Establishment of a reporter line for detecting fragile X mental retardation (*FMR1*) gene reactivation in human neural cells

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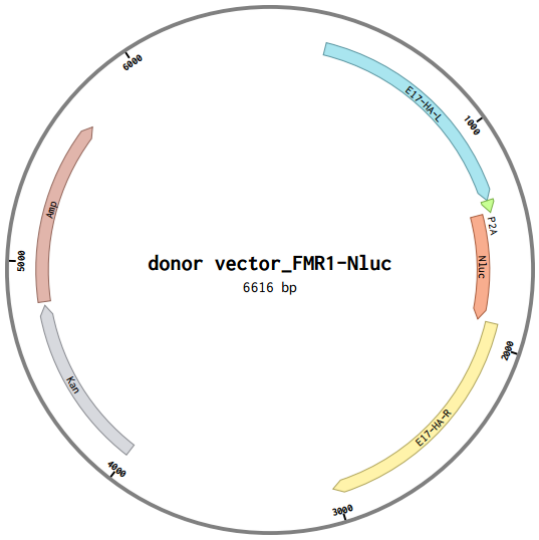
Figure S1.

A



B

P2A; Nluc; Homology arms; Mutated nucleotides (silent mutations)

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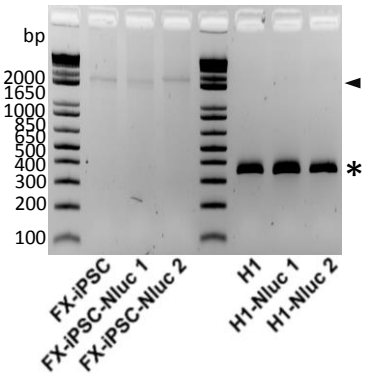
Supplementary Figure S1. Donor plasmid design genome editing.

A) Map of donor vector.

B) Sequence after genome editing. P2A, Nluc, homology arms (HA in Figure 1A; 5' HA, 1072 bp; 3' HA, 1115 bp), and silent mutations are indicated.

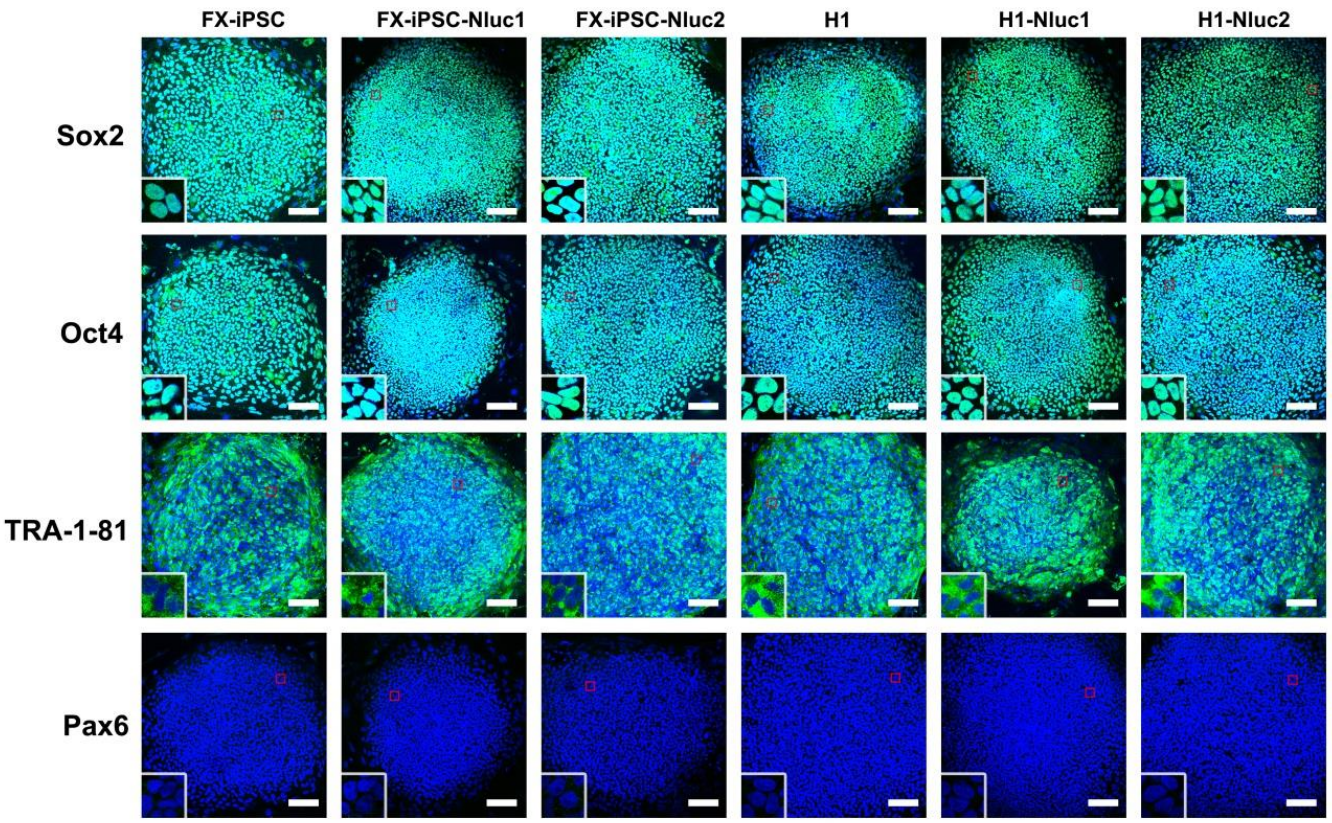
Figure S2.



