CRISPR/Cas9-mediated genome editing in mouse embryonic stem cells and direct analysis of brain phenotypes via neural blastocyst complementation

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Method Article

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Abstract

We provide a protocol for neural blastocyst complementation \(NBC), a method we have developed for studying development and function of specific forebrain regions. We describe all experimental steps from genetically modifying a locus of interest, via CRISPR/Cas9 editing in mouse embryonic stem cells \(ESCs), to generating chimeric mice with ESC-reconstituted forebrain regions that can be directly analyzed. Our _in vivo_ brain organogenesis approach is efficient, allowing functional and systematic analysis of genes \(or other factors) in as little as three months in the context of a whole organism. This protocol is related to Chang _et al._ \(DOI number, _Nature_, publishing date)

Introduction

We designed NBC to facilitate and accelerate _in vivo_ studies of the cerebral cortex and hippocampus. NBC involves molecular biology approaches and techniques involving mouse embryonic stem cells and mouse embryos that fall within the technical repertoire of laboratories addressing neurobiological and developmental questions. In this protocol, we give a detailed overview of the entire approach.

Reagents

Reagents DNA oligonucleotides \(Integrated DNA Technologies, Inc., or other suppliers) FastDigest _BbsI_ and 10x FastDigest buffer \(Thermo Scientific, cat. no. FD1014) Fast alkaline phosphatase \(Thermo Scientific, cat. no. EF0654) T4 polynucleotide kinase \(PNK) and 10x T4 PNK buffer \(NEB, cat. no. M0201S) LigaFast Rapid DNA Ligation System \(contains T4 DNA ligase and 2x Rapid ligation buffer, Promega, cat. no. M8221) 10 mM ATP \(NEB, cat. no. P0756S) Plasmid-Safe DNAse and 10x PlasmidSafe Buffer \(Lucigen, cat. no. E3101K) PureLink Quick Plasmid Miniprep Kit \(Invitrogen, cat. no. K210011) Colcemid \(KaryoMAX solution, Invitrogen, cat. no. 15212-012) Methanol \(Fisher Chemical, cat. no. A412-4) Glacial acetic acid \(Fisher Chemical, cat. no. A38-500) Potassium chloride \(Fisher Chemical, cat. no. P217-500) DAPI Fluoromount-G \(Southern Biotech, cat. no. 0100-20) Puromycin \(Gibco, cat. no. A1113803) TransIT-293 \(Mirus, cat. no. MIR 2705) Dulbecco's phosphate-buffered saline \(DPBS) \(Gibco, cat. no. 14190-144) DMEM with L-glutamine, 4.5 g/L glucose and sodium pyruvate \(Corning, cat. no. 10-013-CV) Heat-inactivated fetal bovine serum \(Atlanta Biologicals, cat. no. S11050H) Leukemia inhibitory factor \(Chemicon, cat. no. ESG1107) L-Glutamine:Penicillin:Streptomycin solution \(Gemini Bio Products, cat. no. 400-110) L-Glutamine \(Gibco, cat. no. 25030081) Non-essential amino acids \(Gibco, cat. no. 11140-050) β-Mercaptoethanol \(Sigma, cat. no. M-6250) Gelatin \(Sigma, cat. no. G-2500). Trypsin-EDTA \(0.5%, 10x) \(Gibco, cat. no. 15400-054) Polybrene \(hexadimethrine bromide, Sigma, cat. no. H9268) Breeder diet for foster females \(LabDiet, cat. no. Picolab mouse diet 20) Standard mouse diet \(LabDiet, cat. no. Prolab Isopro RMH3000) Pregnant mare serum gonadotropin \(PMSG; Biovendor, cat. no. RP1782725000) Human chorionic gonadotropin \(hCG; Sigma, cat. no. CG10-1VL) 1 M HEPES solution \(Corning, cat. no. 25-060-CI) Penicillin-Streptomycin solution \(Gibco, cat. no. 15-070-063) EmbryoMax KSOM mouse embryo medium \(Millipore, cat. no. MR-121-D) Light paraffin mineral oil \(Fisher Scientific, cat. no. 0121-4, CAS 8042-47-5) Ketamine-HCl \(KetaVed, VedCo Inc., NDC

50989-161-06) Xylazine \(AnaSed, Akorn Animal Health, NDC 59399-110-20) Eye lubricant \(Puralube vet ointment, NDC 17033-211-38) Buprenorphine \(0.3 mg/mL in 5% dextrose; NDC 12496-0757-5) **Plasmids** pX330-U6-Chimeric_BB-CBh-hSpCas9 vector \(Addgene #42230) psPAX2 \(Addgene #12260) pMD2.G \(Addgene #12259) FUHGW \(lentiviral hUbc-H2B-EGFP vector; gift from Guo-Liang Xu, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences) **Cells** γ-irradiated murine embryonic fibroblasts \(prepared in-house as per standard protocols or purchased from various suppliers) Mouse embryonic stem cells \(such as TC1; gift from Philip Leder, Harvard Medical School) 293T cells \(ATCC, cat. no. CRL-3216) **Mice** CD-1 vasectomized stud males \(purchased at nine weeks of age; Charles River Laboratories, cat. no. 24101152) CD-1 females \(purchased at six weeks of age; Charles River Laboratories, cat. no. 24101136) Homozygous _R26-DTA_ females \(The Jackson Laboratory, JAX stock no. 010527; 129Sv:C57BL/6) Homozygous _Emx1-Cre_ males \(The Jackson Laboratory, JAX stock no. 005628; C57BL/6) Heterozygous _Emx1-Cre_; hemizygous _DsRed.T3_ males \(generated from JAX stock nos. 005628 and 006051; C57BL/6)

