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A Non-Random Mouse Model for Pharmacological Reactivation of Mecp2 on the Inactive X Chromosome

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Feb 5, 2019

Dear Dr. Weldon

Re: Submission of the revised manuscript titled "A non-random mouse model for pharmacological reactivation of Mecp2 on the inactive X chromosome."

Thank you for your email of January 15th and forwarding us the editorial and reviewers comments. We appreciate the opportunity to revise the manuscript "A non-random mouse model for pharmacological reactivation of Mecp2 on the inactive X chromosome ".

Please find enclosed the revised manuscript where we have addressed all the comments and issues raised by the reviewers. We have also included a rebuttal letter addressing point-by-point response to the reviewers and editorial comments. The revised manuscript accompanies the revised figures, Table and Table of Materials, and permission to use some of the previously published figures.

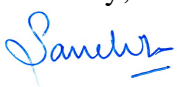
Here, we describe a protocol to generate a viable female murine model with non-random X chromosome inactivation, i.e. the maternally-inherited X chromosome is inactive in 100% of the cells. We also describe a protocol to test feasibility, tolerability, and safety of pharmacological reactivation of the inactive X chromosome in the brain of a living mouse.

Please let us know if any additional information is needed to facilitate the publication of the manuscript.

Many thanks for overseeing our manuscript.

Best Regards
Sanchita

Sincerely,



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TITLE:

A Non-Random Mouse Model for Pharmacological Reactivation of Mecp2 on the Inactive X Chromosome

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KEYWORDS:

Mecp2, inactive X chromosome, mouse model, Rett syndrome, X chromosome reactivation, brain neurons

SUMMARY:

Here, we describe a protocol to generate a viable female murine model with non-random X chromosome inactivation, i.e., the maternally-inherited X chromosome is inactive in 100% of the cells. We also describe a protocol to test feasibility, tolerability, and safety of pharmacological reactivation of the inactive X chromosome in vivo.

ABSTRACT:

X chromosome inactivation (XCI) is the random silencing of one X chromosome in females to achieve gene dosage balance between the sexes. As a result, all females are heterozygous for X-linked gene expression. One of the key regulators of XCI is *Xist*, which is essential for the initiation and maintenance of XCI. Previous studies have identified 13 *trans* acting X chromosome inactivation factors (XCIFs) using a large-scale, loss-of-function genetic screen. Inhibition of XCIFs, such as ACVR1 and PDPK1, using short-hairpin RNA or small molecule inhibitors, reactivates X chromosome-linked genes in cultured cells. But the feasibility and tolerability of reactivating the inactive X chromosome in vivo remains to be determined. Towards this goal, a *XistΔ:Mecp2/Xist:Mecp2-Gfp* mouse model has been generated with non-random XCI due to deletion of *Xist* on one X chromosome. Using this model, the extent of inactive X reactivation was quantitated in the mouse brain following treatment with XCIF inhibitors. Recently published results show, for the first time, that pharmacological inhibition of XCIFs reactivates *Mecp2* from the inactive X chromosome in cortical neurons of the living

mouse brain.

INTRODUCTION:

X chromosome inactivation (XCI) is a process of dosage compensation that balances X-linked gene expression by silencing one copy of the X chromosome in females[1]. As a result, the inactive X chromosome (Xi) accumulates characteristic features of heterochromatin including DNA methylation and inhibitory histone modifications, such as histone H3-lysine 27 trimethylation (H3K27me3) and histone H2A ubiquitination (H2Aub)[2]. The master regulator of X chromosome silencing is the X-inactivation center (Xic) region, around 100−500 kb, which controls the counting and pairing of the X chromosomes, the random choice of the X chromosome for inactivation, and the initiation and spreading of silencing along the X chromosome[3]. The process of X inactivation is initiated by X inactive specific transcript (*Xist*) that coats the Xi in *cis* to mediate chromosome-wide silencing and remodel the three-dimensional structure of the X chromosome[4]. Recently, several proteomic and genetic screens have identified additional regulators of XCI, such as *Xist* interacting proteins[5-12]. For example, a previous study using an unbiased genome-wide RNA interference screen identified 13 *trans*-acting XCI factors (XCIFs)[12]. Mechanistically, XCIFs regulate *Xist* expression and therefore, interfering with XCIFs function causes defective XCI[12]. Together, recent advances in the field have provided important insights into the molecular machinery that is required to initiate and maintain XCI.

Identification of XCI regulators and understanding their mechanism in XCI is directly relevant to X-linked human diseases, such as Rett syndrome (RTT)[13,14]. RTT is a rare neurodevelopmental disorder caused by a heterozygous mutation in the X-linked methyl-CpG binding protein 2 (*MECP2*) that affects predominantly girls[15]. Because *MECP2* is located on the X chromosome, RTT girls are heterozygous for *MECP2* deficiency with ~50% cells expressing wild-type and ~50% expressing mutant *MECP2*. Notably, RTT mutant cells harbor a dormant but wild-type copy of *Mecp2* on the Xi, providing a source of the functional gene, which if reactivated, could potentially alleviate symptoms of the disease. In addition to RTT, there are several other X-linked human diseases, for which reactivation of Xi represents a potential therapeutic approach, such as DDX3X syndrome.

Inhibition of XCIFs, 3-phosphoinositide dependent protein kinase-1 (PDPK1), and activin A receptor type 1 (ACVR1), either by short hairpin RNA (shRNA) or small molecule inhibitors, reactivates Xi-linked genes[12]. Pharmacological reactivation of Xi-linked genes is observed in various ex vivo models that include mouse fibroblast cell lines, adult mouse cortical neurons, mouse embryonic fibroblasts, and fibroblast cell lines derived from an RTT patient[12]. However, whether pharmacological reactivation of Xi-linked genes is feasible in vivo remains to be demonstrated. One limiting factor is the lack of effective animal models to accurately measure the expression of genes from reactivated Xi. Towards this goal, a *XistΔ:Mecp2/Xist:Mecp2-Gfp* mouse model has been generated that carries a genetically labeled *Mecp2* on Xi in all the cells due to heterozygous deletion in *Xist* on the maternal X chromosome[16]. Using this model, the expression of *Mecp2* from Xi has been quantitated following treatment with XCIFs inhibitors in the brain of living mice. Here, the generation of the *XistΔ:Mecp2/Xist:Mecp2-Gfp* mouse model

and methodology to quantitate Xi reactivation in cortical neurons using immunofluorescence-based assays is described.

PROTOCOL:

Work involving mice adhered to the guidelines of the University of Virginia Institutional Animal Care and Use Committee (IACUC; #4112).

