Stem Cell Reports, Volume *10*

Supplemental Information

Loss of MECP2 Leads to Activation of P53 and Neuronal Senescence

Minori Ohashi, Elena Korsakova, Denise Allen, Peiyee Lee, Kai Fu, Benni S. Vargas, Jessica Cinkornpumin, Carlos Salas, Jenny C. Park, Igal Germanguz, Justin Langerman, Contantinos Chronis, Edward Kuoy, Stephen Tran, Xinshu Xiao, Matteo Pellegrini, Kathrin Plath, and William E. Lowry

Supplemental information

Extended Materials and Methods

Generation of isogenic Rett Syndrome iPSCs

Two primary fibroblast lines GM17567 (1461A>G in the gene encoding methyl-CpG binding protein 2 (MECP2)), and GM07982 (frameshift mutation, 705delG, in the gene encoding methyl-CpG binding protein 2 (MECP2)), from patients with Rett Syndrome were obtained from Coriell Cell Repositories (Described in Fig S1). After 8-12 hours, the cells were infected with reprogramming lentivirus that harbors polycystronic human Yamanaka factors (Oct4, Klf4, Sox2, cMyc) in DMEM medium containing 10ug/ml of polybrene and incubated overnight at 37^{o}C in 5% CO2 incubator. On day 6, the culturing media was changed to human ES media containing DMEM/F12 supplemented with L-glutamine, nonessential amino acids (NEAA), penicillin-streptomycin, knockout serum replacement (Invitrogen), and 10 ng/ml basic FGF. Cells were cultured in hiPSC media until iPSC-like colonies were formed.

Generation of teratomas

Generation of teratoma was previously described(Lindgren et al., 2011). Briefly, a single incision was made in the peritoneal cavity of adult SCID mice and the testis was explanted through the incision site. Approximately 60,000 iPSC in a volume of 50 ml 0.5X Matrigel (BD) were transplanted into the testis using a 27-gauge needle. Four to six weeks after surgery, mice were euthanized and the tumors removed for histology.

Differentiation in vitro and analysis

Neural specification with neural rosette derivation, neuroprogenitor (NPC) purification, and further differentiation to neurons and glia were performed as described previously (Karumbayaram et al., 2009; Patterson et al., 2011; Patterson et al., 2014). For spontaneous terminal neuronal differentiation by growth factor withdrawal, NPC cultures were subjected to growth factor withdrawal (removal of EGF and FGF) and cultured in basic medium (DMEMF12 + N2 + B27) with three quarter exchange of media every three days. Neural differentiation efficiency was analyzed four weeks after growth factor withdrawal by counting the number of cells positive for neuronal markers (MAP2 and Tuj1) over the total number of cells visualized by DAPI. NPCs were transfected with *DCX-GFP* reporter one day prior to differentiation using Lipofectamine 2000 (Invitrogen). Sholl analysis of DCX-GFP positive neurites were also measured using ImageJ. All data values were presented as mean +/- SEM. For directed differentiation of interneurons, iPSCs were grown on plates coated with matrigel (Corning) until 80% confluency with mTeSR (Stem Cell Technologies). Cells were then treated with DMEM/F12 (GIBCO) containing NEAA (GIBCO), GlutaMAX (GIBCO), bovine serum albumin (Sigma-Aldrich), ß-mercaptoethanol (Sigma-Aldrich), N2 (GIBCO), B27 (GIBCO), SB431542 (10uM; Cayman Chemical), LDN-193189 (100nM; Cayman Chemical) and XAV939 (2uM; Cayman Chemical) later transitioning to the media containing sonic hedgehog (20ng/mL; R&D) and purmorphamine (1uM; Cayman Chemical) as previously described (Maroof et al., 2013). Cells were further differentiated into interneurons with neurobasal medium (GIBCO) containing N2 (GIBCO), B27 (GIBCO), ascorbic acid (Sigma-Aldrich), GlutaMAX (GIBCO), bovine serum albumin (Sigma-Aldrich), ß-mercaptoethanol (Sigma-Aldrich),

neurotrophin-3 (10ng/mL; R&D), brain-derived neurotrophic factor (10ng/mL; R&D), and glial cell-derived neurotrophic factor (10ng/mL; R&D).