Supplementary Figure S2. CGG repeats were preserved in PSC reporter lines

PCR amplification of CGG repeats from hPSCs. Arrow head indicates the expanded CGG in FXS. Asterisk indicates normal size of CGG repeat in control individuals.

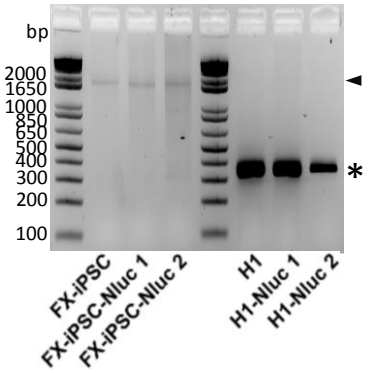
Figure S3.



Supplementary Figure S3. Nluc reporter lines express pluripotent cell markers but not neuroepithelial cell marker.

immunocytochemical analyses of reporter and parental cells using pluripotent cell markers Sox2, Oct2, TRA-1-81, and neuroepithelial cell marker Pax6. (scale bar = 200 μm). The markers are shown in green and DAPI nuclear stain is in blue.

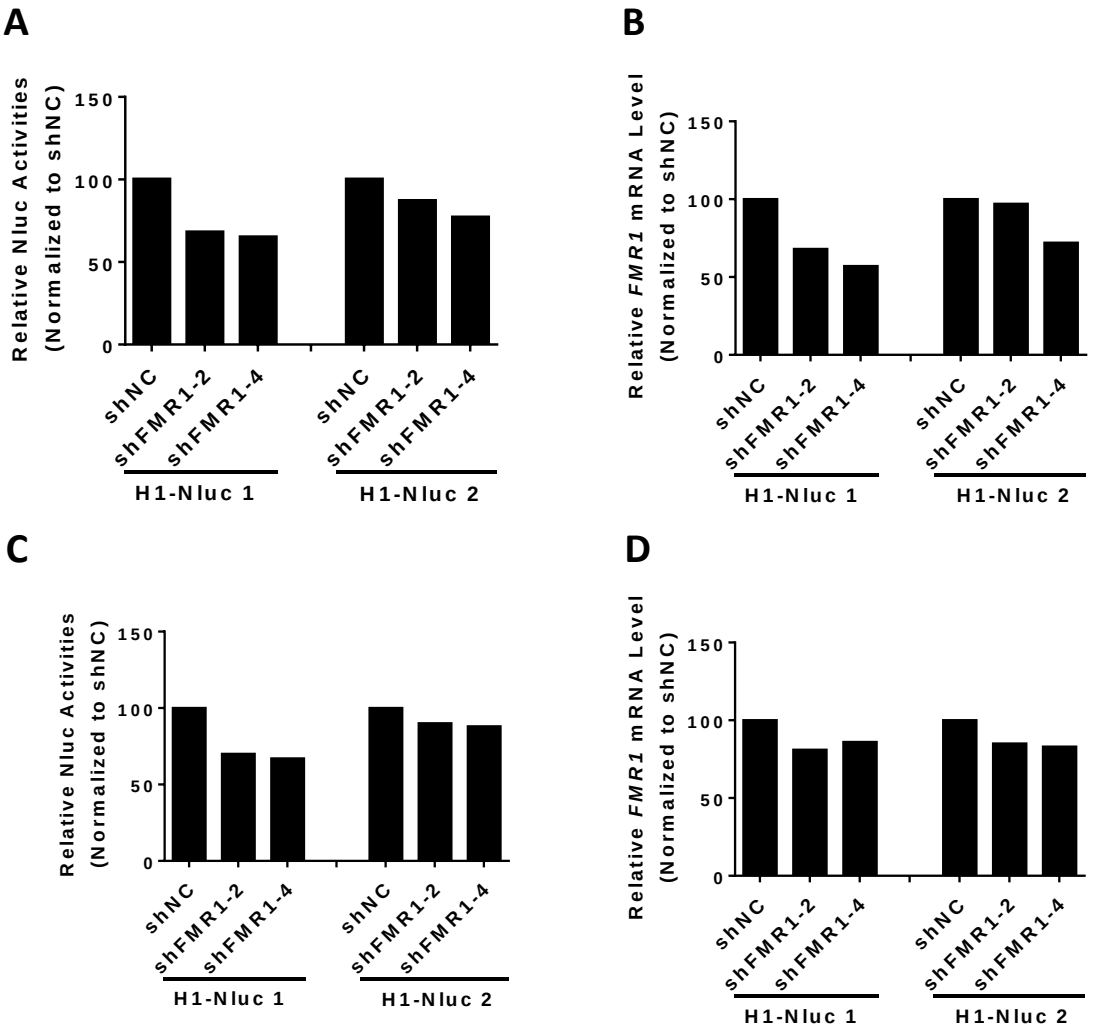
Figure S4.



