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Supplemental Information

MeCP2 and Major Satellite Forward RNA Cooperate for Pericentric Heterochromatin Organization

Salvatore Fioriniello, Eva Csukonyi, Domenico Marano, Arianna Brancaccio, Michele Madonna, Carmela Zarrillo, Alessia Romano, Federico Marracino, Maria R. Matarazzo, Maurizio D'Esposito, and Floriana Della Ragione

Supplemental Information

Figure S1

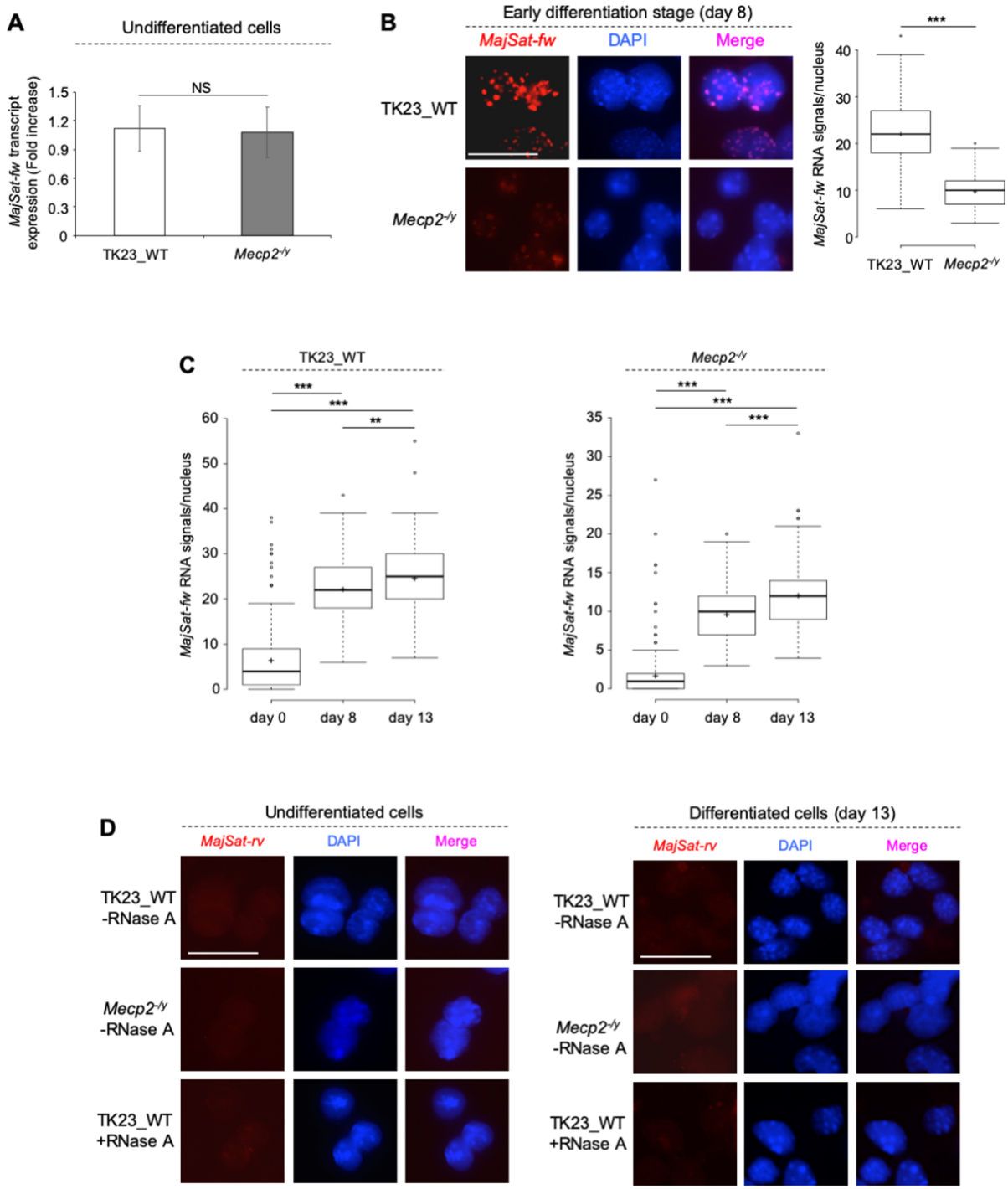

Figure S1. Major satellite forward transcript accumulation at chromocenters progressively increases during neural differentiation (related to Figure 2)

(**A**) Strand-specific RT-qPCR analysis of *MajSat-fw* transcripts in TK23_WT and *Mecp2*$^{-/y}$ undifferentiated cells. Data are normalized to *Gapdh* and are mean ± SD of transcript levels relative to TK23_WT, from three independent experiments. NS, not significant (two-tailed Student's t test).

(**B**) Left: Representative images of i3D-RNA FISH using the *MajSat-fw* LNA probe in TK23_WT and *Mecp2*$^{-/y}$ cells during early stage of neural differentiation (day 8), characterized by the first morphological signs of differentiation (Bertulat et al., 2012). Chromocenters were stained with DAPI. Scale bar, 25 µm. Right: Quantification of *MajSat-fw* RNA signals in 3D space of each nucleus. Data are shown as box and whisker plots with 200 nuclei analyzed per condition, from two replicate slides. ***P<0.001 (two-sample Kolmogorov-Smirnov test).

(**C**) Quantification as for (B) of *MajSat-fw* RNA signals/nucleus in TK23_WT and *Mecp2*$^{-/y}$ cells for undifferentiated state (day 0) and for day 8 and day 13 of neural differentiation. Data are the same reported in Figure 2B and C (day 0 and 13, respectively) and Figure S1B (day 8). Data are shown as box and whisker plots. ***P<0.001; **P<0.01 (two-sample Kolmogorov-Smirnov test with Benjamini-Hochberg correction).
(**D**) Representative images of i3D-RNA FISH using the *MajSat-rv* LNA probe in TK23_WT and *Mecp2*$^{-/y}$ undifferentiated cells and in terminally differentiated neurons. Chromocenters were stained with DAPI. Scale bar, 25 μm.