Western blot

Cells were lysed on ice with RIPA buffer (Pierce) that contains Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). The total protein concentration was measured using BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's protocol. Supernatant was electrophoresed onto NuPAGE 4-12% Bis-Tris Protein Gels (Invitrogen) using MOPS running buffer (Invitrogen). The membrane was blocked with 5% non-fat milk for 1 hr and incubated overnight with primary antibodies at 4°C.

Immunofluorescence and image quantification

Cells on coverslips were washed with PBS, fixed in 4% paraformaldehyde for 15 min at room temperature, blocked for 1 hr at room temperature with 10% serum and 0.1% Triton-X-100, then incubated overnight at 4 °C with primary antibodies. Following primary antibody incubation, the coverslips were incubated with Alexa Fluor (Invitrogen) or Jackson Immunoresearch secondary antibodies at room temperature for 1 hr. Cells were counterstained with DAPI and mounted in Prolong Gold (ThermoFisher). Antibodies used include the following: mouse anti-OCT3/4 (1:100, Santa Cruz Biotechnology Inc., sc-5279), rabbit anti-SOX2 (1:300, Cell Signaling Technology, 3579), rabbit anti-Nanog (1:100, Cell Signaling Technology, 4903), mouse anti-Tra-1-81 (1:250, Chemicon, MAB4381), mouse anti-NESTIN (1:1000, Neuromics, MO15012), chicken anti-MAP2 (1:2000, Biolegend, PCK-554P), chicken anti-GFAP (1:2000, Abcam, ab4647), rabbit

anti-Tubulin β3 (1:500, Covance, MMS-435P), mouse anti-P53 (1:500, Cell Signaling, 2524), rabbit anti-p21 (1:250, Santa Cruz, sc-397), mouse anti-PML (1:100, Santa Cruz, sc-9862), mouse anti-phospho-Histone H2A.X (1:2000, EMD Millipore, 05-636), rabbit anti-5hmc (1:100, Active Motif, 39791), rabbit anti MECP2 (1:1000, Diagenode, pAb-052-050), rabbit anti Foxg1 (1:1000, Abcam, ab18259), and mouse anti NKX2.1 (1:300, Novocastra, NCL-L-TTF-1). Secondary antibodies conjugated with Alexa 488, 568, 594, 647 (1:500, Life Technologies, A-21203, A21202, A31571, A-21207) were used. Mean intensity or a number of foci were quantified using ImageJ (http://rsb.info.nih.gov/ij/). At least 100 cells per condition were used for each independent experiment.

RT-qPCR

RNA from cultured cells was collected using the RNeasy Mini Kit from Qiagen according to the manufacturer's instructions. RNA with an A260/A280 ratio in between 1.8 and 2.0 as well as an A260/A230 ratio in between 2.0 and 2.2 was used. RNA was then reverse transcribed using the Super Script III First-Strand cDNA Synthesis kit with Random Hexamers (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed using SYBR Green master mix (Roche). Reactions were performed in duplicate and duplicate CT values were averaged and then used for standard ΔΔCT analysis. Expression levels were normalized to beta actin.

Primer Sequences

MECP2	*GCTCTGCTGGGAAGTATGATG*
MECP2	*ATGTGTCGCCTACCTTTTCG*
P53_F	*GCCCAACAACACCAGCTCCT*
P53_R	*CCTGGGCATCCTTGAGTTCC*

P21-F	*AAAGAAGAACGGAGCGAACA*
P21-R	*CTCCGCTCAATTTCCAAGAG*
GADD45G-F	*TACGCTGATCCAGGCTTTCT*
GADD45G-R	*AACAGGCTGAGCTTCTCCAA*
DDIT4_F	*GTTTGACCTCTCCACCAGCCT*
DDIT4_R	*GCACACAAGTGTTCATCCTCAGG*
DDB2_F	*TCACTTCCAGCACCTCACAC*
DDB2_R	*ACGTCGATCGTCCTCAATTC*
SFN_F	*GTGTGTCCCCAGAGCCATGG*
SFN_R	*ACCTTCTCCCGGTACTCACG*