1. Generate a non-random XCI mouse model with genetically labeled *Mecp2* on Xi

NOTE: Mouse strains used in the study were as follows: *Mecp2-Gfp/Mecp2-Gfp* (*Mecp2*$^{tm3.1Bird}$, **Table of Materials**) and *Xist/ΔXist* (*B6;129-Xist<tm5Sado>*; provided by Antonio Bedalov, Fred Hutchinson Cancer Center, Seattle). Breeding strategies among the respective strains have been designed to expand the mouse colonies for each strain.

1.1. Perform PCR-genotyping on all mice strains and respective progenies obtained after breeding using gene specific primers listed in **Table 1**.

2. Design the mouse breeding strategy to generate *XistΔ:Mecp2/Xist:Mecp2-Gfp*

2.1. Set up a breeding pair by housing a *Mecp2-Gfp/Y* male and a *XistΔ-Mecp2/Xist-Mecp2* female together (**Figure 1A**). Ideally, set up at least 5 breeding pairs at a time using viable and fertile mice. After the pregnant female gives birth, allow her to raise her first litter with the male.

NOTE: Because paternal *Xist* knockout impairs imprinted XCI, dosage compensation, and differentiation pathways[17], the use of *XistΔ-Mecp2/Y* mice in the breeding will fail to produce the female pups with required genotype.

2.2. After weaning the litter at post-natal day 21 (P21), identify and tag the *XistΔ:Mecp2/Xist:Mecp2-Gfp* female pups using a PCR-based genotyping assay (**Figure 1B**).

NOTE: In terms of the number of animals needed per group, for the results reported below, 5 breeding pairs were set up which generated approximately 10 female *XistΔ:Mecp2/Xist:Mecp2-Gfp* mice**.**

2.3. Use female mouse models for all the proposed experiments.

NOTE: Sex is an important biological variable for XCI studies, and the male model does not account for the confounding effects of random XCI.

2.4. To be consistent throughout the animal studies and rule out any effects of the animal age, perform all the experiments in 5−8-week-old female *XistΔ:Mecp2/Xist:Mecp2-Gfp* mice.

3. Isolate female *XistΔ:Mecp2/Xist:Mecp2-Gfp* mouse embryonic fibroblasts (MEFs)

3.1. Set up timed mating between the *Mecp2-Gfp/Y* male and *XistΔ-Mecp2/Xist-Mecp2* female. Set up at least 3−4 mating cages to increase the likelihood of pregnancy.

3.2. After confirming the vaginal plug the morning after mating, separate the female and this day is considered embryonic day 0.5 (E0.5). Monitor the weight gain of the prospective pregnant female and visually inspect the abdomen of the mice to confirm pregnancy. On E14.5−15.5, euthanize the pregnant female mice via cervical dislocation.

3.3. Under a laminar hood, wipe the abdomen of the pregnant female with 70% ethanol. Using sterile scissors, dissect the abdominal cavity and remove the uterine horns containing the embryos. Using forceps, gently take out the embryos while cutting off the remaining abdominal tissue (6−12 embryos is usually expected).

3.4. Place the uterine horns containing the embryos in a 10 mm tissue-culture dish. Using sterile scissors and forceps, make an incision along the uterine horns to isolate individual sacs carrying an embryo. Carefully, place the uterine horns containing the rest of the embryos in 30 mL of sterile Hanks' balanced salt solution (HBSS) on ice.

3.5. Gently cut open the sac and isolate the embryo using sterile scissors and forceps. Remove the placenta by cutting the umbilical cord.

3.6. Decapitate the embryo.

NOTE: While the rest of the embryonic tissue will be further processed, the tissue from the embryo head will be used to isolate DNA for genotyping.

3.7. Open the abdomen by cutting the midline of the embryo using sterile scissors and forceps. Remove the visceral organs, such as the heart, liver, and lungs.

3.8. Transfer the remaining embryonic tissue into a sterile 60 mm tissue-culture plate and cut into small pieces using scissors or a razor blade. To break open the cell clumps, add 3 mL of trypsin-EDTA (0.05%) and incubate at 37 °C for 15 min.

3.9. Neutralize trypsin-EDTA by adding 5 mL of Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 10 µg/mL penicillin/streptomycin (pen/strep) to the plate and dissociate the tissue by repetitive pipetting (approximately 20−30 times).

3.10. Spin down cells at 300 x *g* for 5 min and re-suspend the cell pellet in 4 mL of DMEM with 10% FBS and 10 µg/mL pen/strep. Plate the cells on a 60 mm culture dish, and culture the cells at 37 °C in the presence of 5% CO_2.

3.11. Carry out steps 3.5−3.10 for each embryo.

3.12. Culture MEFs obtained in step 3.11 for at least 3−4 days.

NOTE: At this stage, MEFs can also be cryopreserved for future experiments.

3.13. To determine the sex and genotype of each embryo, carry out genotyping-PCR using DNA isolated from the heads of the embryos using primers and PCR conditions listed in **Table 1**.

4. Confirm the lack of green fluorescent protein expression in the brain of *XistΔ:Mecp2/Xist:Mecp2-Gfp* mice using a fluorescence activated cell sorting-based assay

4.1. Using scissors, separate the cerebral cortex from the rest of the brain hemispheres, and place in 1 mL of ice-cold nuclei isolation media (NIM) buffer containing 250 mM sucrose, 25 mM potassium chloride (KCl), 5 mM magnesium chloride ($MgCl_2$), 10 mM Tris-Cl, supplemented with 2% paraformaldehyde (PFA), and 0.1% nonionic surfactant (**Table of Materials**).

4.2. Homogenize using an ice-cold glass homogenizer.

4.3. Spin down homogenized tissue at 600 x *g*, 4 °C for 5 min.

4.4. Remove supernatant and resuspend pellet in 1 mL of 25% iodixanol in NIM solution.

4.5. Add 1 mL of 29% lodixanol in NIM solution to a 4 mL ultracentrifuge tube (store on ice until samples are ready) and carefully layer 1 mL of sample (in 25% lodixanol).

4.6. Centrifuge at 9,000 x *g*, 4 °C for 10 min in an ultracentrifuge.

4.7. Aspirate supernatant and resuspend pellet in 500 µL of fluorescence activated cell sorting (FACS) buffer (phosphate-buffered saline [PBS] supplemented with 1 mM EDTA, 0.05% sodium azide, and 2% bovine serum albumin [BSA]).

NOTE: Samples can be stored at 4 °C or up to 1 week.