Figure S2

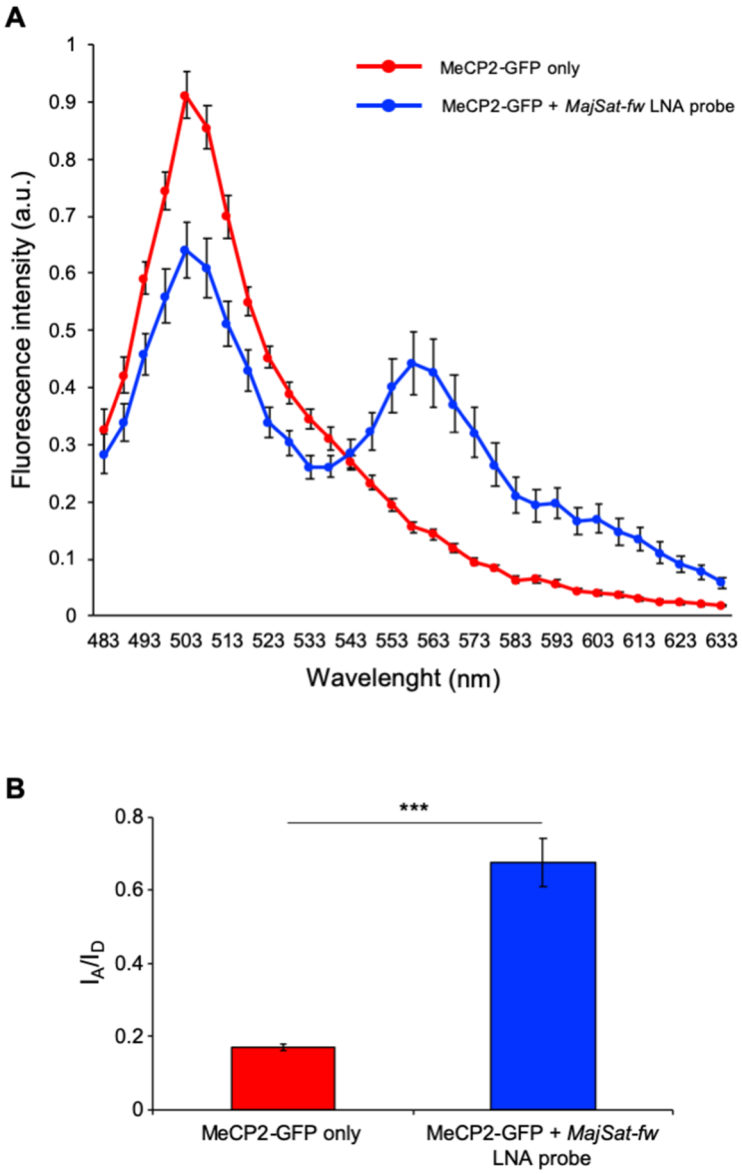

Figure S2. Interaction between MeCP2 and *MajSat-fw* transcript in terminally differentiated neurons analyzed by Fluorescence Resonance Energy Transfer (FRET) (related to Figure 3)

FRET can be observed between the donor EGFP, conjugated to MeCP2 (referred to as MeCP2-GFP), and the acceptor, TYE™563-*MajSat-fw* LNA probe. TYE™563 fluorophore is a direct substitute of Cy3.

(**A**) Emission spectra measured in the presence (MeCP2-GFP + *MajSat-fw* LNA probe) and in the absence (MeCP2-GFP only) of acceptor. λ_{exc}=458 nm. FRET was evaluated measuring fluorescence intensity in the range from 483 to 633 nm after excitation of the donor EGFP (at 458 nm). The occurrence of EGFP-to-TYE™563 FRET in the region of co-localization between MeCP2-GFP and *MajSat-fw* transcript was demonstrated by the decrease of the donor emission peak, at 503 nm, and the concomitant growth of the acceptor emission peak, at 558 nm. Data are mean ± SEM from three independent experiments.

(**B**) FRET ratio calculated as fluorescence intensity of acceptor (IA)/fluorescence intensity of donor (ID), both in the presence (MeCP2-GFP + *MajSat-fw* LNA probe) and in the absence (MeCP2-GFP only) of acceptor. This ratio results strongly and significantly increased in the region of MeCP2-GFP/*MajSat-fw* transcript co-localization. Data are mean ± SEM from three independent experiments; ***P<0.001 (two tailed Student's t test).

Figure S3

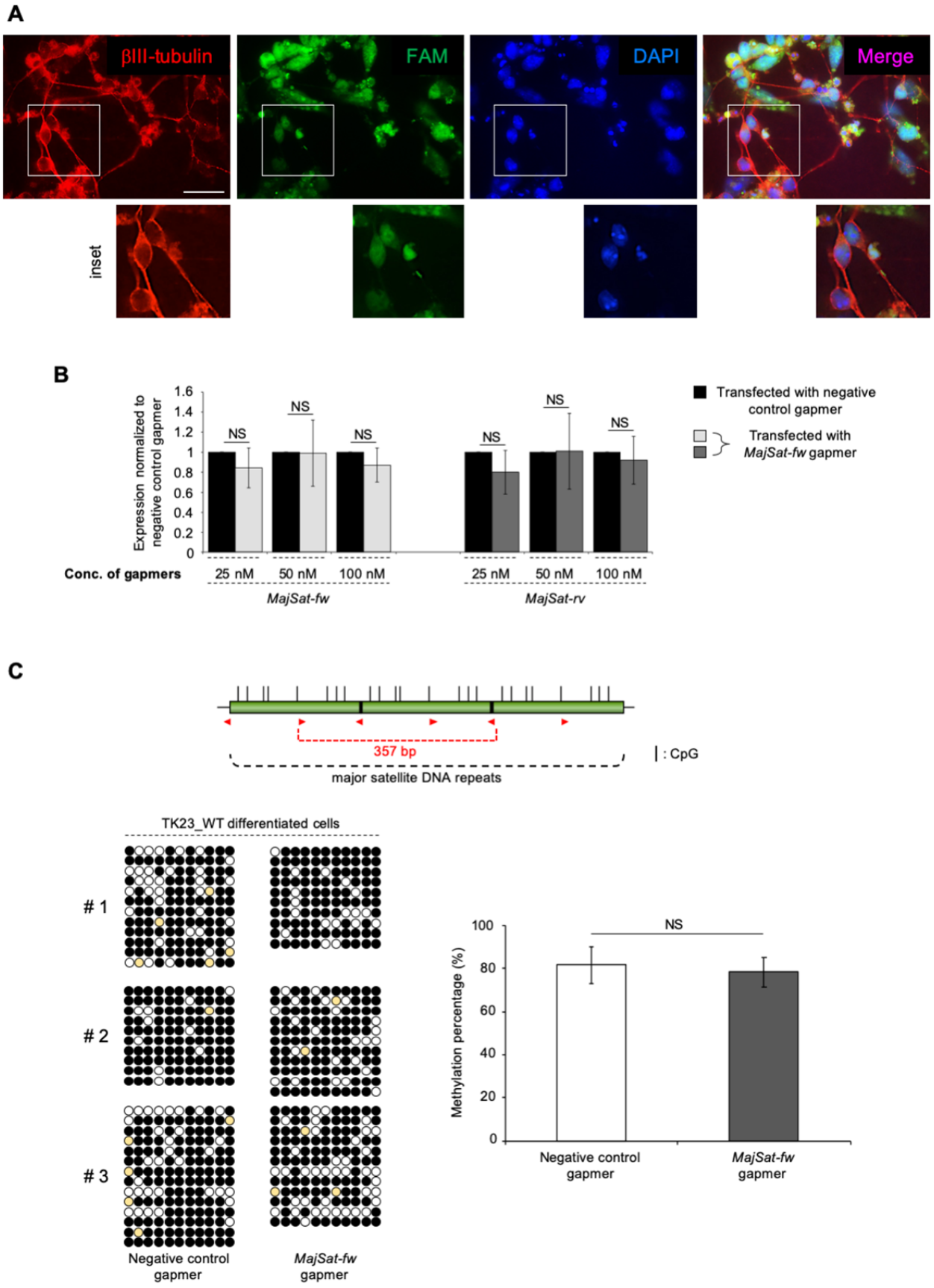
A
βIII-tubulin
FAM
DAPI
Merge
inset
B
Expression normalized to negative control gapmer
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NS
Conc. of gapmers
25 nM
50 nM
100 nM
MajSat-fw
MajSat-rv
Transfected with negative control gapmer
Transfected with MajSat-fw gapmer
C
357 bp
major satellite DNA repeats
: CpG
TK23_WT differentiated cells
1
2
3
Negative control gapmer
MajSat-fw gapmer
Methylation percentage (%)
100
80
60
40
20
0
NS
Negative control gapmer
MajSat-fw gapmer

Figure S3. Characterization of *MajSat-fw* transcript knock-down in terminally differentiated TK23_WT neurons and analysis of its impact on *MajSat* DNA methylation (related to Figure 4)

(**A**) Representative images of neurons from differentiated WT mESCs (not expressing EGFP), transfected with 10 nM *MajSat-fw* gapmer fluorescently labelled with FAM and analyzed by immunifluorescence. An anti-βIII-tubulin antibody was used to stain neurons. Nuclei were stained with DAPI. Insets: neurons at higher magnification. Scale bar, 25 μm.

