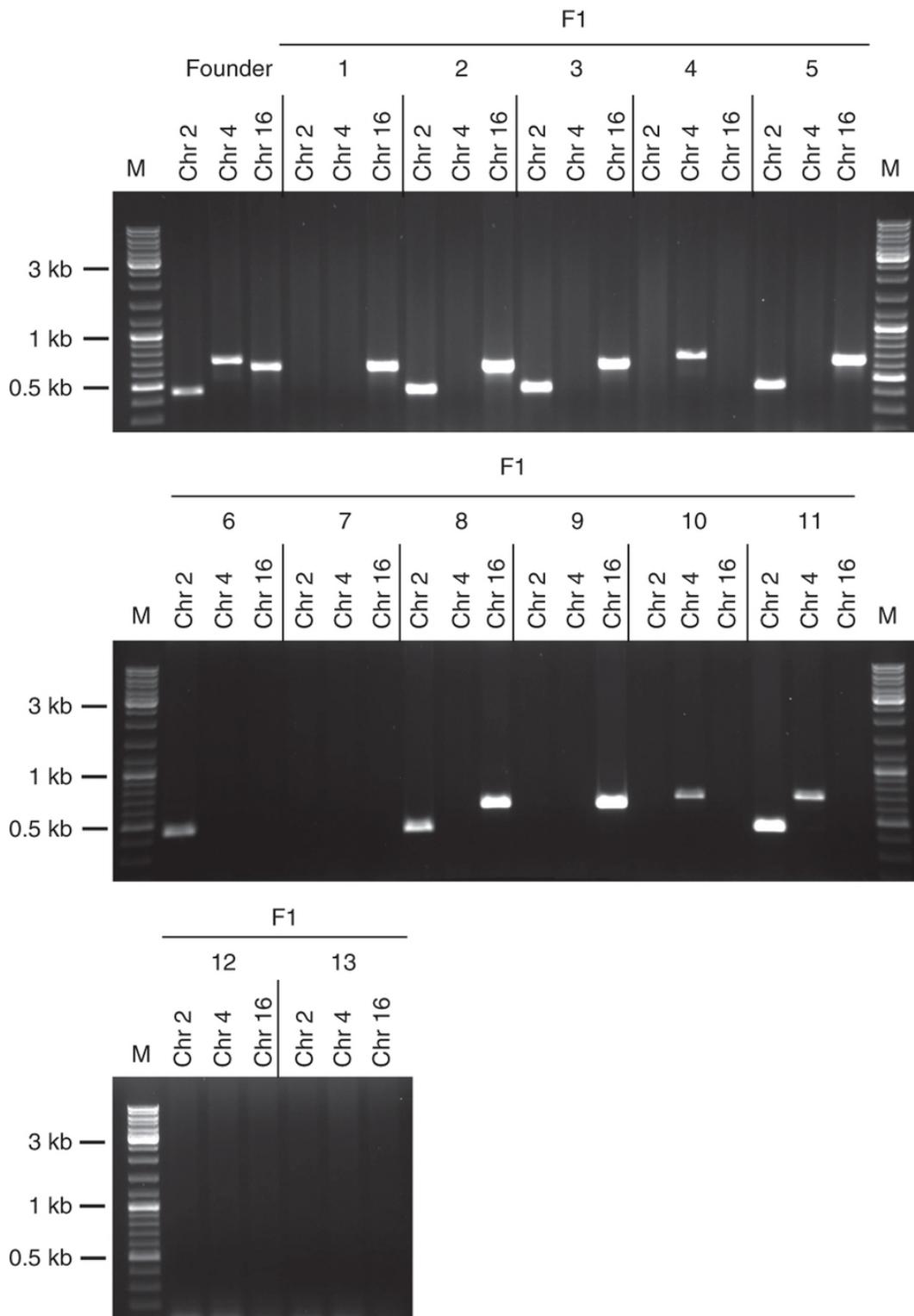
Figure 8. Locus-specific PCR test of a rat founder and its F1 descendants. The founder of these F1 rats carried three SB integrations (in chr2, chr4 and chr16), which were transmitted to the 13 descendants in different combinations. M, DNA size marker.

Table 1. Primer sequences

Oligo designation	Sequence	Description and use
SB short	5'-TACAGTTGAAGTCGGAAGTTTACATAC-3'	Transposon-specific primer used in PCR with Tbal rev. Step 75
Tbal rev	5'-GAATTGTGATACAGTGAATTATAAGTG-3'	Transposon-specific primer used in PCR with SB short. Step 75
Linker(+)	5'-GTAATACGACTCACTATAGGGCTCCG CTTAAGGGAC-3'	Annealed either with Linker(-) Bfal or Linker(-) DpnII to form double stranded linker for LM-PCR. Step 89
Linker(-)Bfal	5'-p-TAGTCCCTTAAGCGGAG-Amino-3'	Annealed with Linker(+). The 3' C7 amino modification prevents polymerase extension. Step 89
Linker(-)DpnII	5'-p-GATCGTCCCTTAAGCGGAG-Amino-3'	Annealed with Linker(+). The 3' C7 amino modification prevents polymerase extension. Step 89
Linker Primer	5'-GTAATACGACTCACTATAGGGC-3'	Linker-specific primer used in the first round of PCR with Tbal rev3s (transposon specific). Step 92
Tbal rev3s	5'-CATGACATCATTTTCTGGAATT-3'	Transposon-specific primer used in the first round of PCR with Linker Primer (linker specific). Step 92
Nested Primer	5'-AGGGCTCCGCTTAAGGGAC-3'	Linker-specific primer used in the second round of PCR with Tbal (transposon specific). Step 94
Tbal	5'-CTTGTGTCATGCACAAAGTAGATGTCC-3'	Transposon-specific primer used in the second round of PCR with Nested Primer (linker specific). Step 94