4.8. Add 5 µL of 7-amino-actinomycin D (7-AAD; 50 mg/mL) for 5 min at room temperature to stain DNA.

4.9. Analyze samples for 7-AAD and green fluorescent protein (GFP) signal using flow cytometry.

5. Determine feasibility of the *XistΔ:Mecp2/Xist:Mecp2-Gfp* mouse model for Xi reactivation

5.1. Seed 1 x 10^5 cells/mL female *XistΔ:Mecp2/Xist:Mecp2-Gfp* MEFs, *Mecp2/Mecp2-Gfp* and *Xist-Mecp2/Y* MEFs obtained in step 3.12 in a 6-well format and in chamber slides, in DMEM with 10% FBS and 10 µg/mL pen/strep, at 37 °C in the presence of 5% CO_2.

NOTE: *Mecp2/Mecp2-Gfp* and *Xist-Mecp2/Y* MEFs are used as positive and negative controls in the experiments.

5.2. Add fresh medium to the cells after 24 h. For *XistΔ:Mecp2/Xist:Mecp2-Gfp* MEFs, add medium supplemented with XCIFs inhibitors (e.g., 0.5 µM LDN193189 and 2.5 µM GSK650394), or vehicle alone. Replace medium supplemented with fresh inhibitor or vehicle alone every 2 days. For *Mecp2/Mecp2-Gfp* and *Xist-Mecp2/Y* MEFs, add fresh medium every 2 days.

5.3. Post 1-week of inhibitor treatment, harvest MEFs either for RNA isolation (6-well plate) or fix cells for immunofluorescence (chamber slides). Use MEFs isolated from *Mecp2/Mecp2-Gfp* embryos as positive and *Xist-Mecp2/Y* embryos as negative controls respectively.

5.3.1. For RNA isolation, isolate total RNA by guanidinium thiocyanate-based RNA extraction reagent, and reverse transcribe using reverse transcriptase. Measure *Mecp2-Gfp* expression by quantitative reverse transcriptase-PCR (qRT-PCR) using *Mecp2-WT* and *Mecp2-GFP,* and primers listed in **Table 1**, as described previously[12].

5.3.2. For immunofluorescence, stain MEFs with an anti-GFP primary antibody (1:100), as described previously[12,16]. Measure GFP intensity by quantitative immunofluorescence in drug treated *XistΔ:Mecp2/Xist:Mecp2-Gfp* MEFs, as described previously[18].

6. Demonstrate the pharmacological Xi reactivation in the brain of the *XistΔ:Mecp2/Xist:Mecp2-Gfp* mouse model

6.1. Prepare drugs and vehicle control.

6.1.1. Prepare a fresh, sterile solution of vehicle (0.9% NaCl, 0.5% methylcellulose, 4.5% dimethyl sulfoxide [DMSO]) for brain injections.

6.1.2. Prepare chemical inhibitors including 1.5 mM LDN193189 (small molecule inhibitor of ACVR1) and 1.6 mM GSK650394 (small molecule inhibitor of SGK1, a downstream effector of PDPK1) re-suspended in vehicle (0.9% NaCl, 0.5% methylcellulose, 4.5% DMSO), or vehicle alone. The total volume of chemical inhibitors or vehicle injected is 10 µL per dose per animal.

6.2. Prepare animal for brain injections.

6.2.1. Prepare the surgical area by wiping the bench and heating pad with disinfectant (10% sodium hypochlorite solution).

6.2.2. Anesthetize the 4-week old mouse with an intraperitoneal injection of ketamine/xylazine mixture at a dose of 140 mg/kg and 10 mg/kg, respectively. Use the pedal withdrawal reflex to determine the level of anesthesia.

6.2.3. Apply ophthalmic ointment to the eyes following induction of anesthesia to prevent

corneal drying.

6.2.4. Shave off the fur from the neck to the top of the head of the mouse.

6.2.5. Position the mouse in the stereotactic platform by hooking the mouse's incisor teeth in the bite bar of the snout restrainer and tightening the nose clamp over the snout while ensuring that the mouse's head is on a level plane.

6.2.6. Adjust the height of the ear bars, as necessary, to reach the caudal portion of the ear canal, securing them such that the mouse's head is in a level plane and immobilized on finger touch.

6.2.7. Disinfect the head of the mouse with alternating wipes of a topical antiseptic, such as povidone-iodine and 70% ethanol.

6.3. Administer drugs.

6.3.1. Using a sterile scalpel, make a 0.75 cm horizontal incision in the mid-scalp.

6.3.2. Using a 0.45 mm burr, drill two symmetrical holes above the right and left cortical hemispheres (2 mm from the sagittal suture and 2 mm from the lambdoid suture, approximately the middle of parietal bone).

6.3.3. Attach a 10 µL syringe to the stereotactic platform firmly.

6.3.4. Mix the solution of chemical inhibitors, and draw 10 µL of solution into the syringe. Avoid any air bubbles in the syringe.

6.3.5. Advance the syringe needle into the burr hole maintaining the needle perpendicular (90°) to the skull. When the needle traverses the skull, zero out the coordinates on the stereotactic digital display and then advance the tip of the needle until it reaches a depth of 2.5 mm.

6.3.6. Withdraw the needle 0.5 mm to the depth for 2 mm.

6.3.7. Slowly inject 10 µL of solution (~1 min). After injection is complete, leave the needle in the brain for ~1 min and then withdraw the needle.

6.3.8. Repeat the injection for the second hemisphere (vehicle only control).

6.3.9. Using sutures or "skin glue," close the skin of the mouse.

6.3.10. Loosen the ear bars and remove the mouse from the stereotactic apparatus.

6.3.11. Place the mouse on a heating pad set to 37 °C until the animal regains consciousness.

6.3.12. Once the mouse is alert and responsive, transfer the animal back to its original cage.

6.3.13. Repeat the procedure every 2 days for 20 days. Repeat drilling of the area is not required for subsequent injections.

6.4. Isolate the mouse brain.

6.4.1. Once the dose regimen is completed, euthanize the mouse in a CO_2 chamber.

6.4.2. Immobilize the mouse on a surface using needles.

6.4.3. Make a lateral incision through the integument and abdominal wall just beneath the rib cage using scissors and forceps. Carefully separate the liver from the diaphragm.

6.4.4. Using scissors, cut the diaphragm and continue cutting along the entire length of the rib cage to expose the pleural cavity.

6.4.5. Using scissors, make an incision to the posterior end of the left ventricle.

6.4.6. Immediately, start injecting the right heart chamber with ~15 mL of PBS over ~2 min. Liver color change from red to pale pink is indicative of good perfusion.

6.4.7. Inject the right chamber of the mouse heart with ~10 mL of 4% paraformaldehyde in PBS over ~2 min.

6.4.8. Decapitate the mouse, and use scissors to make a midline incision of the scalp to expose the skull.

6.4.9. Place one tip of the scissors into the foramen magnum, and cut laterally into the skull toward the eye. Repeat for the other side. Try to keep the end of the scissors as superficial as possible to avoid injury of the brain.