(**B**) Strand-specific RT-qPCR analysis of *MajSat-fw* and *MajSat-rv* transcripts in mESC-derived TK23_WT neurons transfected with different amounts of *MajSat-fw* gapmer (concentration relating to medium) and the negative control gapmer (same concentration as for *MajSat-fw* gapmers). Data are normalized to *Gapdh* and are mean ± SD of transcript levels relative to cells transfected with negative control gapmer from three independent experiments. NS, not significant (one-tailed Student's t test).

(**C**) Top: Schematic representation of mouse *MajSat* DNA repeats (green rectangles). Primers used for PCR analysis following bisulfite modification of genomic DNA are depicted by red triangles. Vertical black lines indicate locations of CpGs. Bottom: Bisulfite genomic sequencing analysis of mouse *MajSat* DNA repeats. Left: Bisulfite sequencing pattern analyzed in three biological replicates (indicated by # 1, # 2 and # 3) of terminally differentiated TK23_WT neurons transfected with 300 nM negative control or *MajSat-fw* gapmers. Circles indicate methylation status: black circles represent methylated CpGs; white circles represent unmethylated CpGs; yellow circles indicate no CpG at the expected site. Each row of circles corresponds to one clone. Following bisulfite treatment and PCR, resulting products (357 bp) were cloned and the DNA of at least 10 independent colonies was sequenced. Right: Percentage of total CpG methylation for all samples illustrated in the left panel, is reported as mean ± SD. NS, not significant (two-tailed Student's t test).

Figure S4

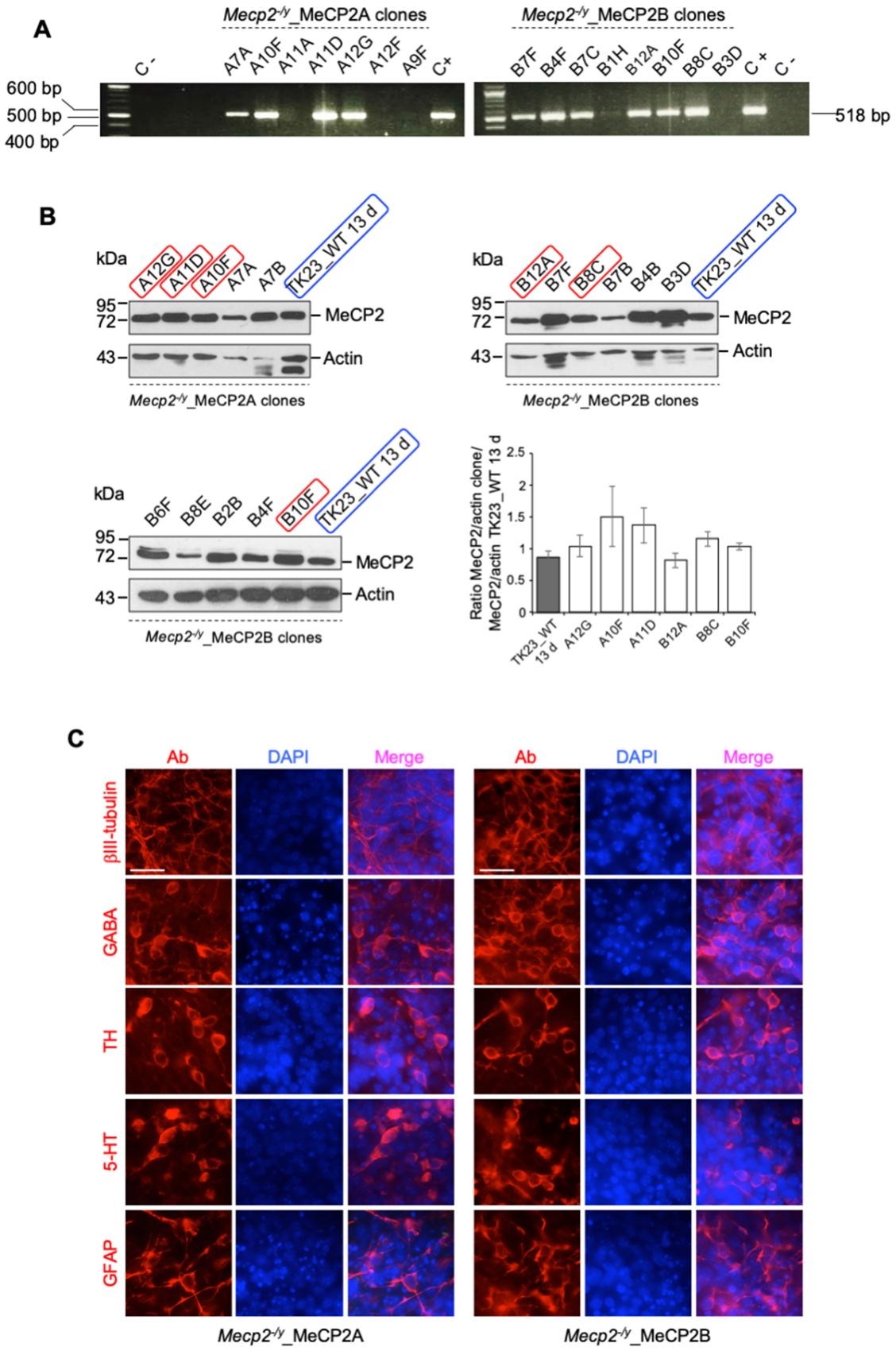
A
Mecp2-/y_MeCP2A clones
Mecp2-/y_MeCP2B clones
C -
A7A
A10F
A11A
A11D
A12G
A12F
A9F
C+
B7F
B4F
B7C
B1H
B12A
B10F
B8C
B3D
C +
C -
600 bp
500 bp
400 bp
518 bp
B
kDa
A12G
A11D
A10F
A7A
A7B
TK23_WT 13 d
95
72
43
MeCP2
Actin
Mecp2-/y_MeCP2A clones
B12A
B7F
B8C
B7B
B4B
B3D
Mecp2-/y_MeCP2B clones
B6F
B8E
B2B
B4F
B10F
Ratio MeCP2/actin clone/ MeCP2/actin TK23_WT 13 d
2.5
2
1.5
1
0.5
0
TK23_WT 13 d
A12G
A10F
A11D
B12A
B8C
B10F
C
Ab
DAPI
Merge
βIII-tubulin
GABA
TH
5-HT
GFAP
Mecp2-/y_MeCP2A
Mecp2-/y_MeCP2B

Figure S4. *Mecp2*$^{-/y}$_MeCP2A and *Mecp2*$^{-/y}$_MeCP2B mESC clones differentiate towards neural fate (related to Figure 6)

(**A**) Representative PCR screening of transfected *Mecp2*$^{-/y}$_MeCP2A and *Mecp2*$^{-/y}$_MeCP2B mESC clones. The 518 bp band indicates insertion of the plasmid containing the coding region of MeCP2A or MeCP2B in the genomic DNA. C+, positive control (plasmid containing coding region of MeCP2A or MeCP2B); C–, negative control.