Equipment

6-well plates \(Corning Costar, cat. no. 3516) 24-well plates \(Corning Costar, cat. no. 3526) 35-mm dishes \(Corning Falcon, cat. no. 353001) Petri dishes \(35 x 10 mm; Falcon, cat no. 351008) 60-mm dishes \(Corning Falcon, cat. no. 353002) 100-mm dishes \(Corning Falcon, cat. no. 353003) 150-mm dishes \(Corning Falcon, cat. no 351058) 50-mL conical tubes \(Sarstedt, cat. no. 62.547.100) 15-mL conical tubes \(Sarstedt, cat. no 62.554.100) 1.5-mL microcentrifuge tubes \(Sarstedt, cat. no 72.690.301) Steriflip-GP sterile centrifuge tube top filter unit \(Millipore, cat. no. SCGP00525) Stericup-GP sterile vacuum filtration system \(Millipore, cat. nos. SCGPU02RE and SCGPU05RE) Steritop threaded bottle top filter \(Millipore, cat. no. SCGPS02RE) QIAquick gel extraction kit \(Qiagen, cat. no. 28706) Amaxa 4D-nucleofector core unit \(Lonza, cat. no. AAF-1002B) with X unit \(Lonza, cat. no. AAF-1002X) P3 primary cell 4D-Nucleofector X kit S \(Lonza, cat. no. V4XP-3032) Vortex Genie 2 \(VWR, cat no. 14216-184) Superfrost Plus microscope slides \(25 x 75 mm; Fisher Scientific, cat. no. 12-550-15) Coverslips \(24 x 40 mm; Fisher Scientific; cat. no. 12-518-108B) Standard fluorescence microscope capable of visualizing DAPI-stained chromosomes Screw cap micro tube \(2 mL, Sarstedt, cat. no. 72.694.006) Parafilm M \(Sigma, cat. no. P7793) Beckman ultracentrifuge \(e.g., Beckman Optima XPN, cat. no. A94469) SW-41 Ti swinging-bucket rotor \(Beckman Coulter, cat. no. 331336) Ultra-Clear centrifuge tubes \(14 x 89 mm; Beckman Coulter, cat. no. BK344059) Countess Automated Cell Counter \(Invitrogen) or hemocytometer Kimwipes \(Kimberly-Clark Professional, cat. no. 34256) 3-mL Luer-Lok syringe \(Becton Dickinson, cat. no. BD309657) 1-mL syringe with 26-G needle \(Becton Dickinson, cat. no. BD 309597) 30-G needle \(Becton Dickinson, BD Precision Glide Needle, cat. no. BD305106) 0.45-μm syringe filters \(GE healthcare, cat. no. 6780-2504) Sterile alcohol prep pads \(Fisherbrand, cat. no. 22-363-750) Povidone-iodine swabsticks \(Medline, cat. no. MDS 093901) Suture material \(Surgical Specialties Corporation; Look Suture, cat. no. 495B) Michel suture clips \(7.5 x 1.75 mm, stainless steel; Fine Science Tools, cat. no. 12040-01) 9-in. borosilicate glass Pasteur pipette \(VWR; cat. no. 14672-412 CTPL) Borosilicate glass \(O.D. 1.0 mm, I.D. 0.75 mm; Sutter Instrument Company, cat. no. B100-75-10)

Suction mouthpiece for micropipette \(HPI Hospital Products, Medtech, cat. no. 1501P-B4036-2) Tygon tubing for micropipette setup \(1/8 in. I.D., 1/4 in. O.D., 1/16 in. wall; Tygon B44-4X) Filter unit for micropipette setup \(Millex-GP filter unit 0.22 µm, Millipore cat. no. SLGP033RB). Mayo-Hegar needle holders \(Roboz Surgical Instrument Co., cat. no. RS-7912) Microdissection forceps \(Biomedical Research Instruments, Inc., cat. no. 10-2850) Graefe forceps \(Roboz Surgical Instrument Co., cat. no. RS-5139) Stevens microdissection scissors \(Roboz Surgical Instrument Co., cat. no. RS-5940) Johns Hopkins clamp \(Roboz Surgical Instrument Co., cat. no. RS-7440) Dumont #55 forceps \(Roboz Surgical Instrument Co., cat. no. RS-5063) Nikon SMZ U 1:10 stereo microscope with ED Plan 1x objective Nikon SMZ 1500 C-DSS115 stereo microscope equipped with a Nikon P-FMD 1007009 focusing mount Nikon Eclipse TE2000-s microscope \(4x, 10x, and 20x objectives) equipped with Leitz manipulator arms Nikon T12-U microscope \(4x, 10x, 20x, and 40x objectives) with Narashige manipulator arms Tissue culture incubator \(Forma Scientific, model no. 3158) Flaming/Brown Micropipette Puller P-87 \(Sutter Instrument Company) Micro-forge MF-1 \(TPI Technical Products International) Heat Therapy Pump HTP-1500 \(Adroit Medical Systems, Inc.) Glass Bead Sterilizer \(Inotech Steri-350, Inotech Biosciences LLC, cat. no. IS-350) Anti-vibration table \(Technical Manufacturing Corporation Ametek Inc., cat. no. MICRO-g 63-541) Medical NF Grade Nitrogen \(for anti-vibration table; Airgas, cat. no. NI NF200)