Supplementary Figure S4. CGG repeats were preserved in NPCs reporter lines .

PCR amplification of CGG repeats from NPC reporter lines and parental lines. Arrow head indicates the expanded CGG in FXS. Asterisk indicates normal size of CGG repeat in control individuals.

Figure S5.

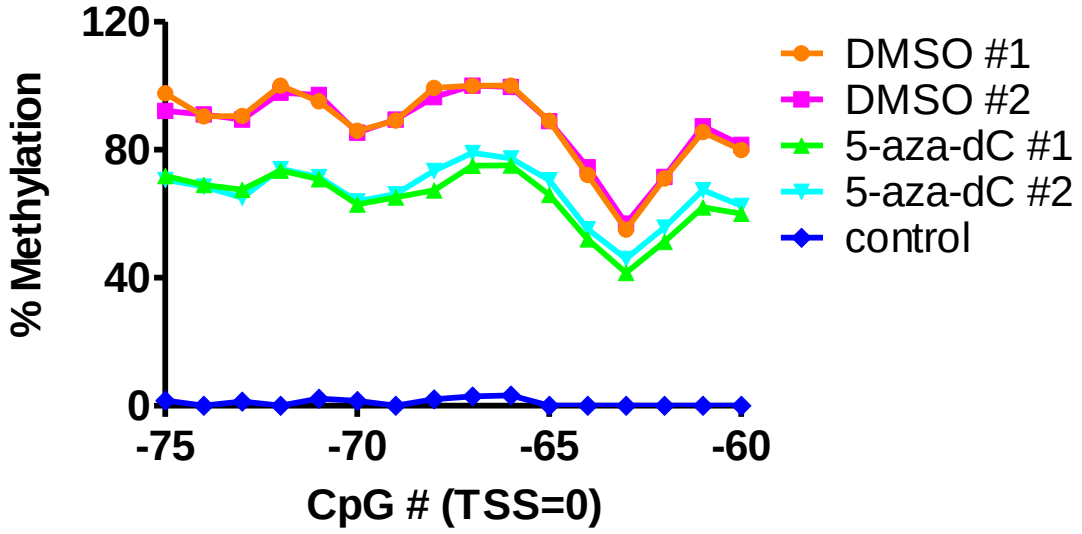


Supplementary Figure S5. shRNA knock-down of *FMR1* in NPCs of H1-Nluc lines.

NPCs of H1-Nluc lines were transfected with FMR1 shRNAs (shFMR1-2 and shFMR1-4) or scrambled shRNA (shNC) followed by luciferase assay (A) or RT-qPCR (B).