(**B**) Representative western blot of MeCP2 in *Mecp2*$^{-/y}$_MeCP2A and *Mecp2*$^{-/y}$_MeCP2B mESC clones screened as in (A). The anti-MeCP2 antibody recognizes a portion of the protein downstream of the TRD. Cells expressing MeCP2A or MeCP2B levels comparable to the endogenous protein levels expressed in mESC-derived TK23_WT neurons (loaded as control; blue boxes) were selected (red boxes). Actin was used as normalizer. Quantification of MeCP2A or MeCP2B protein expression in selected clones is reported. Data are mean ± SD from three independent experiments, as MeCP2/actin ratios for each mESC clone normalized to MeCP2/actin ratio of TK23_WT cells. None of the selected clones showed significant differences compared with TK23_WT cells (two-tailed Student's t test).

A12G and B8C clones were selected for the subsenquent experiments.

(**C**) Representative images of immunofluorescence in *Mecp2*$^{-/y}$_MeCP2A and *Mecp2*$^{-/y}$_MeCP2B clones (A12G and B8C, respectively) at day 13. Generation of mature (βIII-tubulin+), GABAergic (GABA+), dopaminergic (tyrosine hydroxylase+; TH+), and serotonergic (5-hydroxytryptamine+; 5-HT+) neurons and astrocytes (glial fibrillary acidic protein+; GFAP+) was assayed. Nuclei were stained with DAPI. Scale bar, 25 μm.

Figure S5

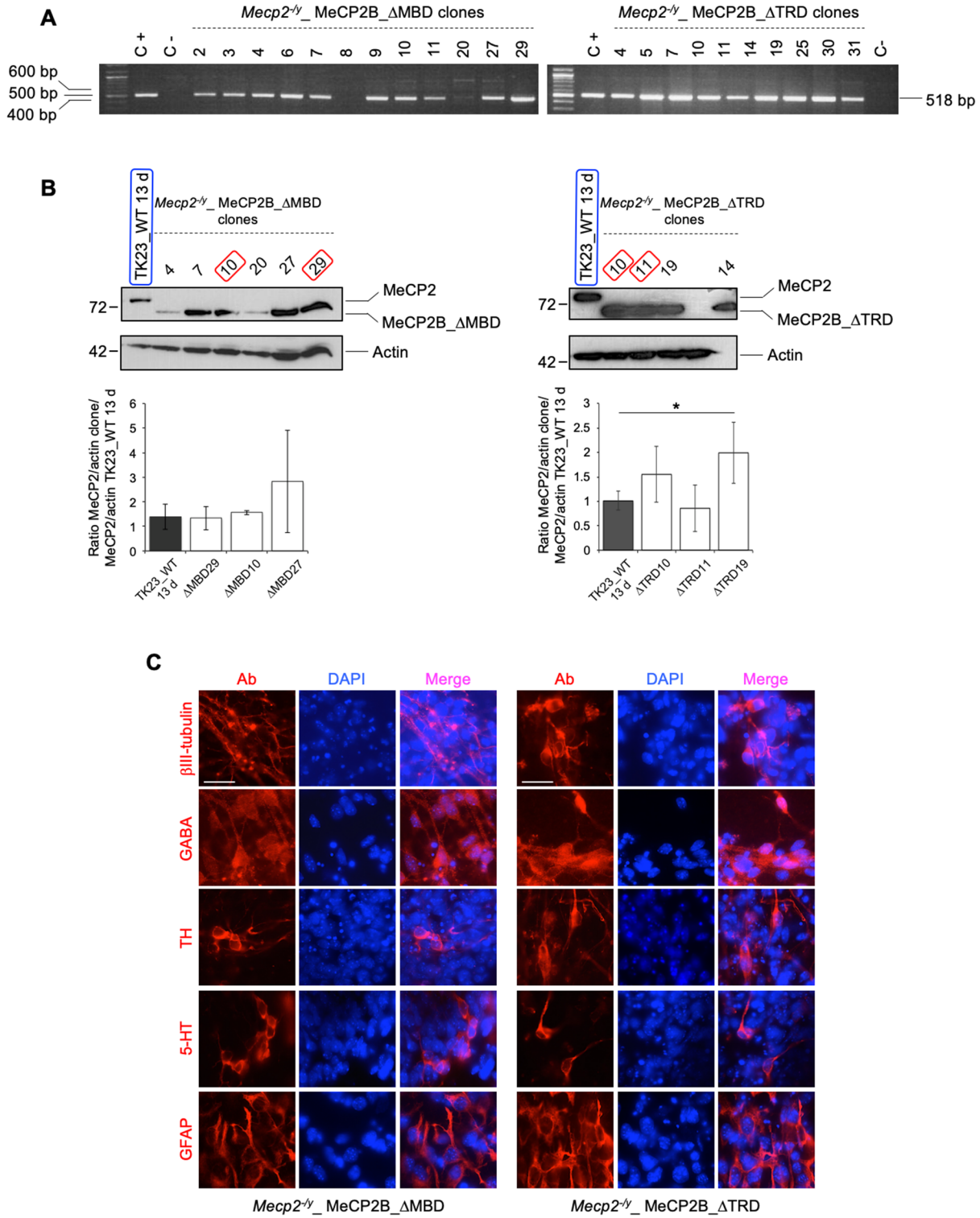
A
Mecp2-/y_ MeCP2B_ΔMBD clones
Mecp2-/y_ MeCP2B_ΔTRD clones
C +
C -
2 3 4 6 7 8 9 10 11 20 27 29
C +
4 5 7 10 11 14 19 25 30 31
C-
600 bp
500 bp
400 bp
518 bp
B
TK23_WT 13 d
Mecp2-/y_ MeCP2B_ΔMBD clones
4 7 10 20 27 29
MeCP2
MeCP2B_ΔMBD
Actin
72
42
Ratio MeCP2/actin clone/ MeCP2/actin TK23_WT 13 d
TK23_WT 13 d
ΔMBD29
ΔMBD10
ΔMBD27
TK23_WT 13 d
Mecp2-/y_ MeCP2B_ΔTRD clones
10 11 19 14
MeCP2
MeCP2B_ΔTRD
Actin
*
TK23_WT 13 d
ΔTRD10
ΔTRD11
ΔTRD19
C
Ab
DAPI
Merge
βIII-tubulin
GABA
TH
5-HT
GFAP
Mecp2-/y_ MeCP2B_ΔMBD
Mecp2-/y_ MeCP2B_ΔTRD

Figure S5. *Mecp2*$^{-/y}$_MeCP2B_ΔMBD and *Mecp2*$^{-/y}$_MeCP2B_ΔTRD mESC clones differentiate towards neural fate (related to Figure 6)

(**A**) Representative PCR screening of transfected *Mecp2*$^{-/y}$_MeCP2B_ΔMBD and *Mecp2*$^{-/y}$_MeCP2B_ΔTRD mESC clones. The 518 bp band indicates the insertion of the plasmid containing the coding region of MeCP2B_ΔMBD or MeCP2B_ΔTRD in the genomic DNA. C+, positive control (plasmid containing coding region of MeCP2B_ΔMBD or MeCP2B_ΔTRD); C–, negative control.