Data collection and statistical analysis

All the experimental data (RT-qPCR, immunostaining, ß-Galactosidase Senescence Assay) were presented as mean +/- SEM based on at least three biological replicates from independent experiments. Student's t-tests were applied to data with two groups. ANOVA analyses were used for comparisons of data with greater than two groups. A *p-value* < 0.05 was considered as statistically significant.

siRNA gene silencing

All knockdown experiments were performed using trilencer siRNAs (from OriGene Technologies) and RNAimax (ThermoFisher) in Opti-MEM media (ThermoFisher). Trilencers were used at a concentration of 20 nM. Transfection media was prepared and then 500,000 cells were plated on top of the transfection media in 6-well plates. The medium was changed to normal NPC media the next day and cells were collected for analysis at the time points indicated.

ß-Galactosidase Senescence Assay

ß-Galactosidase Senescence Assay was performed using the Senescence β-Galactosidase Staining Kit from Cell Signaling according to manufacturer's instructions. Briefly, the cells were fixed on coverslips, incubated with X-gal overnight at 37°C, then mounted on glass slides and imaged using a brightfield microscope. The number of blue cells and number of total cells were quantified using the Cell Counter plugin in ImageJ.

Quantification of Dendritic Arborization

Neuronal cultures were immunostained for Tuj1 in order to identify mature neurons and visualize entire cells. The stained cells were then imaged at 20x and dendritic arbors of individual cells were traced using the Simple Neurite Tracer plugin for ImageJ. The number of process ends per cell were counted using the Cell Counter plugin for ImageJ. The number of process ends per cell are presented as mean ends per cell +/- SEM. Means were compared using the Student's t-Test for data with two groups.

RNA expression profiling

Libraries were prepared according to the manufacturers guidelines using The TruSeq V2 kit (Illumina). For RNA sequencing, the datasets were mapped with RASER and HISAT2. Genes were defined by the exon union from the hg19 ensembl annotations. The function of DESeq in DESeq2 package was used to first normalize the gene read counts data and then identified the differentially expressed genes. The MA plot was generated with the function of plotMA in DESeq2 package. Q-value of 0.05 is regarded as the stringent cutoff of calling DEGs while p-value less than 0.05 is regarded as the low stringency cutoff. For the meta-chromosome plot of DEGs, all the chromosomes (except chromosome Y) were first divided equally into 20bins with different length, and then the number of DEGs in

each bin was counted. GO analysis was performed using DAVID. These data are available from NIH GEO Dataset GSE107399.

Figure S1. Description of patient source for fibroblasts for reprogramming. Supplementary to Figure 1.

Patient data, taken from NIH BioBrainBank repository

Patient 982

Description:
RETT SYNDROME; RTT
METHYL-CPG-BINDING PROTEIN 2; MECP2
Affected: Yes
Gender: Female
Age: 25 YR (At Sampling)

Remarks: Clinically affected; microcephaly; scoliosis diagnosed at age 12; severe kyphoscoliosis at age 25; early milestones were slow; started losing skills at age 2; currently severely retarded; behavioral phenotype includes hand wringing that began at age 2 and became more intense; no sleep problems; no self-injurious behavior; abnormal EEG; CT scan at age 25 showed evidence of atrophy; donor subject carries a frameshift mutation, 705delG, in the gene encoding methyl-CpG binding protein 2 (MECP2); see GM07983 lymphoblast.

Patient data 567

Description:
RETT SYNDROME; RTT
METHYL-CPG-BINDING PROTEIN 2; MECP2
Affected:Yes
Gender:Female
Age:5 YR (At Sampling)

Clinically affected; onset between 15-20 months of age; seizures began at age 3; never walked independently; began to develop repetitive hand movements at 28 months; no hand use; small feet; language regression at 18 months; some sleep problems; nonverbal; significantly abnormal EEG; swallowing difficulties, reflux, and breathing problems; teeth grinding; decelerating head circumference; growth retardation; seizures; donor subject has a missense mutation (A>G) at nucleotide 1461 (1461A>G) in the gene encoding methyl-CpG binding protein 2 (MECP2), resulting in a substitution of a tryptophan for a stop codon at codon 487 [TER487TRP (X487W)].