Procedure

A. CRISPR/CAS9-MEDIATED GENOME EDITING IN MOUSE EMBRYONIC STEM CELLS \(ESCs) In this section, we describe our typical approach for introducing CRISPR/Cas9-mediated modifications into ESCs, by using generation of a _Doublecortin \(Dcx)_ knockout allele as an example. **A.1. Generate Cas9/sgRNA expression plasmids** The following steps for generating Cas9/sgRNA-expressing plasmids are based on protocols from Feng Zhang's lab \(MIT). **Step 1.** Design sgRNAs by entering the genomic region of interest into http://crispr.mit.edu. For example, for generating a _Dcx_ knockout allele, we designed a pair of sgRNA sequences to target genomic regions upstream of exon 2 and downstream of exon 3. _Note:_ we design at least two pairs of sgRNAs for each locus to be modified. We then generate and characterize independent ESC clones from each of these pairs. This is to reduce the likelihood of common off-target mutations that might be present in clones derived from only one pair of sgRNAs. We do not recommend using more than one pair of sgRNAs simultaneously in one nucleofection reaction as that may increase the probability of off-target genomic alterations. **Step 2.** Generate each sgRNA-encoding sequence from a pair of oligonucleotides containing overhangs \(see below) that allow direct insertion of the annealed oligonucleotide duplex into the pX330-U6-Chimeric_BB-CBh-hSpCas9 vector \(Addgene plasmid #42230). Ns indicate the 20 nucleotides corresponding to the genomic target region: Oligonucleotide 1 5’-CACCGNNNNNNNNNNNNNNNNNNN-3’ Oligonucleotide 2 3'-CNNNNNNNNNNNNNNNNNNNNCAAA-5’ **Step 3.** Digest and gel extract the pX330 vector: 1.7 µL pX330 \(0.6 µg/µL) 2 µL 10x FastDigest buffer 1 µL FastDigest _BbsI_ 1 µL Fast Alkaline Phosphatase 14.3 µL water Incubate the reaction at 37°C for 30 min, and gel purify the linearized plasmid using the QIAquick Gel Extraction Kit or a similar product. **Step 4.** Generate oligonucleotide duplex: 1 µL Oligonucleotide 1 \(100 µM stock) 1 µL Oligonucleotide 2 \(100 µM stock) 1 µL 10 mM ATP 1 µL 10x T4

PNK buffer 0.5 µL T4 polynucleotide kinase \(PNK) 5.5 µL water Incubate reaction in a thermocycler using the following settings: 37°C for 30 min, 95°C for 5 min, temperature gradient from 95°C to 20°C \(5°C/min). Once completed, dilute oligonucleotide duplexes 1:250 with ultrapure water suitable for molecular biology. **Step 5.** Ligate the annealed oligonucleotide duplex into the pX330 plasmid: 2.5 µL \(50 ng) _BbsI_-digested pX330 1 µL Annealed oligonucleotide duplex \(1:250) 5 µL 2x Rapid ligation buffer 1 µL T4 DNA ligase 1.5 µL water Mix by pipetting, centrifuge briefly, and ligate at room temperature for 10 min. **Step 6.** Treat the ligated plasmid with Plasmid-Safe ATP-dependent DNase: 11 µL Ligation reaction \(from Step 5) 1.5 µL 10 mM ATP 1.5 µL 10x Plasmid-Safe Buffer 1 µL Plasmid-Safe DNase Incubate at 37°C for 30 min. **Step 7.** Transform 2 µL of ligation reaction into competent bacteria and plate onto LB agar plates containing ampicillin or carbenicillin as per standard protocols. Incubate plates at 37°C overnight and inoculate two to four colonies for plasmid DNA miniprep the next day. Verify that the sgRNA-encoding sequences are free of mutations by sequencing the plasmids from a U6 primer \(5'-ACTATCATATGCTTACCGTAAC-3'). **A.2. Nucleofection of ESCs with CRISPR/Cas9 plasmids** _Note:_ we use the Amaxa 4D-nucleofector core unit with X unit and the P3 primary cell kit for ESC nucleofection. **Step 1.** Thaw ESCs and plate in ESC medium \(DMEM medium supplemented with 15% \(v/v) fetal bovine serum, 20 mM HEPES \(pH 7.4), 2 mM L-glutamine, 50 U/mL penicillin/streptomycin, 0.1 mM MEM non-essential amino acids, 50 µM β-mercaptoethanol, 500 U/mL LIF) onto γ-IR inactivated mouse embryonic fibroblast \(MEF) feeders. We generally start cells in one well of a 6-well plate in 4 mL of ESC medium. It is possible to scale down to smaller well plates. Check that the ESCs form colonies with distinct boundaries and display healthy morphology without signs of differentiation or cell death. ESCs will need to be fed at least daily; never let the ESC medium turn yellow. We grow ESCs at 37°C, 5% CO_2 in a standard tissue culture incubator. _Note:_ we refer readers unfamiliar with stem cell culture to the many excellent protocols \(for example[1,2]) on general aspects of mouse ESC culture and preparation of MEF feeder cells. **Step 2.** Passage the ESCs at least once before nucleofection: feed ESCs \(and one recipient well with feeder cells) by replacing medium with 4 mL of pre-warmed ESC medium about one to two hours before passaging. Rinse ESCs with 5 mL of DPBS, then add 0.5 mL of 0.05% \(v/v) trypsin-EDTA/DPBS. Incubate for 5 min at 37°C. Triturate ESC suspension gently with a P1000 pipette tip. Add 2 mL of ESC medium to inactivate trypsin. Centrifuge cell suspension at 180 x g for 5 min. Remove supernatant from cell pellet and resuspend thoroughly \(but gently) in 1 mL of fresh ESC medium. Triturate again with a P1000 pipette tip to generate a single-cell suspension. Passage ESC suspension 1:6 into the feeder well containing 4 mL ESC medium. **Step 3.** On the day of nucleofection, trypsinize ESCs and generate a single-cell suspension \(as above). Add cell suspension to a gelatinized 60-mm dish \(pre-treated for at least 15 min with 0.2% \(w/v) gelatin/DPBS) for 1 h to deplete the MEF feeder cells. **Step 4.** Prepare the nucleofection mixture: 1 µL pX330-sgRNA 1 \(1 µg/µL) 1 µL pX330-sgRNA 2 \(1 µg/µL) 100 µL Nucleofector solution P3 _Note:_ include 0.2 µg of a plasmid containing a puromycin resistance marker in the nucleofection reaction to transiently allow for enrichment of nucleofected cells. Do not linearize plasmids before nucleofection as that may result in integration of plasmid sequences into the ESC genome. **Step 5.** Collect the ESCs from the supernatant \(A.2. Step 3 above) and determine cell number by using a hemocytometer. Per nucleofection, place 2 x 10^6 ESCs into a fresh 15-mL conical tube and centrifuge cells for 5 min at 180 x g. **Step 6.** Discard the supernatant,