6.4.10. Use scissors to cut the region between the eyes and above the nose of the mouse.

6.4.11. Use forceps to gently peel the cranial bones from the brain hemispheres.

6.4.12. Lift the brain with a spatula, and use scissors to carefully dissect the cranial nerve fibers that fix it to the skull. Place the brain into a 15 mL tube filled with 4% PFA in PBS.

6.4.13. Place the brain on a plastic dish, cut out the cerebellum and olfactory bulbs, and separate the hemispheres.

6.5. Cryo-section the mouse brain.

6.5.1 Fix brain in 4% paraformaldehyde in PBS at 4 °C overnight.

6.5.2. Rinse the brain with PBS at 4 °C at least 3x for 5 min each.

6.5.3. Label the disposable molds for cryopreserving the tissues.

6.5.4. Cut out the front of the brain using scissors and forceps (start making ~5 mm sections from injection sites).

6.5.5. Transfer the brain to the cryomold with the front of the brain facing down to obtain coronal sections. Submerge the mold containing brain in the optimal cutting temperature compound (OCT).

6.5.6. Pour liquid nitrogen into a 10 mm plastic Petri dish and place the brain in the cryo-mold into the nitrogen.

NOTE: It is important to orient the tissue as described in step 6.5.5 to guide the sectioning of the brain.

6.5.7. When the OCT is solid white, place the frozen brain into an -80 °C freezer for storage.

6.5.8. Equilibrate the brain to -20 °C for at least 30 min prior to sectioning with cryostat and cryosection (5−6 µm thickness) the brain, mounting 2−3 sections per slide. Slides can be stored at -80 °C for later use.

6.6. Determine Xi reactivation using an immunofluorescence-based approach.

6.6.1. Before starting the staining procedure, dry the brain sections overnight at 4 °C.

6.6.2. Immerse slides in antigen retrieval solution (0.1 M citric acid, 0.1 M Tris-base, pH = 6.0) on a 100 °C heat block for 5 min.

6.6.3. Wash slides 4x with 1x PBS for 5 min each at room temperature.

6.6.4. Immerse slides in blocking solution (0.1 M NH_4Cl/PBS/0.2% gelatin/0.05% nonionic surfactant) for 20 min at room temperature.

6.6.5. Wash slides 3x with wash buffer (PBS/0.2% gelatin) at room temperature for 5 min each.

6.6.6. Stain brain sections with anti-GFP-AlexaFluor647 (1:100) and anti-MAP2 (1:1000) antibodies in incubation medium (PBS/0.2% gelatin/1% BSA) and incubate at 4 °C overnight.

6.6.7. Collect primary antibodies (can be reused). Wash slides 4x with wash buffer for a total of

30 min at room temperature.

6.6.8. Incubate brain sections with goat anti-chicken fluorescein isothiocyanate (FITC)-labeled secondary antibody (1:1000) in incubation medium and incubate 1−2 h at room temperature in the dark.

6.6.9. Wash slides 4x with wash buffer for a total of 30 min at room temperature.

6.6.10. Place a drop of mounting medium with 4',6-diamidino-2-phenylindole (DAPI) on a 22 mm x 50 mm coverslip, then invert the coverslip onto a slide, covering all tissue sections.

6.6.11. Image on a microscope and adjust images for contrast and brightness. Capture images and quantify the number of GFP-positive cells for both drug-treated and vehicle treated *XistΔ:Mecp2/Xist:Mecp2-Gfp* mouse brain hemispheres.

REPRESENTATIVE RESULTS:

To demonstrate the feasibility of the *XistΔ:Mecp2/Xist:Mecp2-Gfp* mouse model for Xi reactivation studies, XCIF inhibitor-mediated reactivation of Xi-linked *Mecp2-Gfp* was tested in mouse embryonic fibroblasts (MEFs). Female MEFs were isolated from day 15.5 *XistΔ:Mecp2/Xist:Mecp2-Gfp* embryos as described in section 3 (**Figure 1A**). The genotypes of female *XistΔ:Mecp2/Xist:Mecp2-Gfp* MEFs were confirmed by genotyping-PCR, as described previously[19] (**Figure 1B**), and FACS-based assay (**Figure 1C**). MEFs were treated with either DMSO or the two drugs LDN193189 and GSK650394 (0.5 µM and 2.5 µM, respectively) for 7 days. Following drug treatment, the expression of *Mecp2-Gfp* was monitored by qRT-PCR. As shown in **Figure 1D**, the drug treatment, but not DMSO, reactivated expression of Xi-*Mecp2-Gfp.* Next, quantitative immunofluorescence was carried out to determine the extent of Xi-*Mecp2-Gfp* expression in individual MEFs, as described in step 5.3.2. Signal from negative-control MEFs isolated from male embryos (*Mecp2/Y*) was set as the background, and ~66% of positive control *Mecp2-Gfp/Mecp2* MEFs had a nuclear GFP signal. As expected, *XistΔ:Mecp2/Xist:Mecp2-Gfp* MEFs treated with DMSO had a very low level of nuclear GFP (~3%). By contrast, ~31% of drug-treated *XistΔ:Mecp2/Xist:Mecp2* MEFs were positive for nuclear GFP (**Figure 1E**). Together, these results demonstrate that XCIF inhibitors reactivate Xi-linked *Mecp2* in MEFs, but the extent of Xi reactivation varies in the cell population.

To assess the feasibility of pharmacological Xi reactivation-based approach in vivo, whether drug treatment reactivates Xi-linked *Mecp2* in the brain of *XistΔ:Mecp2/Xist:Mecp2-Gfp* female mice was investigated. 10 µL of vehicle or 10 µL of XCIF inhibitors (1.5 mM LDN193189 and 1.6 mM GSK650394) was administered in opposite brain hemispheres of 4-week-old *Xi-Mecp2-Gfp* female mice by intracerebroventricular injection using stereotactic surgical procedures (**Figure 2A,B**). The procedure was repeated every second day (**Figure 2C**), and three weeks later, animals were euthanized, and brains were isolated. One subset was used for qRT-PCR and another was analyzed by immunohistochemistry. The expression of wild-type *Mecp2* and Xi-*Mecp2-Gfp* in the vehicle- and drug-infused hemispheres was determined by qRT-PCR (sequences of primers listed in **Table 1**). As shown in **Figure 2D**, drug treatment reactivated Xi-

Mecp2-Gfp in ~30% of cells in the drug-infused brain hemisphere, whereas Xi- *Mecp2-Gfp* was not detected in the vehicle-infused hemisphere. A large number of MAP2, a neuronal marker, positive neurons were also GFP positive (~45%) in the drug-treated hemisphere, indicative of Xi-*Mecp2-Gfp* expression. Approximately 20% of MAP2 negative brain cells expressed GFP, confirming Xi-*Mecp2-Gfp* reactivation in non-neuronal cells (**Figure 2E)**.