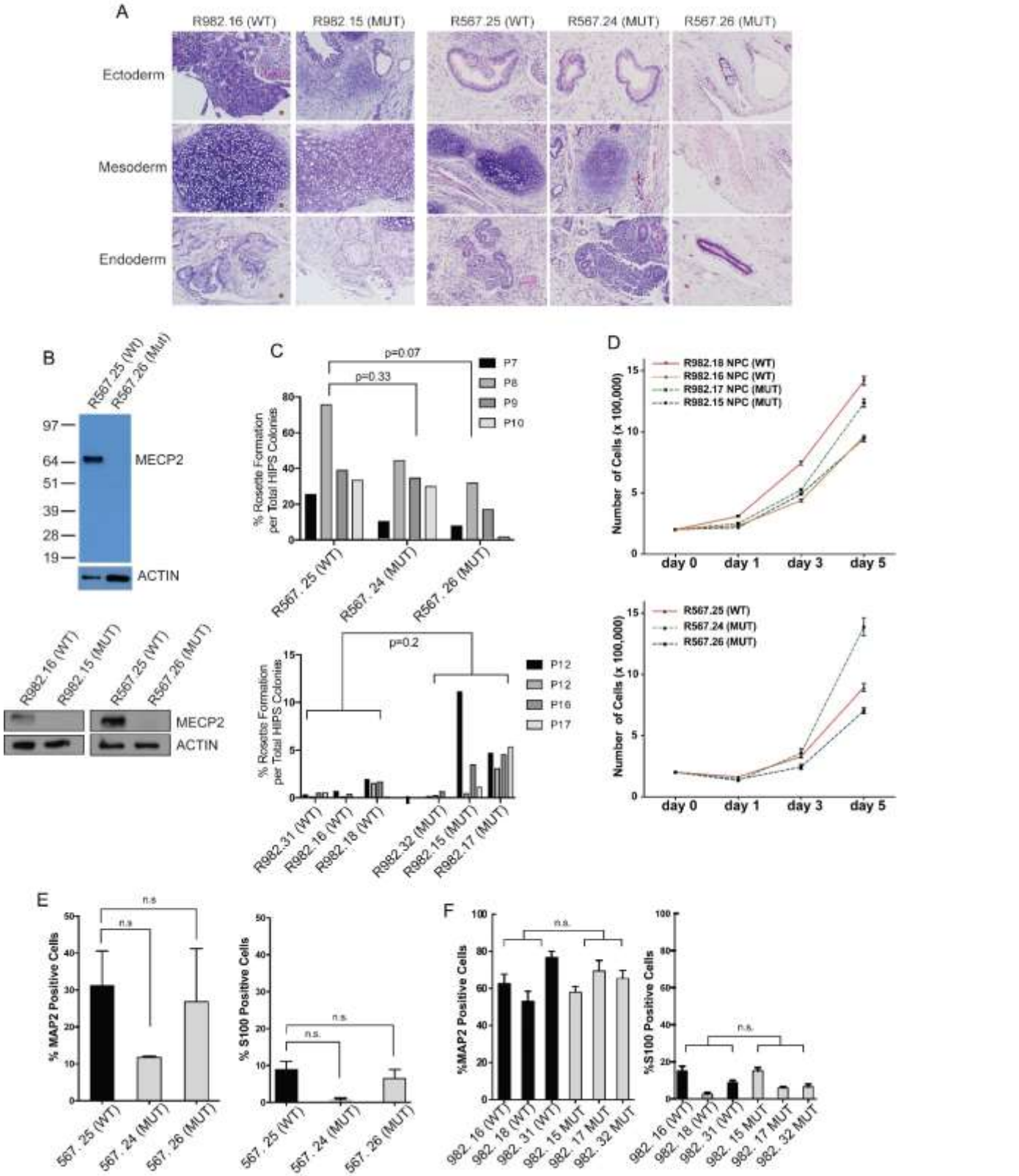
A
R982.16 (WT)
R982.15 (MUT)
R567.25 (WT)
R567.24 (MUT)
R567.26 (MUT)
Ectoderm
Mesoderm
Endoderm
B
R567.25 (Wt)
R567.26 (Mut)
97
64
51
39
28
19
MECP2
ACTIN
R982.16 (WT)
R982.15 (MUT)
R567.25 (WT)
R567.26 (MUT)
MECP2
ACTIN
C
p=0.07
p=0.33
P7
P8
P9
P10
% Rosette Formation per Total HIPS Colonies
R567. 25 (WT)
R567. 24 (MUT)
R567. 26 (MUT)
p=0.2
P12
P12
P16
P17
% Rosette Formation per Total HIPS Colonies
R982.31 (WT)
R982.16 (WT)
R982.18 (WT)
R982.32 (MUT)
R982.15 (MUT)
R982.17 (MUT)
D
R982.18 NPC (WT)
R982.16 NPC (WT)
R982.17 NPC (MUT)
R982.15 NPC (MUT)
Number of Cells (x 100,000)
day 0
day 1
day 3
day 5
R567.25 (WT)
R567.24 (MUT)
R567.26 (MUT)
Number of Cells (x 100,000)
day 0
day 1
day 3
day 5
E
n.s
n.s
% MAP2 Positive Cells
567. 25 (WT)
567. 24 (MUT)
567. 26 (MUT)
n.s.
n.s.
% S100 Positive Cells
567. 25 (WT)
567. 24 (MUT)
567. 26 (MUT)
F
n.s.
%MAP2 Positive Cells
982. 16 (WT)
982. 18 (WT)
982. 31 (WT)
982. 15 MUT
982. 17 MUT
982. 32 MUT
n.s.
%S100 Positive Cells
982. 16 (WT)
982. 18 (WT)
982. 31 (WT)
982. 15 MUT
982. 17 MUT
982. 32 MUT