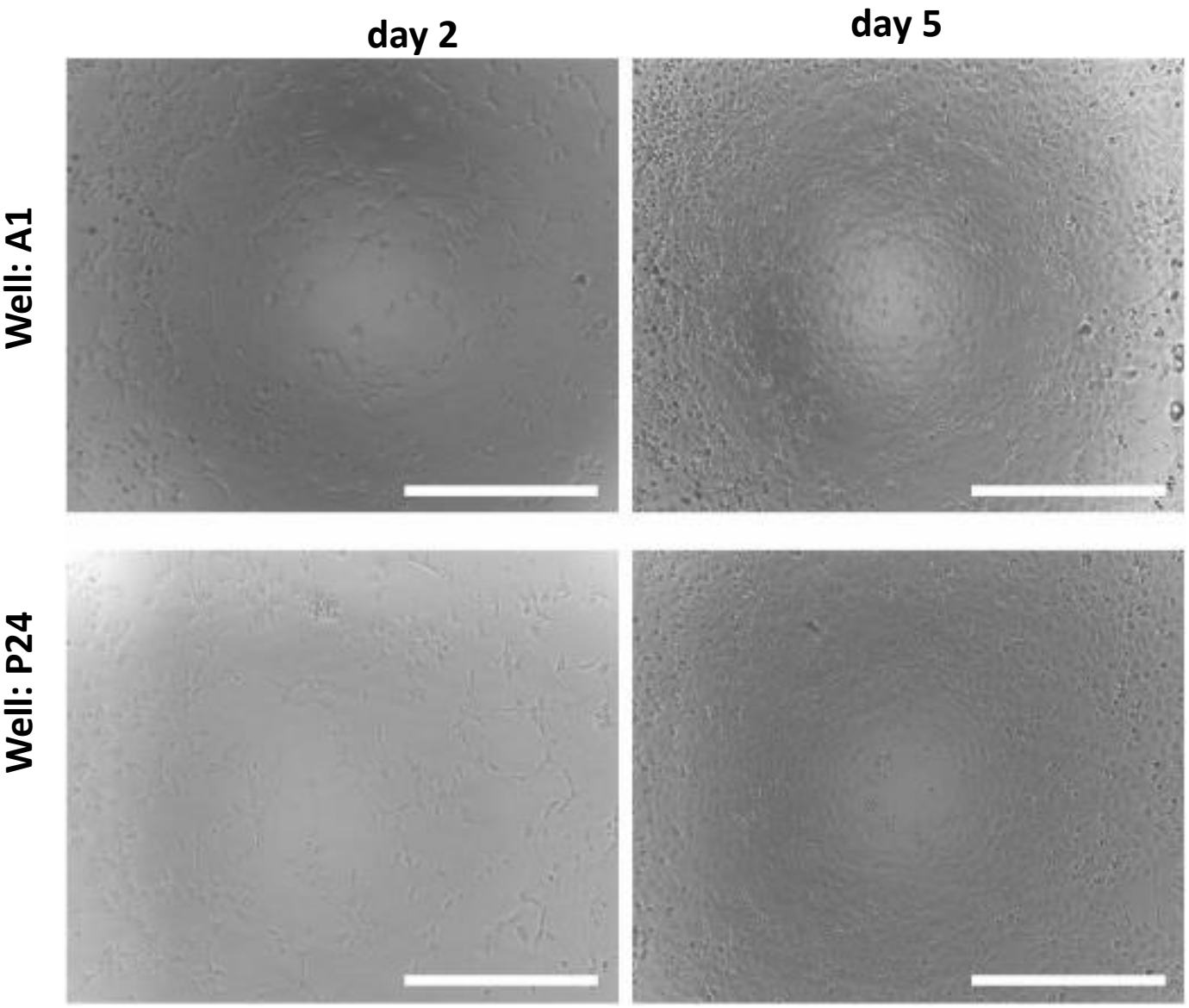
(C) and (D) are repeats of (A) and (B) using different sets of cells and transfection.

Figure S6.



Supplementary Figure S6. 5-aza-dC treatment reduced DNA methylation of *FMR1* promoter in FX-iPSC-Nluc 1 NPCs. FX-iPSC-Nluc 1 NPCs were treated with a single dose of 0.03 uM 5-aza-dC or DMSO carrier for three days followed by pyrosequencing analysis of DNA methylation of 16 CpG positions (-328bp to -256bp to TSS) in FMR1 promoter (n=2). H1-Nluc 1 NPCs without DMSO or 5-aza-dC treatment were assayed as a control.