FIGURE AND TABLE LEGENDS:

Figure 1: Generation and validation Xi-*Mecp2* mouse model. (**A**) Schematic of the breeding strategy for generating *XistΔ:Mecp2/Xist:Mecp2-Gfp* mice. (**B**) PCR genotyping of *Xist:Mecp2-Gfp/Y*, *XistΔ:Mecp2/Xist:Mecp2* and *XistΔ:Mecp2/Xist:Mecp2-Gfp* mice. Mice were monitored for the presence of *Mecp2-Gfp*, *Mecp2* and sex-determining region Y (*SRY*). (**C**) Flow cytometry analysis of nuclei isolated from the mouse cortex. *Mecp2/Mecp2-Gfp* mouse cortex show ~50% of GFP-positive nuclei while *XistΔ:Mecp2/Xist:Mecp2-Gfp* shows no GFP-positive nuclei. (**D**) qRT-PCR analysis monitoring the expression of *Mecp2-Gfp* and wild-type *Mecp2* transcripts in female *XistΔ:Mecp2/Xist:Mecp2-Gfp* MEFs following treatment with DMSO or drug (LDN193189 and GSK650394). *Gapdh* was monitored as a loading control. (**E**) Quantitative immunofluorescence monitoring GFP intensity in female *XistΔ:Mecp2/Xist:Mecp2-Gfp* MEFs following treatment with DMSO or the drugs LDN193189 and GSK650394. MEFs isolated from *Mecp2/Y* or *Mecp2/Mecp2-Gfp* mice were used as negative and positive controls, respectively. Each dot represents an MEF, and the dashed line indicates the maximum background signal obtained in *Mecp2/Y,* which was set to 1. Lower panel shows representative pictures of nuclei. This figure has been modified from Przanowski et al.[16].

Figure 2: Pharmacological reactivation of X-linked *Mecp2* in cerebral cortical neurons of living mice. (**A**) Schematic of a mouse skull and **(B)** brain showing the site of injection for vehicle or drug in the left or right hemispheres of the brain. (**C**) Schematic of the drug regimen. **D**) Representative immunofluorescence images showing endogenous GFP signal (green) in coronal brain sections from vehicle- or drug-treated hemispheres. DAPI staining is shown in blue. (**E**) Representative immunofluorescence images of the coronal brain sections monitoring the expression of GFP (anti-GFP; red) and MAP2 (green) in drug-treated hemisphere. DAPI staining is shown in blue. This figure has been modified from Przanowski et al.[16].

Table 1: List of primers used for genotyping and quantitative real-time RT-PCR.

DISCUSSION:

Previously, XCIFs that are selectively required for silencing of Xi-linked genes in mammalian female cells were identified[12]. We further optimized potent small molecule inhibitors to target XCIFs, such as ACVR1 and downstream effectors of PDPK1, which efficiently reactivate Xi-linked *Mecp2* in mouse fibroblast cell lines, mouse cortical neurons, and a human fibroblast cell line derived from a RTT patient. These results suggest that Xi reactivation is a plausible therapeutic approach to rescue the gene deficiencies in X-linked disease patients; however, the in vivo feasibility remains to be determined. Recently, XCIF inhibitors were shown to reactivate Xi-linked *Mecp2* in vivo, for which a non-random *XistΔ:Mecp2/Xist:Mecp2-Gfp* mouse model was

generated.

An attractive feature of the *XistΔ:Mecp2/Xist:Mecp2-Gfp* model is that it allows an accurate quantitation of the Xi-linked *Mecp2* reactivation for several reasons. First, due to the deletion of *Xist* on the maternal X chromosome, the *XistΔ:Mecp2/Xist:Mecp2-Gfp* mouse has non-random XCI. As a result, the genetically labeled *Mecp2* is silent in 100% of cells (**Figure 1C**), unlike an expected 50:50 expression of X-linked genes in random XCI mice models, such as *Xist:Mecp2/Xist:Mecp2-Gfp.* Therefore, the results are not precluded by the mosaic expression of GFP, and 100% cells carry *Mecp2-Gfp* on *Xi* in the *XistΔ:Mecp2/Xist:Mecp2-Gfp* model. Second, the genetic labeling of *Mecp2* permits direct visualization of individual neurons with reactivated GFP, thereby minimizing the experimental manipulations in neuronal analysis.

A recent study found that intracerebroventricular injection of the XCIF inhibitors in the mouse brain hemisphere reactivates Xi-linked *Mecp2* using immunofluorescence analysis of the mouse brain[16]. Importantly, drug treatment had no adverse effect on the general health, such as weight, grooming, or mobility[16]. Moreover, there is no toxicity detected by drug treatment in the liver or spleen. Together, this study provides an essential proof-of-principle to demonstrate that interfering with the function of XCIFs leads to de-repression of Xi in vivo.

In summary, a sensitive mouse model can be used to evaluate the reactivation of Xi. This animal model design can also be adapted for generating an improved RTT mouse model that harbors *Mecp2* mutations (probably less symptomatic) on the active X chromosome and wild-type *Mecp2* on the Xi in all cells. Due to non-random XCI, while the phenotypic symptoms may be more pronounced, it is expected that this model will also allow for better evaluation and accurate assessment of the reversal of symptoms due to Xi reactivation. Additionally, *XistΔ:Mecp2/Xist:Mecp2-Gfp* can also be modified to study Xi reactivation in other X-linked disease models, such as the DDX3X syndrome.

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DISCLOSURES:

The authors have nothing to disclose.