Figure S2. Validation of disease in a dish model for Rett Syndrome. Supplementary to Figure 1.

A, Teratoma assay was performed to establish pluripotency of hiPSCs made from Rett patient fibroblasts. The resulting tumors each showed evidence of differentiation towards all three embryonic germ layers. **B,** NPCs were produced from isogenic hiPSCs of Rett patient, and assessed by western blot to validate loss of MECP2 and specificity of antibody. Top panel shows that the antibody only recognizes MECP2. Bottom panel shows that in NPCs from both patients, individual clones either express or lack MECP2. **C**, The ability of hiPSCs to generate NPCs was assayed in Rosette formation assay. Lack of MECP2 did not affect rosette formation across multiple lines from both patients. N=4 independent experiments. *p value < 0.05 according to student's t test (for patient R567) or ANOVA (for patient R982). Bar graphs represent mean +/- SEM. **D**, Growth curves show that loss of MECP2 does not affect proliferation of NPCs made from either patient. **E**, 3 weeks of growth factor withdrawal drives NPCs to differentiate into neurons and glia as measured here by immunostaining for MAP2/Tuj1 or S100/GFAP in patient 567 derived cultures. There was no consistent difference in differentiation potential across lines from either patient. N=2 independent experiments. Bar graphs represent mean +/- SEM. **F**, Patient 982 derived cultures also do not show dramatic differences in the presence of neurons or astrocytes as measured by MAP2 and S100. N=3 independent experiments. Bar graphs represent mean +/- SEM. Scale Bars represent 20 microns.