(**B**) Representative western blot of MeCP2 in *Mecp2*$^{-/y}$_MeCP2B_ΔMBD and *Mecp2*$^{-/y}$_MeCP2B_ΔTRD mESC clones screened as in (A). The anti-MeCP2 antibody recognizes a portion of the protein downstream of the TRD. Cells expressing MeCP2B_ΔMBD or MeCP2B_ΔTRD levels comparable to the endogenous protein levels expressed in mESC-derived TK23_WT neurons (loaded as control; blue boxes) were selected (red boxes). Molecular weights of MeCP2B_ΔMBD and MeCP2B_ΔTRD were smaller than endogenous MeCP2 detected in TK23_WT, as expected. Actin was used as normalizer. Quantification of MeCP2B_ΔMBD or MeCP2B_ΔTRD expression in selected clones is reported. Data are means ± SD from three independent experiments, as MeCP2/actin ratios of each mESC clone normalized to MeCP2/actin ratio of TK23_WT cells. *P<0.05 (two tailed Student's t test).

ΔMBD10 and ΔTRD10 clones were selected for the subsenquent experiments.

(**C**) Representative images of immunofluorescence in *Mecp2*$^{-/y}$_MeCP2B_ΔMBD and *Mecp2*$^{-/y}$_MeCP2B_ΔTRD clones (ΔMBD10 and ΔTRD10, respectively) at day 13. Generation of mature (βIII-tubulin+), GABAergic (GABA+), dopaminergic (tyrosine hydroxylase+; TH+) and serotonergic (5-hydroxytryptamine+; 5-HT+) neurons, and astrocytes (glial fibrillary acidic protein+; GFAP+) was assayed. Nuclei were stained with DAPI. Scale bar, 25 μm.

Figure S6

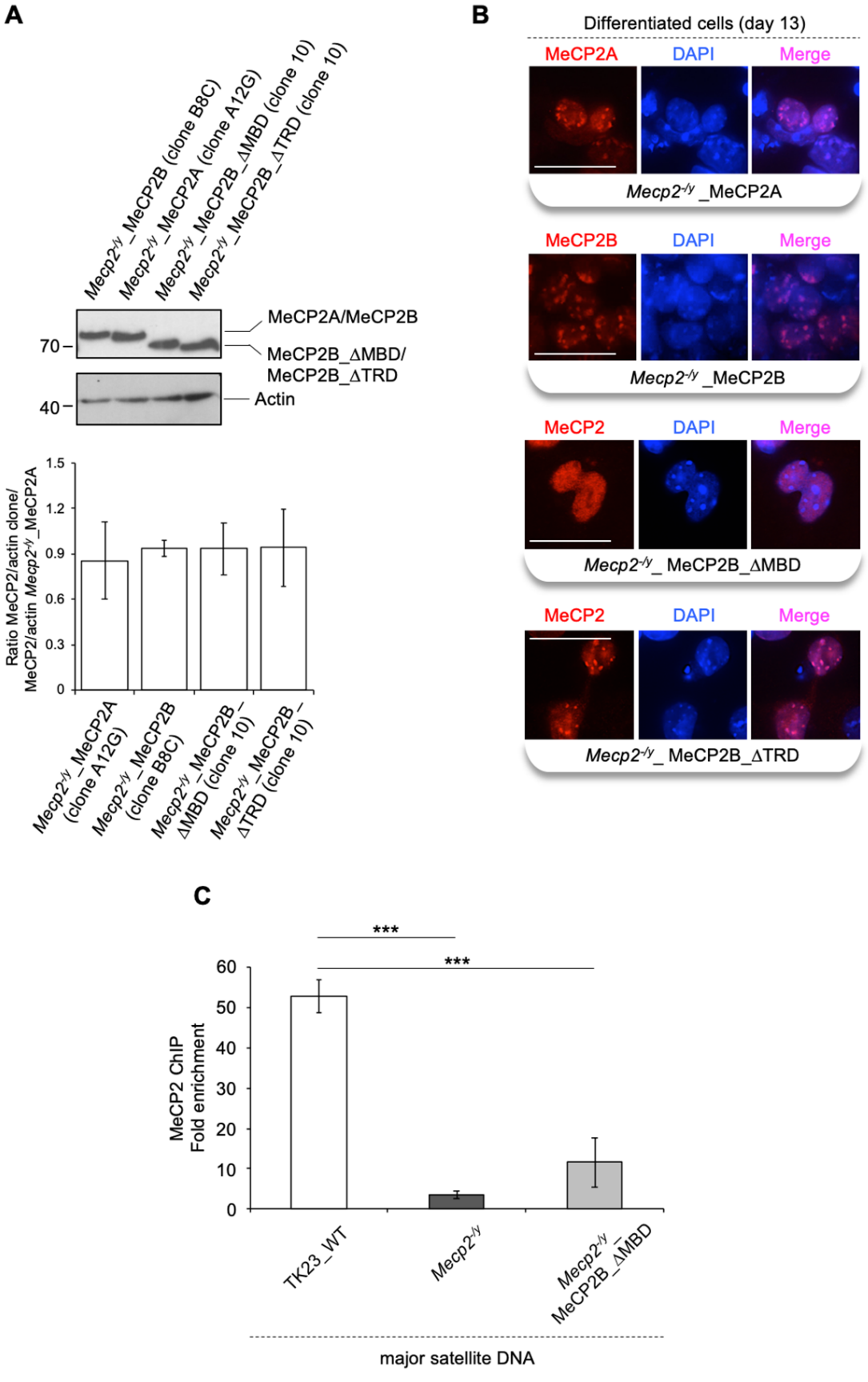
A
Mecp2-/y _MeCP2B (clone B8C)
Mecp2-/y _MeCP2A (clone A12G)
Mecp2-/y _MeCP2B_ΔMBD (clone 10)
Mecp2-/y _MeCP2B_ΔTRD (clone 10)
70
40
MeCP2A/MeCP2B
MeCP2B_ΔMBD/
MeCP2B_ΔTRD
Actin
Ratio MeCP2/actin clone/
MeCP2/actin Mecp2-/y_MeCP2A
1.5
1.2
0.9
0.6
0.3
0
Mecp2-/y _MeCP2A (clone A12G)
Mecp2-/y _MeCP2B (clone B8C)
Mecp2-/y _MeCP2B_ΔMBD (clone 10)
Mecp2-/y _MeCP2B_ΔTRD (clone 10)
B
Differentiated cells (day 13)
MeCP2A
DAPI
Merge
Mecp2-/y _MeCP2A
MeCP2B
DAPI
Merge
Mecp2-/y _MeCP2B
MeCP2
DAPI
Merge
Mecp2-/y_ MeCP2B_ΔMBD
MeCP2
DAPI
Merge
Mecp2-/y_ MeCP2B_ΔTRD
C
***
***
MeCP2 ChIP
Fold enrichment
60
50
40
30
20
10
0
TK23_WT
Mecp2-/y
Mecp2-/y _MeCP2B_ΔMBD
major satellite DNA