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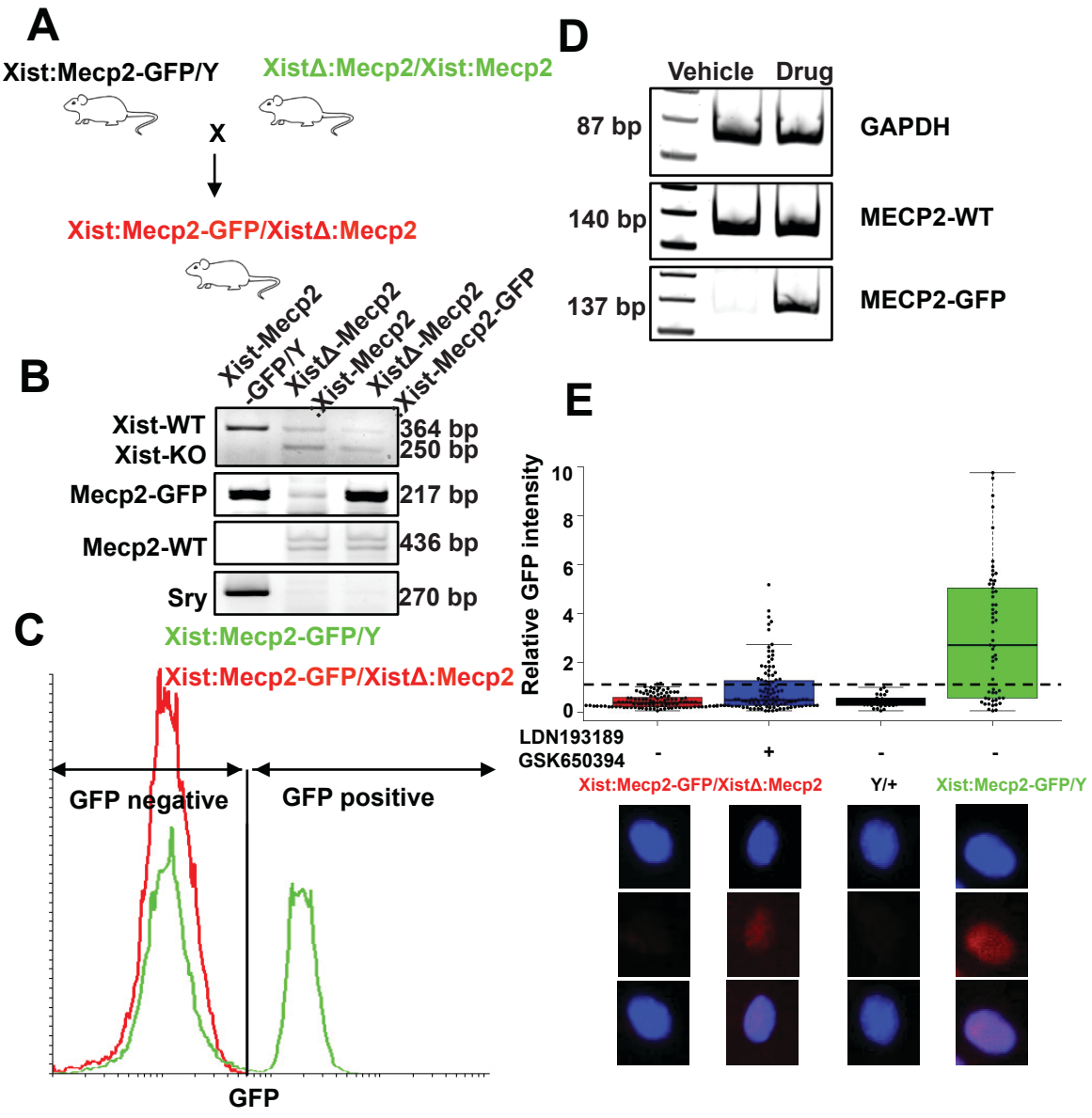

Figure 2

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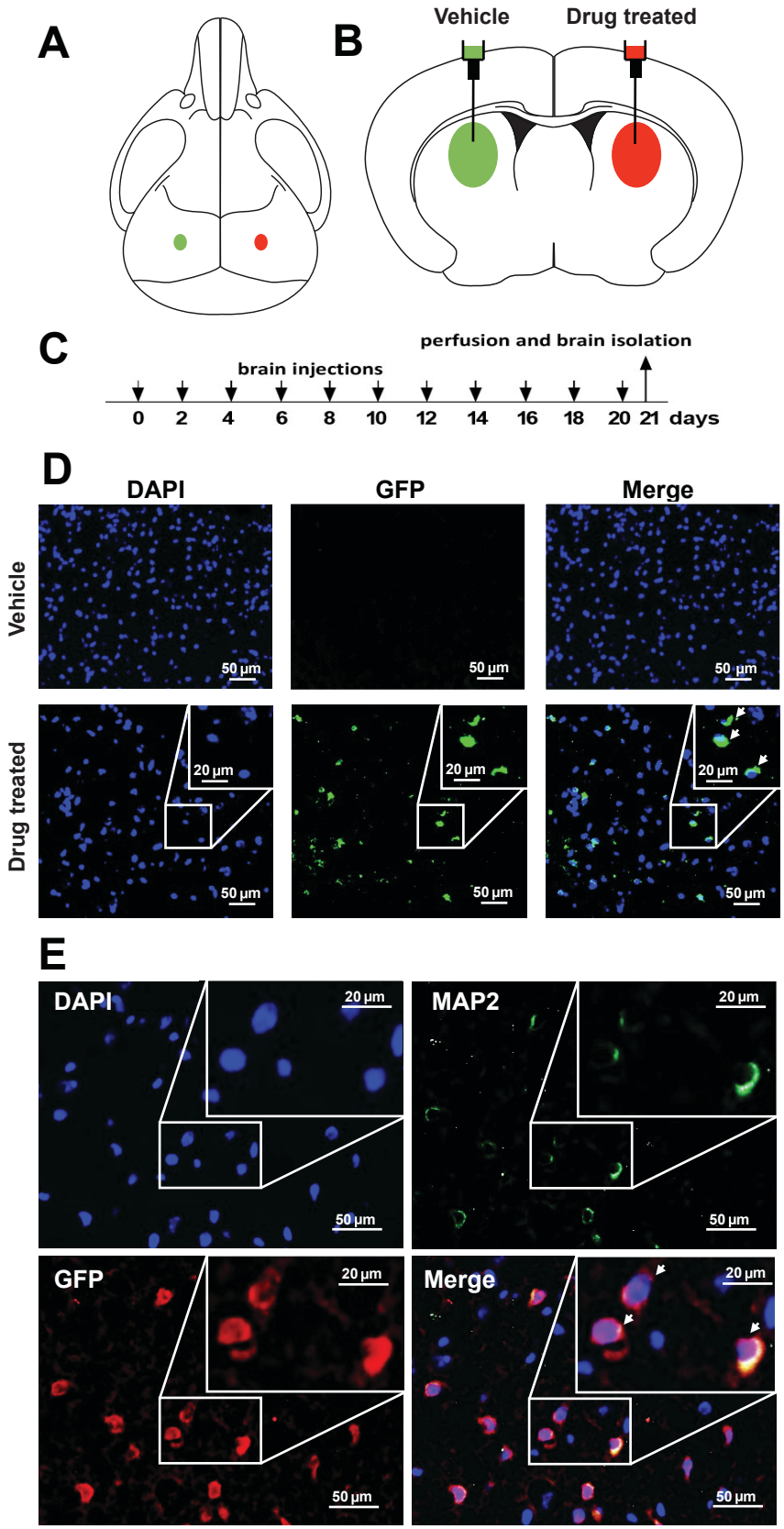