immediately resuspend the ESC pellet in the nucleofection mixture and transfer to a nucleofection cuvette. Place the cuvette into the nucleofector. Use program CG-104 for nucleofection. **Step 7.** Upon completion of nucleofection, remove the cells immediately from the cuvette by using the supplied plastic Pasteur pipette, and transfer to 10 mL of prewarmed ESC medium in a 15-mL conical tube. Resuspend cells and aliquot 0.2 mL, 2 mL, or 7.8 mL of cell suspension into three 60-mm dishes seeded with feeders. Return the dishes quickly to the incubator. **Step 8.** 12 h after nucleofection, remove the ESC medium completely, and add 3-4 mL of ESC medium containing 1 µg/mL puromycin for selection. Perform a full medium change every day with puromycin-containing medium \[see section A.3. for expected timeline]. **A.3. Isolation and validation of mutant ESC clones** _Note:_ monitor ESC dishes daily for appearance of positively selected clones. Depending on the growth rate of the ESC line used and the efficiency of the nucleofection, the dish seeded at the highest density \(7.8 mL) should have many colonies three to four days after nucleofection. On the dishes seeded at lower density \(0.2 mL or 2 mL), individual colonies should become visible under a microscope \(4x or 10x objective) five or six days after nucleofection. **Step 1.** To obtain an estimate of gene modification efficiency, collect genomic DNA from the plate seeded at the highest density on day three or four. Perform a genotyping PCR to determine if the desired genomic modification is present. If it is, proceed to picking clones from the dishes seeded at lower density, usually six to eight days after nucleofection. **Step 2.** ESC colonies are ready for picking when they have the approximate size of the opening of a standard P10 pipette tip. Place the ESC dish under a light microscope equipped with a 10x objective. Use a P10 pipette tip to aspirate individual ESC colonies and transfer them one-by-one to individual 96-well plate wells containing 10 µL of DPBS. When identifying colonies to pick, select those with a 3D morphology \(i.e., non-flattened) and clear, distinct edges that do not show signs of differentiation. **Step 3.** After 8 to 12 clones have been picked and transferred into the 96-well plate, add 50 µL 0.1% \(v/v) trypsin-EDTA/DPBS to each well. Incubate for 5 min at 37°C, then triturate with a P200 pipette tip to dissociate the colony. _Note:_ use a multichannel pipette to simultaneously process 8 to 12 clones. **Step 4.** Use the multichannel pipette to transfer the single-cell suspensions to a fresh 96-well plate that has been seeded with feeders and contains 200 µL ESC medium per well. Pipette gently to mix. Return plate to tissue culture incubator. **Step 5.** Repeat Steps 2-4 until the desired number of ESC clones has been collected. We typically collect at least 96 ESC clones. **Step 6.** After three to four days of culture, passage ESC clones from the master 96-well plate into two replicate 96-well plates. One plate \(gelatinized but without feeders) will be used for genomic DNA preparation; the other plate \(containing feeders) will be used to maintain the cells. **Step 7.** Isolate genomic DNA from the gelatinized 96-well plate and perform a genotyping PCR to identify positive clones that contain the desired genomic modification. **Step 8.** Expand positive clones from the 96-well replicate plate to a 6-well plate to collect genomic DNA for Southern blotting, and to freeze down ESC clones for future use, as per standard protocols. **Step 9.** Assess positive ESC clones by Southern blotting with specific probes \(see standard molecular biology protocols for details on Southern blotting and hybridization). We also routinely perform Southern blotting with probes against the pX330 plasmid backbone and the puromycin resistance plasmid to confirm that these sequences have not integrated into the genome of positive clones. **A.4. Metaphase karyotyping of ESC clones** **Step 1.** Passage ESC clones to be karyotyped into one gelatinized well of a 6-well plate \(or a gelatinized 35-mm dish). When