Figure S6. Both MeCP2A and MeCP2B isoforms accumulate at PCH and the MeCP2B_ΔMBD deleted protein is widespread in the nucleoplasm (related to Figure 6)

(**A**) Top: Western blot analysis of MeCP2 in *Mecp2*$^{-/y}$_MeCP2A, *Mecp2*$^{-/y}$_MeCP2B, *Mecp2*$^{-/y}$_MeCP2B_ΔMBD and *Mecp2*$^{-/y}$_MeCP2B_ΔTRD selected mESC clones (A12G, B8C, ΔMBD10 and ΔTRD10, respectively). Molecular weights of MeCP2B_ΔMBD and MeCP2B_ΔTRD were smaller than MeCP2 detected in *Mecp2*$^{-/y}$_MeCP2A and *Mecp2*$^{-/y}$_MeCP2B mESC clones, as expected. Actin was used as normalizer. Bottom: Quantification of MeCP2 protein expression in selected clones. Data are means ± SD from three independent experiments, as MeCP2/actin ratios of each mESC clone normalized to MeCP2/actin ratio of *Mecp2*$^{-/y}$_MeCP2A cells. None of the selected clones showed significant differences (one-way ANOVA followed by Tukey post-hoc test).

(**B**) Representative immunofluorescence of MeCP2 in terminally differentiated *Mecp2*$^{-/y}$_MeCP2A, *Mecp2*$^{-/y}$_MeCP2B, *Mecp2*$^{-/y}$_MeCP2B_ΔMBD and *Mecp2*$^{-/y}$_MeCP2B_ΔTRD neurons (A12G, B8C, ΔMBD10 and ΔTRD10, respectively). Chromocenters were stained with DAPI. Scale bar, 25 μm.

(**C**) ChIP-qPCR of MeCP2 at *MajSat* DNA in differentiated *Mecp2*$^{-/y}$_MeCP2B_ΔMBD, TK23_WT (positive control) and *Mecp2*$^{-/y}$ (negative control) neurons. Data are means ±SD of four independent qPCR replicates from two ChIP experiments. ***P<0.001 (one-tailed Student's t test).

The anti-MeCP2 antibody used in the experiments showed in A, B and C recognizes a portion of the protein downstream of the TRD.

Figure S7

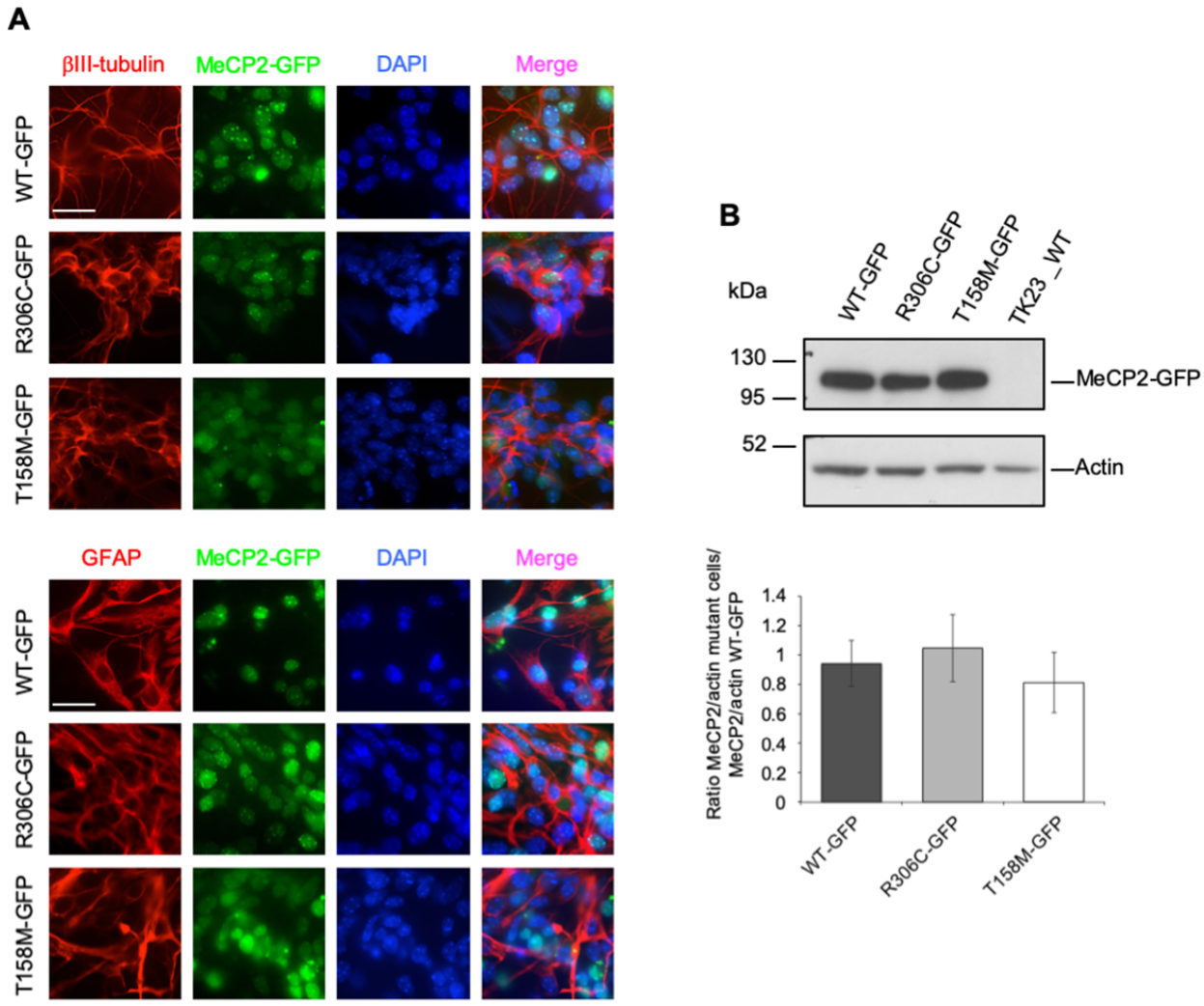

Figure S7. T158M-GFP and R306C-GFP mESCs differentiate towards neural fate as WT-GFP and express similar levels of the MeCP2 protein (related to Figure 7)

(**A**) Representative images of immunofluorescence in terminally differentiated WT-GFP, R306C-GFP and T158M-GFP neurons (day 13). Generation of mature neurons (top: βIII-tubulin+) and astrocytes (bottom: glial fibrillary acidic protein+; GFAP+) was assayed. EGFP (green) highlights wild-type or mutant MeCP2 nuclear localization in WT-GFP, R306C-GFP and T158M-GFP cell lines. Chromocenters were stained with DAPI. Scale bar, 25 μm.