Table 1

	Forward primer (5’ -> 3’)	Reverse primer (5’ -> 3’)
qRT-PCR		
Gapdh	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
Mecp2-GFP	CCAAACAGAGAGGAGCCTGTG	GCTGAACTTGTGGCCGTTTA
Mecp2-WT	CCAAACAGAGAGGAGCCTGTG	TGTCAGAGCCCTACCCATAAG
Mouse genotyping		
Xist-WT	CGGGGCTTGGTGGATGGAAAT	GCACAACCCCGCAAATGCTA
Xist-KO	GGTCCCTCGAAGAGGTTCACTAG	GCACAACCCCGCAAATGCTA
Mecp2-WT	AACAGAGAGGAGCCTGTGGA	AATTGCCCTAACGAGCACAC
Mecp2-GFP	AACAGAGAGGAGCCTGTGGA	GAACTTCAGGGTCAGCTTGC
Sry	TTGTCTAGAGAGCATGGAGGGCCATGT	CTCCTCTGTGACACTTTAGCCCTCCGA

Anealing temperature/PCR product length
60 °C / 87 bp
62 °C / 137 bp
62 °C / 140 bp
62 °C / 364 bp
62 °C / 250 bp
62 °C / 436 bp
62 °C / 217 bp
66 °C / 270 bp

Name of Material/ Equipment	Company
MICE	
Mecp2 $^{tm3.1Bird}$	The Jackson Laboratory
B6;129-Xist<tm5Sado>	provided by Antonio Bedalov, Fred Hutchinson Cance
REAGENTS	
22x22 mm coverslip	FISHERfinest (Fisher Scientific)
32% Paraformaldehyde	Electron Microscopy Sciences
50 ml syringe	Medline Industries
60mm culture dish	CellStar
7-AAD	BioLegend
ammonium chloride (NH_4Cl)	Fisher Chemical
anti-GFP-AlexaFluor647	Invitrogen
anti-MAP2	Aves Labs
BSA	Promega
citric acid	Sigma
DMSO	Fisher Bioreagents
Dulbecco's Modified Eagle Medium (DMEM)	Corning Cellgro
Ethanol	Decon Labs
fetal bovine serum (FBS)	VWR Life Science
gelatin	Sigma-Aldrich
glass slides	Fisherbrand
goat anti-chicken FITC-labeled secondary antibody	Aves Labs
GSK650394	ApexBio
hamilton 10µl syringe	Hamilton Sigma-Aldrich
Hank's Balanced Salt Solution (HBSS)	Gibco
Ketamine/xylazine mixture	Ketaset
Large blunt/blunt curved scissors	Fine Science Tools
LDN193189	Cayman Chemicals
lodixanol	Sigma
magnesium chloride (MgCl2)	Fisher Chemical
Methylcelulose	Sigma
mounting medium with DAPI	Vectashield
Needle tip, 26 GA x 1.25"	PrecisionGlide

ophthalmic ointment	Refresh Lacri-Lube
optimal cutting temperature (O.C.T.)	ThermoFisher
PCR mix	
Penicillin/Streptomycin (Pen/Strep)	Corning
Phosphate buffered saline pH 7.4 (PBS)	Corning Cellgro
Potassium chloride (KCl)	Fisher Scientific
scalpel blades	
Shallow glass or plastic tray	
skin glue/tissue adhesive	3M Vetbond
sodium azide	Fisher Scientific
Sodium chloride (NaCl)	Fisher Chemical
standard hemostat forceps	Fine Science Tools
Standard tweezers	Fine Science Tools
Straight iris scissors	Fine Science Tools
sucrose	Fisher Scientific
Tris-base	Fisher Bioreagents
Triton X-100	Fisher Bioreagents
Trypsin-EDTA	Gibco
EQUIPMENT	
Zeiss AxioObserver Live-Cell microscope	Zeiss
0.45mm burr	IDEAL MicroDrill
BD FACScalibur	
centrifuge	
glass homogenizer	
cell culture incubator	Thermo Scientific HERACELL VIOS 160i
Leica 3050S research cryostat	
stereotactic platform	
thermocycler	
Timer	
ultracentrifuge	Beckman Coulter Optima L-100 XP
Water bath (37 °C)	Fisher Scientific Isotemp 2239

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Reviewer #1:
We thank reviewer for accepting our manuscript for publication.

Reviewer #2:

We thank reviewer 2 for his/her positive comments, “The steps of the protocol are properly described and could be replicated elsewhere. I believe this protocol provides important information on how to manipulate mice, isolate cell lines and treat them to achieve X chromosome reactivation.” The reviewer has recommended publication with minor revision. We have addressed all the comments of reviewer and our point-by-point response is detailed below:

Minor/Major Concerns:

In 1.1, I would state the background of the Mecp2tm3.1Bird mice. At the end of the paragraph, it is written that Table I states the strains and respective progenies, although this Table I is nowhere to be found in the document.

We have indicated the background in the Table of Materials and correctly cited in text.

In 3.11, the authors state 'Repeat steps 3.5-3.12 for next embryo', which reads as all the previous steps have to be done sequentially. Is this correct? If not, I would write it differently in such a way that all embryos are processed in parallel, not in sequence.

We have revised the text to clarify the steps of harvesting embryo.

In 5.2, please add here the concentration at which the inhibitors are used (on top of what is stated in Results).

The text has been revised to include inhibitor concentrations.

In 5.5, the authors say that the staining of MEFs with an anti-GFP primary antibody has been described previously in ref 12. I could not find that description in that paper. Maybe the authors wanted to refer to ref 18? (Although within ref 18, the authors refer to ref 7).

We thank reviewer for pointing the reference. We have revised to include the correct citation.

In 6.1.2, it is stated that the inhibitors LDN193189 and GSK650394 were used to inject the mouse brains. LDN193189 is an inhibitor of ACVR1. However, GFK650394 is an inhibitor of SGK1, not PDPK1 as is suggested throughout the paper in the abstract, results and discussion sections. In fact, I would clearly state, either in 6.1.2 (line 184) or in line 69 which inhibitors are used and which

factors they inhibit. I had to look into ref 18 to make the connection between the inhibitors and their targets.

We have revised the text to clarify that GSK650394 inhibits downstream effector of PDPK1.

Lines 299-301 state that within the population of skewed MEFs, there is a 3% of cells that are GFP positive and that there is 31% in the drug-treated conditions. Those values are not shown in Fig.1E. You can add them to the figure as you did in ref 18 Fig5B. On another note, how come that 3% population cannot be seen in Fig1C by FACS? Because the IF is more sensitive than FACS?

To clarify, Fig.1C shows the GFP signal in the brain cells. However, Fig.1E are the results from the MEFs isolated from the mouse embryo. Because signal is weaker in MEFS, we utilized anti-GFP antibody, which has some background associated with staining. Therefore, we see ~3% GFP positive signal. We choose not to show the percentage of the cell to minimize the confusion to the readers.