cells reach about 70-80% confluency, treat with 0.05 µg/mL colcemid for six hours in the tissue culture incubator. **Step 2.** Trypsinize cells \(as in A.2. Step 2) and transfer cell suspension to a 15-mL conical tube. Collect cells by centrifugation at 200 x g for 5 min. Discard supernatant and wash the cell pellet once with 5 mL DPBS. **Step 3.** Pre-warm 75 mM potassium chloride \(KCl) solution to 37°C. Add 5 mL of 75 mM KCl solution dropwise \(very slowly) to resuspend the ESC pellet while shaking the tube gently on a vortex mixer set to the lowest speed. Make sure to prevent aggregation. Flick tube after 1 mL of 75 mM KCl solution has been added, then add the remaining 4 mL of 75 mM KCl solution. Bring the volume up to 12 mL with more 75 mM KCl solution. Incubate at 37°C for 20 min. **Step 4.** Collect cells by centrifugation at 90 x g for 8 min at 4°C. Discard supernatant. Loosen the cell pellet by gently flicking the tube, then slowly \(about 1 drop per second) add 5 mL of freshly prepared, ice-cold Carnoy's fixative \[75% \(v/v) methanol, 25% \(v/v) glacial acetic acid] while shaking the tube gently on a vortex mixer set to the lowest speed. Bring the volume to 12 mL by adding more Carnoy's fixative. Collect cells by centrifugation at 90 x g for 8 min at 4°C. Discard supernatant. **Step 5.** Repeat the fixation \(Step 4) two more times, for a total of three fixation steps. On the final fixation step, do not centrifuge the cells, but store them in 12 mL of Carnoy's fixative at -20°C overnight. **Step 6.** The next day, collect cells by centrifugation at 90 x g for 8 min at 4°C. Discard the supernatant, leaving a small volume \(~0.5 mL) of Carnoy's fixative in the tube. Flick the tube to gently resuspend the pellet. **Step 7.** Use a P10 pipette tip to drop 7-10 µL of the cell suspension onto a Superfrost Plus microscope slide. Position the pipette about an inch above the glass slide for optimal metaphase dropping. Allow slides to dry overnight at room temperature. **Step 8.** Place a drop of DAPI Fluoromount-G mounting medium onto the slide and add a 24 x 40 mm coverslip. Store slides protected from light. **Step 9.** Visualize the metaphase chromosomes on a microscope under UV light. Use a 63x \(or 100x) oil immersion objective to acquire images of chromosomes. Analyze chromosome spreads in widely separated fields. Acquire at least 50 metaphase spreads and count the chromosomes. Euploid mouse ESCs contain 40 chromosomes. **B. FLUORESCENT LABELING OF DONOR ESCs** This section describes generation of stably H2B-EGFP-expressing ESCs via lentiviral transduction. **B.1. Lentivirus production** \! CAUTION: All steps involving lentiviruses must be performed in accordance with your local regulations and guidelines regarding work with lentiviral vectors. We do not comment on operational/safe work practices as they may differ between institutions. Please contact your local biosafety officer for biological agent risk assessment and biosafety operational practices prior to initiating work with lentiviral vectors. **Step 1.** Plate approximately 5.5 x 10^6 293T cells per 100-mm dish in 10 mL of 293T growth medium \(DMEM with 10% \(v/v) fetal bovine serum and 2 mM L-glutamine; add penicillin-streptomycin if desired). At time of transfection \(approximately 18 to 24 h later) cells should be at about 70% confluency. **Step 2.** Per 100-mm dish to be transfected, mix 30 µL TransIT-293 with 570 µL DMEM \(without serum or any additives) in a sterile 1.5-mL microcentrifuge tube \(see Mirus TransIT-293 manual for details). Vortex briefly or pipette to mix thoroughly. Incubate for 10 min at room temperature \(RT) while preparing the following three-plasmid mix in sterile 1.5-mL microcentrifuge tubes: _Plasmid ratios/amounts per 100-mm dish_ 4.5 µL psPAX2 \(1 µg/µL) 0.5 µL pMD2.G \(1 µg/µL) 5 µL FUGHW \(1 µg/µL) **Step 3.** Add 600 µL of TransIT-293/DMEM mix to tubes containing 10 µL of the three-plasmid mix. Mix gently by pipetting with a P1000 pipette tip and incubate for 30 min at room temperature. **Step 4.** Add

transfection mix dropwise to 293T cells. Rock dish gently back and forth while adding the transfection complexes. Place 100-mm dish inside of a 150-mm dish with a lid to prevent spills and return to tissue culture incubator. **Step 5.** 12 h after addition of transfection complexes, carefully remove the growth medium \(discard into a container with 10% bleach) and add 10 mL of fresh 293T medium. **Step 6.** 24 h after medium change, collect virus-containing supernatant \(harvest 1) and transfer to a sterile 50-mL conical tube. Collect supernatant carefully as 293T cells detach easily at this point. Gently add 10 mL of fresh 293T growth medium to the 100-mm dish and return to incubator \(inside of a 150-mm dish with lid). Store harvest 1 in a 50-mL conical tube at 4°C until harvest 2 is collected the next day. **Step 7.** 24 h after collection of harvest 1, collect supernatant \(harvest 2) and add to 50-mL conical tube from Step 5. **B.2. Lentivirus concentration by ultracentrifugation** \! CAUTION: Ultra-Clear SW41 tubes cannot be autoclaved. Sterilize by filling with 70% \(v/v) ethanol and incubating in biosafety cabinet for 10 min. Discard ethanol and let tubes air dry. _Note:_ must use SW41 Ti rotor buckets with intact seals and autoclave buckets before use. Also autoclave Kimwipes on dry cycle for later steps. **Step 1.** Centrifuge virus-containing supernatant \(pooled harvest 1 and 2) at 300 x g for 5 min, 4°C. Filter through a 0.45-µm syringe filter into a fresh 50-mL conical tube. **Step 2.** Add filtered viral supernatant to Ultra-Clear SW41 tubes \(maximum volume per tube is about 12 mL; add DPBS to fill tube to about 5 mm from top to prevent tubes from collapsing during ultracentrifugation). Weigh tubes to ensure they are balanced. **Step 3.** Centrifuge viral supernatant for 90 min at 107,000 x $g_{\(max)}$ \(25,000 RPM) at 4°C. **Step 4.** Transfer centrifuge buckets to biosafety cabinet before opening. Remove tubes from rotor and discard supernatant. Invert tubes onto autoclaved Kimwipe tissue for 1 min. Wipe tube walls with sterile Kimwipe. **Step 5.** Resuspend pellet with 100 µL DPBS by pipetting up and down about 20 times with a P200 filter pipette tip. Seal top of tube with Parafilm M, place tube inside a 50-mL sterile conical tube and incubate overnight at 4°C to let viral particles resuspend. **Step 6.** After overnight incubation, prepare 20-µL aliquots of concentrated virus suspension in sterile screw cap micro tubes and store at -80°C \(for up to 1 year). **B.3. Lentiviral transduction of ESCs** **Step 1.** Grow ESCs in ESC medium \(see A.2. Step 1) on feeder cells in a 35-mm dish to about 60-70% confluency. **Step 2.** Four hours prior to lentiviral transduction, aspirate the ESC medium and add 4 mL of pre-warmed, fresh ESC medium. Return cells to tissue culture incubator. **Step 3.** Gelatinize one 60-mm dish and three 35-mm dishes by covering them with 0.2% \(w/v) gelatin/DPBS solution. Incubate for at least 10 min in tissue culture incubator \(or for at least 20 min at room temperature in the biosafety cabinet). **Step 4.** Four hours after feeding \(Step 2), aspirate medium from ESCs, rinse cells twice with 2 mL DPBS and incubate with 0.5 mL 0.05% \(v/v) trypsin-EDTA/DPBS solution for 5 min in tissue culture incubator. Inactivate trypsin by adding 3 mL of ESC medium. Centrifuge the cell suspension for 5 min at 180 x g at 4°C. **Step 5.** Resuspend cell pellet in 5 mL of fresh ESC medium by pipetting up and down several times to obtain a single-cell suspension. Transfer dissociated ESCs to a gelatinized 60-mm dish and incubate for 30 min in the tissue culture incubator to deplete the feeder cells. **Step 6.** Carefully recover ESCs from the supernatant \(without dislodging the settled feeder cells). Pipette up-and-down several times to obtain a single-cell suspension. Determine cell number with an automated cell counter or by using a hemocytometer. **Step 7.** Plate single ESCs onto three gelatinized 35-mm dishes: 10,000 ESCs are plated per dish in 2 mL of fresh ESC medium containing 8 µg/mL polybrene. **Step 8.** Remove frozen