Figure S7.



Supplementary Figure S7. Enlarged images of Figure 6B
Cells in a corner well (A1) and a center well (P24) in 1536-well plates (Fig. 6A) were imaged in bright field on day 2 (the day after plating) and day 5 (the day of cell collection and luciferase assay), showing good cell survival and growth in 1536-well plates (scale bar = 400 µm).

Supplementary Materials and methods

PCR amplification of CGG repeats: Amplification of CGG repeats and flanking sequences was performed with AccuPrime Pfx DNA Polymerase (Thermo Fisher Scientific) in 15 ul reactions containing 0.15 ul of Pfx polymerase, 1x Reaction Mix, 2.5 M Betaine (Sigma), 7.5% DMSO (Sigma), 50 ng of genomic DNA, and 0.3 uM of each primer CGG-F (TCAGGCGCTCAGCTCCGTTTCGGTTTCA) and CGG-R (AAGCGCCATTGGAGCCCCGCACTTCC) (Filipovic-Sadic et al., 2010). Samples were amplified with an initial denaturation step of 98°C for 5 min; 35 cycles of 98°C for 35 s, 64°C for 35 s, and 68°C for 2 min; and a final extension step at 68°C for 5 min. The PCR products were resolved by 2% agarose gels. Specific amplification of CGG repeats was confirmed by sequencing of PCR products.

Karyotyping: G-banding was performed by WiCell cytogenetics (Madison, WI).

RNA isolation and qPCR analysis. Total RNA from cells was isolated with TRIzol reagents (Thermo Fisher Scientific) following manufacturer's instruction. 1ug of total RNA was converted to cDNA in a 20ul reaction using Transcriptor First Strand cDNA Synthesis Kit (Roche) and oligo dT primer. qPCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) and StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). For pluripotent stem cells, data were collected from cells of three different passages. Data for NPCs were from three batches of differentiated cells. Primers that were used to quantify *FMR1* mRNA, *Nluc* mRNA, and housekeeping gene *GAPDH* are listed below. Statistical significance was analyzed by either student T-test (Fig. 2A, 2D, and 3D) or one-way ANOVA with post-hoc tests (Fig. 4F).

PCR primers

Name	Application	Sequence (5'-3')
sgFMR1-F	sgRNA cloning	CACCGTAGGGTACTCCATTCACGAG
sgFMR1-R		AAACCTCGTGAATGGAGTACCCTAC
Nluc-F	amplifying P2A-Nluc	GCCACTAACTTCTCCCTGTTGAAACAAGCAGGGGAT GTCGAAGAGAATCCTGGTCCAATGGTCTTCACACTCGAAGATT
P2A-F		TCTAGTTAACGGAGTACCCGCCACTAACTTCTCCCTGTTGA
Nluc-R		CCATTCACTTACGCCAGAATGCGTTCGCACA
HA-L-F	amplifying 5' homology arm	TACCGAGCTCGGATCCACATGCACATACCCACTACTT
HA-L-R		ACTCCGTTAACTAGAGGTTGCTGACCATCCACGCTGTC
HA-R-F	amplifying 3' homology arm	ATTCTGGCGTAAGTGAATGGAGTACCCTAAACTGC
HA-R-R		CATGCTCGAGCGGCCACCTGCTTCCAATGTTTCTTAGAC
Red-F	colony screening and sequencing	TGGATGCAGTGAGATGACCAG
Red-R		TCTGCCCTGAAGTGCTAAGT
Green-F	colony	ACTGAATCCTTGGTAACGAGACA

Green-R	screening	CACCGCTCAGGACAATCCTT
Black-F	detection of random insertion of donor vector	TGTAAAACGACGGCCAGT
Black-R		ACCATTTTCCTGCATTGGGTGA
Grey-F	detection of random insertion of donor vector	CATTAGGCACCCCAGGCTTT
Grey-R		ACTCCGTTAACTAGAGGTTGCTGACCATCCACGCTGTC
hGAPDH-qF1	qPCR for *GAPDH**	AGCCACATCGCTCAGACACC
hGAPDH-qR1		AACTGGGATAACCGGATCGAT
FMR1-qF5	qPCR for *FMR1*	GCAAATGTGTGCCAAAGAGGC
FMR1-qR5		CTCCGAAAGTGCATGTCAATCAG
Nluc-qF2	qPCR for *Nluc*	CACTGGTAATCGACGGGGTT
Nluc-qR2		TTTTGTTGCCGTTCCACAGG

*, Note Human hGAPDH primers were published previously (Bar-Nur et al., 2012).