Related to Fig 2C and D, how come the GFP signal is not nuclear as one would expect from an Mecp2-GFP fusion protein?
In Fig2C, endogenous GFP fluorescence is measured, if I read correctly. But in Fig2D, an anti-GFP (far red) and anti-MAP2 (green) antibodies are used. Can't the endogenous GFP signal confound the MAP2 signal?

To clarify, the endogenous GFP signal is majorly nuclear. However, due to the antigen retrieval step in the staining of brains sections with anti-GFP, we see the bleaching of Mecp2-GFP in the cytosol.

In line 92, it is said Table I, although in line 172 it is written Table 1 (which should be Table S1). Lines 313 and 347 should also read Table S1. Make sure you use Table I, Table 1 or Table S1 correctly.

We have corrected throughout the manuscript.

In line 96: Paternal inheritance of a Xist KO allele is not deleterious to random XCI, which is why the authors should add 'imprinted' before XCI.

We have corrected in the manuscript.

Ioxidinol should read ioxidanol throughout the manuscript.

We have corrected throughout the manuscript.

Line 158: Supernatant instead of supernate.

We have corrected throughout the manuscript.

Line 256: -20ºC, not approximately 20ºC.

We have corrected throughout the manuscript.

As a general comment, I would carefully review the usage or absence of definite and indefinite articles.

We have corrected throughout the manuscript.

Reviewer #3:
We thank reviewer 3 for his/her positive comments, “The authors have described the useful protocol for visualizing reactivation from the inactive X-chromosome in MEF and brain. It enables to evaluate the feasibility and tolerability of drugs these are candidates for alleviating symptoms of the X-linked gene diseases such as Rett syndrome and DDX3X syndrome. I think the protocol described here are very useful and beneficial in general and the protocol itself is well written for enabling the reproducibility. Therefore, I recommend that the report is suitable for publishing in the journal.”

Minor comments:
1) At line 137, the authors described the speed of centrifuge as 1000 RPM. However, the gravity is different between centrifuges even though if the same 1000 RPM is selected. For the reproducibility, gravity is much important than the centrifuge speed. Therefore, I recommend for describing the degree of centrifuge, not only RPM, but also the gravity, for example, 1,000 rpm (2,000g). Describing the gravity at all of the other RPM parts, as well.

We have corrected throughout the manuscript.

2) At line 169, it might be, "as negative control, respectively".

We have corrected throughout the manuscript.

3) Table S1, I recommend to add columns of "expected length" of the PCR products.

We have included the expected length in the Table1.

4) Figure1, A. It's better to show that the mother is right, and the father is left. The genotype of the pups must be "Xist∆:Mecp2/Xist:Mecp2-GFP" but not "Xist:Mecp2-GFP/Xist∆:Mecp2".

We respectfully disagree with the reviewer as we have previously published the same figure.

5) Figure1, B The length of the PCR product must be described.

We have included the expected length in the Figure 1B.

6) Figure1, C. The arrow of GFP negative and red peak are overlapped. They should be separated, but not overlapped.

We have corrected in the Figure 1.

7) Figure 1D. The length of the PCR product must be described.

We have included the expected length in the Figure 1D.

8) Figure 2C. I imagine that the upper panel is "vehicle" and the lower is "drug". The information must be described.

We have included the information in the Figure 2C.

9) Figure 2D. I imagine that the figure is only "drug". However, the figure legend was written as "vehicle- and drug- treated himispheres." (line 344). Which is correct? They must show the result of both of vehicle/drug at the figure 2D or the figure legend must be corrected.

We have corrected the figure legend for Fig.2D.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have carefully revised the manuscript.

2. Please revise lines 40-44, 116-119, 210-212, 218-221, 227-231, 237-243 to avoid previously published text.

We have revised the text.

3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

The permission has been obtained from PNAS and include in the revised material.

4. Figure 1: Please line up the panel labels better.

The figures have been corrected.

5. Figure 2: Please include a space between the number and the units of the scale bar.

The figures have been corrected.

6. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

The appropriate.ai files have been uploaded.

7. Table 1: Please upload it to your Editorial Manager account as an .xls or .xlsx file.

The appropriate.xls files have been uploaded.

8. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

The required changes have been made.

9. Please provide an email address for each author.

We have included emails for all the authors.

10. Please add a Summary section before the Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

We have added the summary.

11. Please define all abbreviations before use.

We have revised the text.

12. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u. Please abbreviate liters to L to avoid confusion.

We have revised the text.

13. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.

We have revised the text.

14. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

We have revised the text.

15. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: The Jackson Laboratory, Thermo Fisher Scientific, Sigma, Beckman, BioLegend, BD FACScalibur, Life Technologies, Styrofoam, Zeiss AxioObserver, etc.

We have included all the information in Table of Materials.

16. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have revised the text.

17. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

We have revised the text.

18. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

We have revised the text as recommended.

19. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
20. Line 121, 6.4.5: How large is the incision?

We have included the information.

21. Please specify all surgical tools used throughout the protocol.

We have included all the information in text.

22. 3.11: Do you mean “3.5-3.10” instead of “3.5-3.12”?

We have revised the text.

23. 3.12: Please specify the primers and conditions used for genotyping-PCR.

We have included all the information in table.

24. 5.1: Please mention the conditions for growing these cells.

We have revised the text.

25. 5.2: Please describe how the treatment is actually done.

We have revised the text.

26. 5.3: Please specify how cells are harvested.

The steps have been included in 5.4 and 5.5.

27. 6.2.1: Please specify the disinfectant used in this step.

We have revised the text.

28. 6.2.2: Please mention how proper anesthetization is confirmed.

We have revised the text.

29. Please include single-line spaces between all paragraphs, headings, steps, etc.

We have revised the text.

30. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted the text for video filming.

31. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.

We have highlighted the text for video filming.

32. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have revised the text.

33. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

We have revised the text.

34. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

We have included the disclosure statement.

35. References: Please do not abbreviate journal titles.

We have corrected the references

UNIVERSITY of VIRGINIA

Sanchita Bhatna

RE: Request for re-using some figures

1 message

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We recently published a manuscript in PNAS " Proc Natl Acad Sci U S A. 2018 Jul 31;115(31 10.1073/pnas.1803792115. Epub 2018 Jul 16. Pharmacological reactivation of inactive X-link cortical neurons of living mice."

We are submitting a Methods manuscript in JOVE regarding the mouse model that was inclu and will like to re-use a part of the figure included in the manuscript. I am writing to ask for the the modified version of the figures. Can you please approve my request.

Thanks in a advance.

Best Regards

Sanchita

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