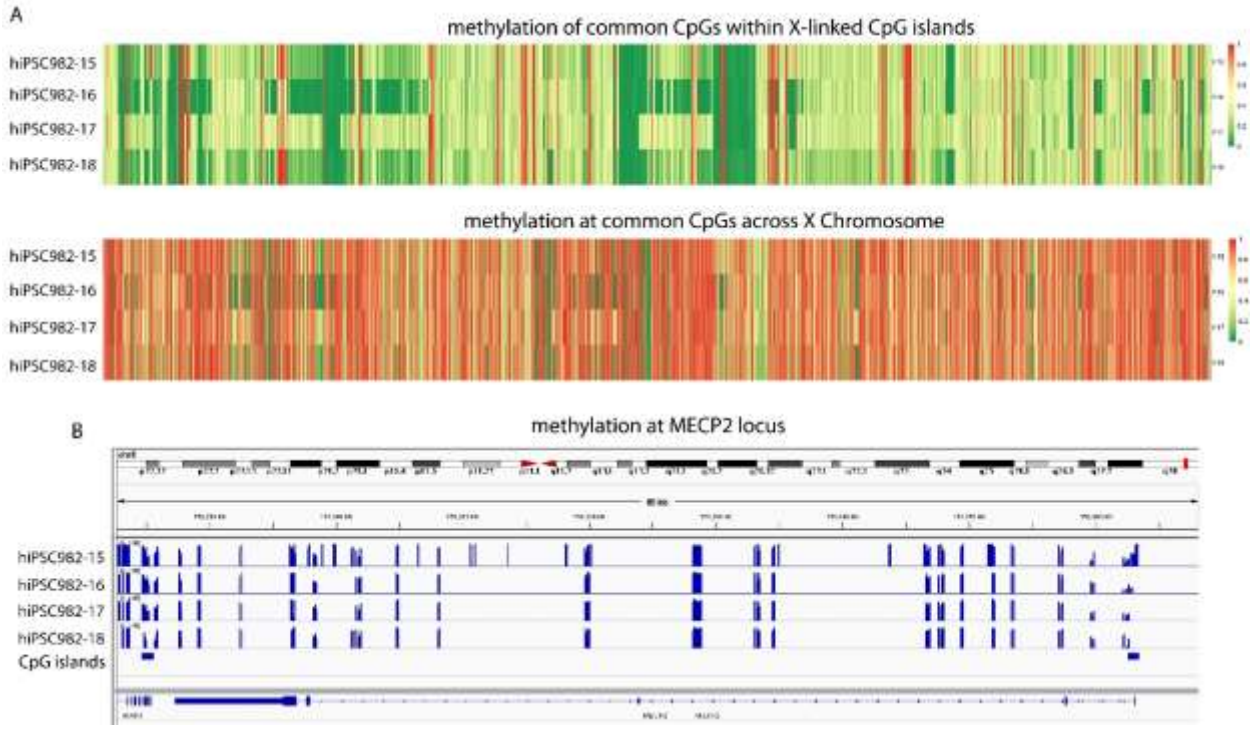

Figure S3. Methylation analysis to measure XCI erosion in hiPSC lines made from Rett Patients. Supplementary to figure 1.

A, The silencing of the X chromosome is accompanied by the gain of DNA methylation at CpG islands (CGIs). We utilized reduced representation bisulfite sequencing (RRBS) to examine methylation levels across the X chromosome in indicated hiPSC lines. Methylation level of 1 indicates 100% methylation and 0 absence of methylation. Top: Heatmap of RRBS-based methylation levels for CpGs within X-linked CGIs showing that most CGIs are hemi-methylated (intermediate methylation level) indicative of XCI on one X chromosome and no methylation on the other (which results in hemi-methylation when measured across both X chromosomes). Erosion (no methylation) is only seen for a small subset of CGIs (compare to data in (Patel et al., 2017)). Only CpGs with coverage across all samples are shown (including across Patel et al samples). Bottom: As above, except for all CpGs on the X chromosome with coverage in all samples, demonstrating the globally high methylation level in all hiPSCs. **B,** IgV view of the

methylation data within the MECP2 locus. The CGI of MECP2 is indicated and hemi-methylated in all hiPSC lines, indicative of XCI at this locus. RRBS and data analysis was performed as described in(Patel et al., 2017).