(**B**) Top: Western blot analysis of MeCP2 with anti-EGFP antibody in terminally differentiated WT-GFP, R306C-GFP and T158M-GFP neurons (day 13). mESC-derived TK23_WT neurons are the negative control. Actin was used as normalizer. Bottom: Quantification of $MeCP2^{WT\text{-}GFP}$, $MeCP2^{R306C\text{-}GFP}$ and $MeCP2^{T158M\text{-}GFP}$ protein expression. Data are means ± SD from three independent experiments, as the MeCP2/actin ratios for each mutant cell line normalized to MeCP2/actin ratio of WT-GFP cells. The R306C-GFP and T158M-GFP cell lines did not show significant differences in comparison with WT-GFP cells (two tailed Student's t test).

Table S1. Primers used in this study (related to Figures 1, 2, 3, 4, 5, 6, S1, S3, S4, S5 and S6).

Primer name	Nucleotidic sequence (5'-3')
1A/3A	AAAGAATTCGGCCGGCCATGGCCG
1B	TTTCTGCTCTCTCCTGGSGGGTTCTGGCACTGCTGGGGCGAGCC
2A	CCCTCCAGGAGAGAGCAGAAACCA
2B/4B	AGTGAATTCTCAGCTAACTCTCTCG
3B	TTGACCTCGATGCTGACCGTACCTTCTGATGCTGCTGCCTTTGGT
4A	ACGGTCAGCATCGAGGTCAAGGAA
UP-TGA	CCCACCTAAGAAGGAGCACC
L-pal	GAAGTCAGATGCTCAAGGGGC
major satellites (For)	AAATACACACTTTAGGACG
major satellites (Rev)	TCAAGTGGATGTTTCTCATT
GAPDH F2	ACGGCAAATTCAACGGCACA
GAPDH R2	TCCACGACATACTCAGCACCG
GAPDH prom F2	TGAATGCTGCTTCCCGAGTA
GAPDH prom R2	CTCAACTTTTCCGCAGCCTT
MALAT1 mas1 Forward	GGCTGGGGAGTGTTCCAGTGA
MALAT1 mas1 Reverse	TGGTGGCTGGCACTCCTGGT
Liu_MajSat_fw	GGAATATGGTAAGAAAATTGAAAATTATGG
Liu_MajSat_rv	CCATATTCCAAATCCTTCAATATACATTTC

Table S2. Strand-specific LNA fluorescent probes and antisense LNA gapmers (related to Figures 2, 3, 4, 6, 7, S1 and S2).

LNA fluorescent probes		
Name	**Fluorophore**	**Sequence**
major 1 (specific for MajSat-fw strand) Cat. no. EX500150-27479	TEX 615	TCTTGCCATATTCCACGTCC
major 2 (specific for MajSat-rv strand) Cat. no. EX500150-26917	TEX 615	GCGAGGAAAACTGAAAAAGG
major 3 (specific for MajSat-fw strand) Cat. no. 339406YCO0196697	TYE 563	TCTTGCCATATTCCACGTCC
Antisense LNA gapmers		
Name	**Fluorophore**	**Sequence**
LNA gapmer MajSat-fw ID DESIGN: 531668-1	6FAM	AAGTCGTCAAGTGGAT
LNA gapmer MajSat-fw ID DESIGN: 531668-1	-	AAGTCGTCAAGTGGAT
Negative control A gapmer	-	AACACGTCTATACGC

Table S3. List of antibodies used (related to Figures 1, 3, 4, 5, 6, S3, S4, S5, S6 and S7).

Antibody	Application	Company	Cat. N°
Anti-MeCP2	IF (1:500) WB (1:3500) ChIP (5 µg) RIP (5 µg)	Sigma-Aldrich	M9317
anti-TH (anti-tyrosine hydroxylase)	IF (1:200)	Millipore	AB152
anti-5-HT (anti-5 hydroxytryptamine)	IF (1:200)	Sigma-Aldrich	S5545
anti-GFAP (anti-glial fibrillary acidic protein)	IF (1:300)	Dako Cytomation	Z0334
anti-GABA (anti-γ-aminobutyric acid)	IF (1:400)	Sigma-Aldrich	A2052
anti-βIII-tubulin	IF (1:750)	Sigma-Aldrich	T8660
anti-GFP	WB (1:10000)	Abcam	Ab290
anti-H3K9me3	IF (1:500) WB (1:2500) ChIP (5 µg)	Abcam	Ab8898
anti-H4K20me3	IF (1:500) WB (1:1000) ChIP (5 µg)	Millipore	07-463
anti-actin	WB (1:3500)	Sigma-Aldrich	A2066
anti-histone H3	WB (1:30000)	Abcam	Ab1791

Supplemental Experimental Procedures

Animals

The *Mecp2*$^{tm1.1Bird}$ (*Mecp2*$^{-/y}$) and wild-type C57BL/6J mice were originally from Jackson Laboratories, and were then bred in the Animal Facility at IRCCS Neuromed. Hemizygous mutant males were generated by crossing heterozygous knock-out females with C57BL/6J males. Genotyping was performed by PCR, according to the Jackson Laboratory protocols. All animal experiments were approved by the IRCCS Neuromed Animal Care Review Board and by the Italian Ministry of Health (permit number: 988/2015-PR), and were conducted according to EU Directive 2010/63/EU for animal experiments. Nine-week-old mice (symptomatic and age-matched wild-type) were anaesthetized with ketamine/xylazine injection and sacrificed by cervical dislocation. The brains were removed from the skull and immediately processed for RNA immunoprecipitation.

Plasmids

The coding regions of mouse MeCP2A and MeCP2B were excised by restriction digestion from the MTmmMeCP2AEGFP and MTmmMeCP2BEGFP plasmids, kindly provided by C. Schanen **(**Kumar et al., 2008), and sub-cloned into the pallino βA expression vector (Parisi et al., 2003), under the control of the constitutive chicken β-*actin* promoter. As the coding sequences of MeCP2A and MeCP2B were fused in-frame with EGFP in the original plasmids, TGA stop codons were inserted at the end of the MeCP2 coding sequences. The cloning procedure was performed using standard PCR-based techniques and restriction digestion. The MeCP2B_ΔMBD sequence was created by fusing two PCR products together (ΔMBD-up and ΔMBD-lw). The ΔMBD-up was amplified with 1A/3A and 1B primers and the ΔMBD-lw with 2A and 2B/4B primers. The MeCP2B_ΔTRD sequence was also created by fusing two PCR products (ΔTRD-up and ΔTRD-lw) together. ΔTRD-up was amplified with the 1A/3A and 3B primers and ΔTRD-lw with the 4A and 2B/4B primers. The fused fragments were digested as needed and sub-cloned into the pallino βA expression vector (Parisi et al., 2003), under the control of the chicken β-*actin* promoter. All plasmids were checked by direct sequencing. Primers are listed in Table S1.

Generation and screening of stable mESC clones

The murine embryonic stem cells (mESCs) lacking MeCP2 (tEG) (Bertulat et al., 2012) were electroporated at 250 mV and 500 μF with the linearized pallino βA vector (10 μg) that contained the coding sequences for MeCP2A, MeCP2B, MeCP2B_ ΔMBD or MeCP2B_ΔTRD. After 3 days with 1.5 μg/ml puromycin, the resistant clones were selected, expanded and screened by PCR, using the UP-TGA and L-pal primers (Table S1). The positive clones were analyzed by western blot. The cell lines selected for the experiments had MeCP2 expression similar to the endogenous protein expressed in the terminally differentiated TK23_WT neurons.