aliquots of concentrated virus preparation \(see B.2. Step 6) from the -80°C freezer. Quickly thaw aliquots in 37°C water bath. **Step 9.** Briefly centrifuge tube containing virus preparation \(2,000 x g, 10 s). Add 10 µL, 20 µL, or 40 µL of lentivirus preparation to each of the 35-mm dishes containing ESCs and 8 µg/mL polybrene. Gently rock the dishes to mix. Incubate cells in the incubator for 12-16 h. **Step 10.** Remove virus-containing medium from ESCs and gently rinse cells with 2 mL of DPBS. Remove DPBS and add 2 mL pre-warmed ESC medium per dish. Return cells to incubator. **Step 11.** Prepare five 60-mm dishes with feeder cells. Continue to culture the ESCs for another 24 h on the gelatinized 35-mm dishes. **Step 12.** Check EGFP expression by fluorescence microscope. Choose the dish with the highest number of EGFP-positive ESCs for further passaging. **Step 13.** Collect transduced ESCs from the selected dish by trypsinization \(as in B.3. Step 4). Resuspend ESCs in 1 mL of fresh ESC medium. Dilute single-cell suspension at ratios of 1:25, 1:50, 1:100, 1:200, and 1:400, respectively, into the five 60-mm dishes containing feeder cells and 4 mL ESC medium \(prepared in Step 11). **Step 14.** Isolated colonies should become visible three to four days later. Observe dishes under a fluorescence microscope and mark position of EGFP-positive clones on the dish with a marker pen. Pick EGFP-positive colonies into a 24-well plate with feeder cells \(see A.3. Steps 2-5 for details on clone picking). Passage the clones twice and select clones with good ESC morphology and stable EGFP expression for further experiments. **C. CULTURE AND PREPARATION OF ESCs FOR BLASTOCYST INJECTION** This section describes culture and preparation of mutant and wild-type ESCs for a given blastocyst injection day. **C.1. Culture of ESCs for injection** **Step 1.** On Day -9 \(before blastocyst injection), prepare one 6-well plate containing feeder cells. This will provide enough feeder cells for culturing the ESCs until the day of blastocyst injection. _Note:_ time frame can be adjusted and ESCs can be grown in smaller wells or dishes if desired. ESCs should be passaged at least once or twice before injection into blastocysts. **Step 2.** On Day -8, thaw a frozen vial of ESCs and plate ESCs into one well of a 6-well plate with feeder cells. **Step 3.** On Day -7 \(about 18 h after initial ESC plating) replace growth medium with 4 mL of fresh ESC medium. **Step 4.** Subculture ESCs on Day -6 \(Passage 1): feed cells by replacing medium with 4 mL of pre-warmed ESC medium about one to two hours before passaging. Also replace medium on one well with feeder cells with 4 mL of fresh ESC medium. **Step 5.** Rinse ESCs with 5 mL of DPBS, then add 0.5 mL of 0.05% \(v/v) trypsin-EDTA/DPBS. Incubate for 5 min at 37°C. Triturate ESC suspension gently with a P1000 pipette tip. Add 2 mL of ESC medium to inactivate trypsin. Centrifuge the cell suspension at 180 x g for 5 min. **Step 6.** Remove supernatant from cell pellet and resuspend thoroughly \(but gently) in 1 mL of fresh ES cell medium. Triturate again with P1000 pipette tip to generate a single-cell suspension. Passage ESC suspension 1:6 into the feeder well containing 4 mL ESC medium. **Step 7.** Check cells on Day -5 under a microscope and replace growth medium with 4 mL of fresh ESC medium. **Step 8.** Passage cells on Day -4 into another feeder well \(same procedure as Steps 4-6 above). **Step 9.** Check cells on Day -3 and replace growth medium with 4 mL of fresh ESC medium. **Step 10.** On Day -2 \(Passage 3), feed ESCs with 4 mL of pre-warmed ESC medium about one to two hours before passaging. Passage cells \(as in Step 4-6 above) but split cells at 1:3 and 1:6 ratios into two new wells with feeders. **Step 11.** Check cells on Day -1 and replace growth medium with 4 mL of fresh ESC medium. **C.2. Harvest of ESCs for blastocyst injection** **Step 1.** At about 8 a.m. on the blastocyst injection day \(Day 0), warm 15-mL aliquots of ESC medium and DPBS to 37°C.