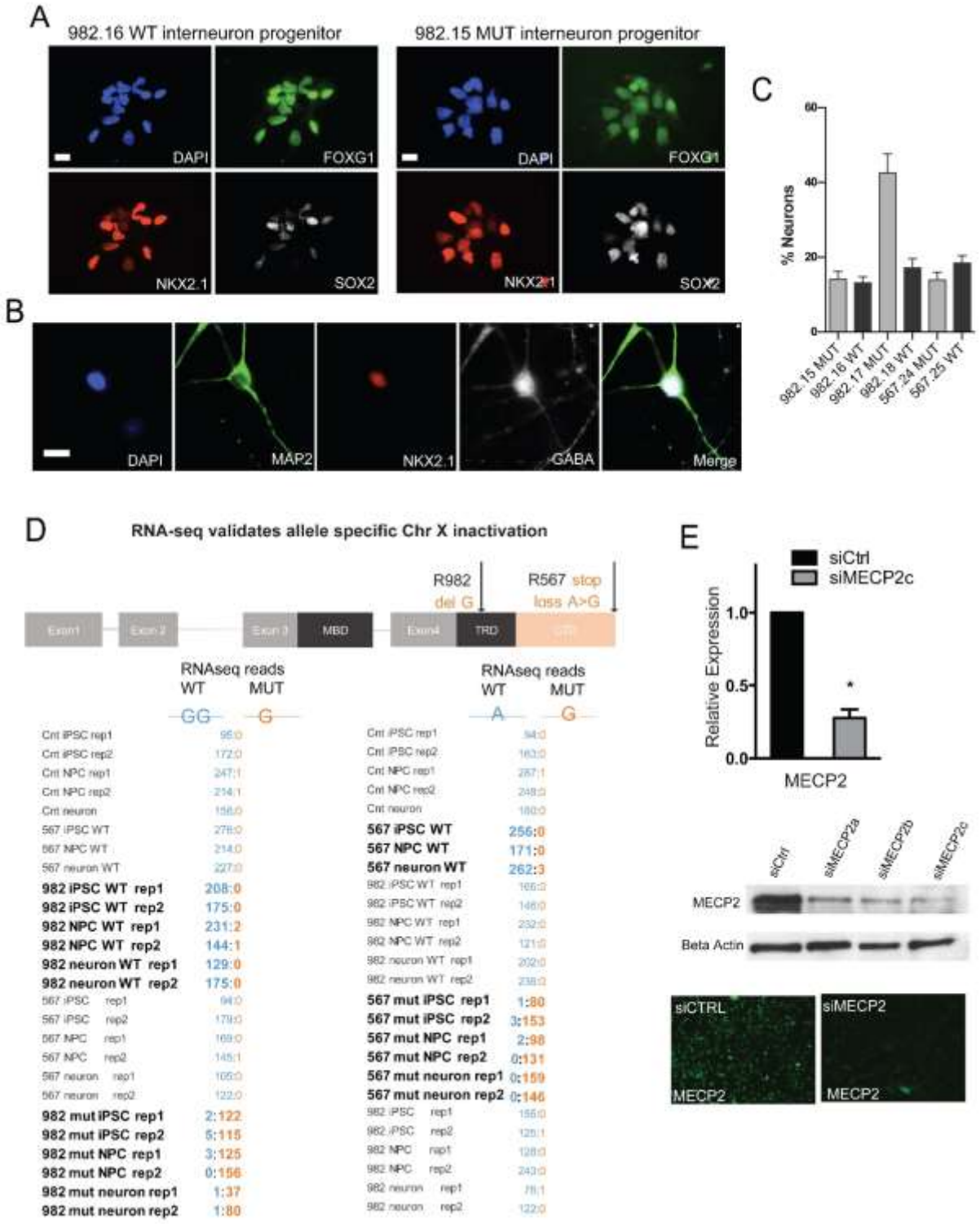

Figure S4. RNA-seq analysis to determine the relative ration of WT versus MUT transcripts of MECP2 in Rett patient derived lines. Supplementary to figure 2.

A and B, immunostaining to demonstrate the efficiency of directed differentiation towards neural progenitors (A) and then onto interneurons (B) with markers typical of each stage. **C,** Quantification of the percentage of interneurons in the cultures used to perform the RNA-seq, as measured by immunostaining. **D,** Detection of WT and MUT transcripts from each of the lines indicated demonstrated a clear bias towards individual alleles in each patient derived line. This analysis indicates XCI status for each allele, and demonstrates that XCI status is unchanged, even after differentiation to neurons. **E**, MECP2 was downregulated by RNA interference, quantified by RT-PCR (left), for protein by western blot (middle), and as demonstrated by immunostaining for MECP2 (right). N=3 independent experiments. *p value < 0.05 according to student's t test. Bar graphs represent mean +/- SEM. Scale bars represent 10 microns.

Patel, S., Bonora, G., Sahakyan, A., Kim, R., Chronis, C., Langerman, J., Fitz-Gibbon, S., Rubbi, L., Skelton, R.J.P., Ardehali, R.*, et al.* (2017). Human Embryonic Stem Cells Do Not Change Their X Inactivation Status during Differentiation. Cell Rep *18*, 54-67.