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.