Western blotting Primary antibodies used in this study are listed below. Cells were lysed in RIPA buffer (50 mM Tris, pH8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS) supplemented with protease inhibitor (Sigma). After centrifugation for 15 min at 4 degrees, supernatants were resolved by SDS-PAGE, transferred to PVDF membrane, blocked with 5% nonfat milk, and probed with primary antibodies. Secondary antibodies used were IRDye 800CW (LI-COR, Cat #: 92632210) for FMRP and IRDye 680LT (LI-COR, Cat #: 92668021) for GAPDH at dilution of 1:10,000. Bands were visualized with an Odyssey Imager (LI-COR).

Immunofluorescence and confocal imaging. Primary antibodies used in this study are listed below. Cells on coverslips were fixed in 4% PFA for 10 min. Then cells were washed with PBS and blocked with PBST (PBS containing 0.2% Triton X-100) plus 10% normal goat or donkey serum (Sigma), followed by incubation with primary antibodies diluted in PBST plus 10% serum for overnight at 4°C. Cells were then washed 3 x 5 min with PBS. Fluorescently labeled secondary antibodies (Thermo Fisher Scientific) were diluted in PBST plus 5% serum and incubated with cells for 1 hour at room temperature. Cells were washed 2 x 5 min with PBS and counterstained with DAPI. Cells on coverslips were then washed 2 x 5 min with PBS and mounted on slides with DABCO. Images of the cells were collected with Nikon A1 confocal microscope. .

Primary antibodies.

Name	Application	Dilution	Company	Cat. #	Host
FMRP	WB/ICC	1:1000	Thermo Fisher Scientific	MA5-15499	mouse

GAPDH	WB	1:5000	Thermo Fisher Scientific	TAB1001	rabbit
Nestin	ICC	1:1000	Santa Cruz	SC-21247	goat
PAX6	ICC	1:1000	Covance	PRB-278P	rabbit
OCT4	ICC	1:1000	Santa Cruz	SC-5279	mouse
SOX2	ICC	1:1000	R&D	MAB2018	mouse
TRA-1-81	ICC	1:1000	Millipore	MAB4381	mouse

shRNA knock-down of *FMR1* in H1-Nluc NPCs. *FMR1* and control shRNAs used in this study were from GeneCopoeia, including a scrambled control shRNA (Cat #: CSHCTR001-LVRU6GH; shNC, gcttcgcgccgtagtctta) and two FMR1 shRNAs (Cat #: HSH005998-LVRU6GH; shFMR1-2, tggtggttagctaaagtga; shFMR1-4, ttgcctcgagatttcatga). Knock-down experiments were performed in 24-well plates. On day 0, 3×10^5 NPCs/well of H1-Nluc lines were plated in Matrigel-coated plates. On day 1, a transfection solution, consisting of 23.5 ul of NPC medium, 1.5 ul of FuGENE HD (Promega), and 0.5 ug of shRNA plasmid, was added to cells. Medium was changed 24 hours after transfection. Luciferase assay, CellTiter assay, and qPCR analysis were performed on day 6 as described above.

DNA methylation assay of *FMR1* promoter. 5×10^5 cells/well of FX-iPSC-NLuc 1 NPCs were plated in Matrigel-coated 6-well plates. 24 hours later, 0.03 uM 5-aza-dC or DMSO (0.01% v/v) was added to cells. Three days after adding 5-aza-dC or DMSO, cells were collected for DNA methylation assay of *FMR1* promoter (EpigenDx, Assay ID: ADS1451-FS2). Non-treated H1-Nluc 1 NPCs were also assayed as a control.

References

Bar-Nur, O., Caspi, I., and Benvenisty, N. (2012). Molecular analysis of FMR1 reactivation in fragile-X induced pluripotent stem cells and their neuronal derivatives. Journal of Molecular Cell Biology *4*, 180-183.

Filipovic-Sadic, S., Sah, S., Chen, L., Krosting, J., Sekinger, E., Zhang, W., Hagerman, P.J., Stenzel, T.T., Hadd, A.G., Latham, G.J., *et al.* (2010). A novel FMR1 PCR method for the routine detection of low abundance expanded alleles and full mutations in fragile X syndrome. Clinical chemistry *56*, 399-408.