Bring 0.05% \(v/v) trypsin-EDTA/DPBS to room temperature. **Step 2.** Select the well of ESCs to be used for injection based on cell morphology. Feed cells by replacing medium with 4 mL of pre-warmed ESC medium. **Step 3.** 1 h after feeding, remove ESC medium and rinse ESCs once with 5 mL DPBS \(warmed to 37°C). Add 0.5 mL 0.05% \(v/v) trypsin-EDTA/DPBS and incubate for 5 min in tissue culture incubator. **Step 4.** Pipette up-and-down with a P1000 pipette tip to triturate ESCs \(set pipette to 450 µL to avoid air bubbles). Stop the trypsin reaction by adding 4 mL of pre-warmed ESC medium. Produce a single-cell suspension, but do not pipette more than necessary or too harshly. Centrifuge the cell suspension at 180 x g for 5 min. Remove supernatant from cell pellet and resuspend thoroughly \(but gently) in 1 mL of fresh ESC medium. Triturate again with a P1000 pipette tip to generate a single-cell suspension. Add another 3 mL of pre-warmed ESC medium to the ESC suspension. **Step 5.** To deplete feeder cells, add the cell suspension to a gelatinized 60-mm dish, and return to the tissue culture incubator for 30 min. Most feeder cells will become loosely attached to the dish bottom whereas the ESCs will mostly remain in suspension. **Step 6.** Very carefully tilt the dish and collect medium containing ESCs. Centrifuge the cell suspension at 130 x g for 3 min. **Step 7.** Remove supernatant and resuspend the cell pellet thoroughly \(but gently) in 800 µL of ice-cold ESC medium. Transfer the suspension to a 15-mL conical tube containing 5 mL of fresh ice-cold ESC medium and place tube on ice. ESCs are now ready for blastocyst injection. Cells should be injected within two hours of collection. **D. NEURAL BLASTOCYST COMPLEMENTATION** This section describes generation of blastocysts and pseudopregnant fosters, ESC injection into blastocysts, and transfer of injected blastocyst into fosters. _Note:_ all work involving mice must be carried out in compliance with protocols and guidelines established by your local Institutional Animal Care and Use Committee \(IACUC) and Institutional Biosafety Committee \(IBC). We maintain all mice on a 12-h light-dark schedule in a temperature-controlled environment, with food and water provided _ad libitum_. **D.1. Generation of blastocysts for ESC injection and pseudopregnant fosters** **Step 1.** At 12 p.m. \(six days before blastocyst injection) superovulate 4- to 5-week-old homozygous _R26-DTA_ donor females by intraperitoneal \(i.p.) injection of 5 U \(0.1 mL) of 50 IU/mL pregnant mare serum gonadotropin \(PMSG). Use a 1-mL syringe with a 26-G needle. **Step 2.** 48 h later \(12 p.m., four days before blastocyst injection) administer 5 IU \(0.1 mL) of 50 U/mL human chorionic gonadotropin \(hCG) via i.p. injection. Immediately set females 1:1 with homozygous _Emx1-Cre_ males or heterozygous _Emx1-Cre_; hemizygous _DsRed.T3_ males. We usually set up 10 to 20 mating pairs for blastocyst generation. _Note:_ we recommend using males that are at least eight weeks old for this step and replace males when they are more than eight months old. **Step 3.** Before 9 a.m. the next morning \(three days before blastocyst injection), check superovulated females for presence of coital mating plugs and separate them from the males. Females displaying a coital mating plug are considered 0.5 days post coitum \(dpc) and are group-housed at up to 10 mice per large mouse cage until blastocyst isolation three days later. **Step 4.** To obtain pseudopregnant fosters, set up CD-1 females with vasectomized CD-1 stud males at a ratio of 1:1 three days before blastocyst injection. We routinely set up 50 males with 50 females. _Note:_ single-house CD-1 vasectomized stud males \(purchased at nine weeks of age from Charles River Laboratories or prepared in-house) before use. We recommend replacing vasectomized CD-1 stud males every six months. CD-1 females are replaced weekly \(or less frequently if doing fewer NBC experiments). Unplugged CD-1

females can be kept and reused after 10 days if desired. CD-1 pseudopregnant females are used as foster recipients at 2.5 dpc \(if not enough females with plugs are obtained, 0.5 dpc CD-1 females can be used as fosters). Foster mothers are fed a breeder diet \(Picolab mouse diet 20) during pregnancy and while nursing. **Step 5.** Before 9 a.m. the next morning, check CD-1 foster females for presence of a coital mating plugs and separate them from the males. Females displaying a coital mating plug are group-housed at up to 10 mice per large mouse cage until the day of transfer surgery \(section D.4). **D.2. Blastocyst collection** **Step 1.** Between 8 a.m. and 10 a.m. at 3.5 dpc \(three days after D.1. Step 3), euthanize superovulated blastocyst donor females via CO_2 asphyxiation \(must follow local IACUC guidelines and approved protocols). **Step 2.** Place donor females on their backs. Clean the abdominal area with sterile alcohol prep pads. Expose the abdominal cavity, remove uterine horns, and transfer them to a 35-mm Petri dish \(Falcon 351008) containing 2 mL of blastocyst flushing medium \[435 mL DMEM with L-glutamine, 4.5 g/L glucose and sodium pyruvate, 50 mL heat-inactivated FBS, 10 mL 200 mM HEPES, and 5 mL 5,000 U/mL penicillin-streptomycin]. Blastocyst flushing medium should be 0.22-µm filtered and can be stored for one month at 4°C. **Step 3.** Place the tip of a 26-G or 30-G \(0.5-in.) needle attached to a 3-mL syringe containing blastocyst flushing medium inside the uterine horn. Flush embryos from each uterine horn with about 0.5 mL blastocyst flushing medium into a fresh 35-mm Petri dish. **Step 4.** For blastocyst collection, prepare two to four 35-mm Petri dishes containing up to six 20-µL drops of EmbryoMax KSOM mouse embryo medium and cover with light paraffin mineral oil. Use enough oil to completely cover the height of the 20-µL drops \(we use about 3 mL per 35-mm dish). Pre-equilibrate dishes in a 5% CO_2, 37°C tissue culture incubator. **Step 5.** Collect blastocysts and morulae by using a 9-in. borosilicate glass Pasteur transfer pipette \(hand-pulled using a Bunsen burner to about 50 to 100 µm) equipped with a suction mouthpiece, Tygon tubing \(1/8 in. I.D., 1/4 in. O.D., 1/16 in. wall), and a 0.22-µm filter \(Millex-GP filter unit) under a Nikon SMZ U 1:10 stereo microscope, using an ED Plan 1x objective at highest power. **Step 6.** Place up to 20 collected blastocysts or morulae \(sorted into different drops, i.e., one drop with blastocysts and another drop with morulae) per each 20-µL drop in 35-mm Petri dishes \(prepared in D.2. Step 4). Record the total number of embryos. Dishes are placed into a tissue culture incubator for up to several hours before injection. **D.3. ESC injection into blastocysts** **Step 1.** Perform blastocyst injection on either a Nikon Eclipse TE2000-s microscope equipped with Leitz manipulator arms or a Nikon T12-U microscope with Narashige manipulator arms \(or use an equivalent setup) situated on an anti-vibration lab table. Prepare injection micropipette needles and holding micropipette needles from borosilicate glass \(O.D. 1.0 mm, I.D. 0.75 mm). Pull injection micropipette needles on a Flaming/Brown Micropipette Puller using the following settings: H450, P200, V100, T50, pressure 20. This should result in an internal diameter of 10 to 20 µm. **Step 2.** Hand-cut pulled injection micropipette needles under a stereo microscope to create a curved/shovel scoop tip. Next, bend injection micropipette needles 5° to 20° on a Micro-forge MF-1 or equivalent. **Step 3.** Make holding micropipette needles by hand-pulling over a Bunsen burner flame to blunt break to a diameter that is half the diameter of a blastocyst \(20 to 50 µm). The tip is then fire polished using the micro-forge and the needle is also bent 5° to 20°. **Step 4.** Perform blastocyst injection in the lid of a 35-mm Petri dish containing 2 mL of blastocyst flushing medium. Lower injection needle and holding needle into the medium, then overlay the medium with 2 mL of light paraffin mineral oil. Use the Pasteur transfer pipette

mentioned above to add 10 blastocysts at a time. Add an aliquot of about 10 µL ESC suspension \(stored on ice from C2. Step 7) by using the same transfer pipette. **Step 5.** Inject 10-15 blastocysts with 12 ESCs each \(we have found a range of 10-12 ESCs per blastocyst to be optimal for NBC). Use a 20x objective \(200x magnification) for blastocyst injection. Blastocysts should not be left in the injection dish for more than 30 min. **Step 6.** Use a transfer pipette equipped with a suction mouthpiece \(see D.2. Step 5) to place injected blastocysts back into EmbryoMax KSOM mouse embryo medium under light paraffin mineral oil. Incubate in a 5% CO_2, 37°C tissue culture incubator until transfer surgery about two to three hours later. **D.4. Blastocyst transfer surgery** **Step 1.** Anesthetize 2.5-dpc pseudopregnant recipient females \(or, if necessary, 0.5-dpc pseudopregnant recipient females) by i.p. injection of a mix of 100-120 mg/kg ketamine and 5-10 mg/kg xylazine, and check for desired plane of anesthesia \(must follow local IACUC guidelines and approved protocols). **Step 2.** Place eye lubricant in each eye. Shave fur from an approximately 1 x 1.5 in. rectangle on the back. Disinfect skin by alternating application of sterile alcohol prep pads and Povidone-iodine three times, ending on Povidone-iodine. **Step 3.** Transfer mouse in ventral position \(i.e., in sternal recumbency) to a sterile surgical area. Use microdissection scissors to make a 1- to 2-cm midline incision on the back and a 0.5-cm incision in the peritoneal wall over the ovaries. Use Graefe forceps to remove the ovarian fat pad and hold with a Johns Hopkins clamp to expose the ovary, oviduct, and uterine horns. **Step 4.** Place the mouse on the base of a Nikon SMZ 1500 C-DSS115 stereo microscope equipped with a Nikon P-FMD 1007009 focusing mount. While holding the uterine horn securely with Dumont #55 forceps, pierce a small hole into the uterus with a 30-G needle. Use the hand-pulled transfer mouth pipette \(see D.2. Step 5) to transfer five to eight ESC-injected blastocysts into each uterine horn of the 2.5-dpc pseudopregnant recipients. Alternatively, if using 0.5-dpc pseudopregnant fosters, pierce a small hole into the ampulae of the oviduct, and transfer five to eight blastocysts. **Step 5.** Reintroduce uterus, oviduct, and fat pad into the intraperitoneal cavity. Close the body wall with dissolvable suture material \(Look Suture, Cat. No. 495B, PolySyn suture, 18 in., size 5-0, 3/8 circle, precision reverse cutting) with Mayo-Hegar needle holders and Graefe forceps. Repeat this process on the other oviduct or uterine horn. Close the skin with 7.5 x 1.75 mm stainless steel Michel suture clips. Administer 0.1 mg/kg Buprenorphine \(0.3 mg/mL in 5% dextrose) i.p. and place the mouse into a small cage. Place the cage on a circulating water cage warmer \(Heat Therapy Pump HTP-1500). Monitor each mouse until fully conscious before returning it to the holding room. Sterilize all surgical tools between female recipients by using a glass bead sterilizer. **Step 6.** Pups are born 17 days after blastocyst transfer to uterus \(or 19 days after transfer to oviduct). Postnatal procedures are the same as for conventional mouse pups. Analysis of embryos at various prenatal developmental stages is also an option.

Timing

1) Genome editing in ESCs and validation: ~4 to 8 weeks **2)** Expansion of mutant and parental wild-type ESCs for NBC \(simultaneous generation of blastocysts and fosters): 9 days **3)** Blastocyst collection, ESC injection, and transfer into fosters: 1 day **4)** Chimeric pups born: 17 to 19 days

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