Stem cell culture and differentiation

mESCs (all derived from E14 cell line) were cultured without feeder on gelatin (Sigma-Aldrich)-coated plates as previously reported (Bertulat et al., 2012). Neural differentiation was performed as already described (Bertulat et al., 2012: Fico e al., 2008), by cell seeding at 1000 cells/cm^2 (1500 cells/cm^2 for tEG, to compensate for their slower growth). The differentiation medium was Knock-out Dulbecco's minimal essential medium (Life Technologies) supplemented with 15% Knock-out Serum Replacement (Life Technologies), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 0.1 mM 2-mercaptoethanol. The cells were grown for up to 13 days. For i3D-DNA FISH and immunofluorescence for neuronal and glial markers, the mESCs were seeded and differentiated on poly-D-lysine/laminin (both from Sigma-Aldrich)-coated glass slides (Bertulat et al., 2012). For all the other experiments, the cells were plated and differentiated on gelatin-coated plates. For i3D-RNA FISH, immuno-RNA FISH, immunofluorescence for MeCP2, H3K9me3 and H4K20me3, fluorescence resonance energy transfer and antisense LNA gapmer transfection, the cells at an early differentiation stage (day 8, used only for i3D-RNA FISH) and the terminally differentiated neurons were dissociated with Accutase (Sigma-Aldrich), plated at a 1:3 dilution on poly-D-lysine/laminin-coated glass slides, and left to adhere for 24 h, to obtain well separated cells.

Immunofluorescence

For the MeCP2, H3K9me3 and H4K20me3 immunofluorescence, different procedures were applied, according to the required antibodies. For MeCP2 and H3K9me3 immunofluorescence, the cells were fixed in 4% PFA and permeabilized with 0.1% Triton X-100/PBS for MeCP2, or with 0.5% Triton X-100/PBS for H3K9me3. For H4K20me3 immunofluorescence, the cells were washed with CSK buffer (10 mM Pipes, pH 7, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$) supplemented with protease inhibitors (PI) (Roche), permeabilized with 0.5% Triton X-100/CSK buffer, washed with CSK buffer and PBS, and finally fixed in 2% PFA. For the detection of neuronal (βIII-tubulin, γ-aminobutyric acid, tyrosine hydroxylase, 5-hydroxytryptamine) and glial markers (glial fibrillary acidic protein), the terminally differentiated neurons were fixed in 4% PFA and permeabilized with 0.1% Triton X-100/PBS. Cells were,

then, blocked with 10% normal goat serum (NGS, Agilent)/PBS (for MeCP2, H3K9me3, neuronal and glial markers), or with 2.5% BSA/PBS (for H4K20me3). Then, the cells were incubated with the primary antibodies (i.e., anti-MeCP2, anti-H3K9me3, anti- βIII-tubulin, anti-γ-aminobutyric acid, anti-tyrosine hydroxylase, anti-5-hydroxytryptamine, anti-glial fibrillary acidic protein, anti-H4K20me3). After washes with 0.1% Triton X-100/PBS (for MeCP2, H3K9me3), PBS (for neuronal and glial markers) or 0.1% Tween20/PBS (for H4K20me3), the cells were incubated with the appropriate secondary antibodies (Alexa-Fluor 594 donkey anti-mouse or anti-rabbit; Thermofisher Scientific).
For the immunufluorescence for MeCP2 without or with RNase A treatment, the cells were washed with CSK buffer supplemented with PI, permeabilized with 0.5% Triton X-100/CSK buffer, washed with CSK buffer, with PBS, and then incubated with RNase A (1 mg/ml; Roche)/PBS or with PBS (mock) for 10 min. Then, the cells were fixed with 2% PFA for 10 min. Immunofluorescence for MeCP2 was performed following procedures already described (see above).
The detection of MeCP2 protein has been performed using an anti-MeCP2 antibody that recognizes a portion of the protein downstream of the TRD.
The slides were mounted using Vectashield/DAPI. The quantification of MeCP2, H3K9me3 and H4K20me3 enrichment at chromocenters, represented as proportions of nuclei with MeCP2, H3K9me3 and H4K20me3 spotted to PCH, were obtained by counting at least 200 cells for each condition.
Details of the antibodies are given in Table S3.

Bisulfite sequencing

Genomic DNA was isolated from terminally differentiated TK23_WT neurons transfected with 300 nM LNA gapmer *MajSat-fw* or the negative control A gapmer (Exiqon), using Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's protocol. Genomic DNA was sonicated in H_2O to obtain fragments of ~500 bp in length, then, 500 ng of DNA were subjected to bisulfite modification using the Epitect Bisulfite kit (Qiagen), according to the manufacturer's protocol. PCR amplification of major satellite DNA was performed by using Liu_MajSat_fw and Liu_MajSat_rv primers (Liu et al., 2013), listed in Table S1. The thermal cycling conditions were: 95 °C for 1 min, followed by 95 °C for 30 s, 60 °C for 30 s and 72 °C for 10 s for 40 cycles. Amplified DNA fragments (357 bp) were extracted from agarose gel using QIAquick Gel Extraction Kit (Qiagen), following the manufacturer's instructions and cloned by using TA cloning kit (Invitrogen). Sequencing of at least 10 clones for each group has been performed by using M13 forward and reverse primers by Eurofins Genomics.

Western blot

Total cell extracts were prepared by resuspending the cells in lysis buffer (100 mM Tris-HCl, pH 8, 140 mM NaCl, 20 mM EDTA, 0.2% SDS, 1% NP-40) supplemented with PI. The extracts enriched in histone proteins were obtained by isolating the nuclei in TEB buffer (0.5% Triton X-100, 0.02% NaN3, in PBS) supplemented with PI, and then with acid extraction with 0.2 N HCl, overnight at 4 °C. Total protein extracts have been used to detect MeCP2 and H3K9me3, whereas extracts enriched of histone proteins were used to analyze H4K20me3. Protein lysates were quantified using the Bradford protein assay (Bio-Rad), separated by SDS-PAGE, and transferred to PVDF membranes. Conventional western blotting assays were performed, by using the primary antibodies listed in Table S3. The detection of MeCP2 protein has been performed using an anti-MeCP2 antibody that recognizes a portion of the protein downstream of the transcriptional repression domain (TRD). Densitometric analysis was performed using the Typhoon Scan and ImageQuant software.

RNA extraction and reverse-transcription qPCR

Total RNA was isolated from mESCs and the terminally differentiated neurons using the QIAzol lysis reagent (Qiagen) and DNase treated with Turbo DNA-freeTM kits (Ambion), according to the manufacturer's instructions. cDNA was obtained by reverse transcription with strand-specific primers for major satellite transcripts [major satellites (For) or (Rev) (Maison et al., 2011)] and for the *Gapdh* transcript (GAPDH R2) (Table S1), using the SuperScript III First-Strand Synthesis system (Life Technologies). Quantitative PCR (qPCR) was performed using the primers for *MajSat* [major satellites (For) and (Rev) (Maison et al., 2011)] and *Gapdh* (GAPDH F2/R2) transcripts (Table S1), with SsoAdvance Universal SYBR Green supermix (Bio-Rad) on a Real Time PCR system (CFX96, Bio-Rad), according to the manufacturer's protocols. The $2^{-\Delta\Delta Cq}$ method was used to determine the relative quantitative levels, and the data were normalized with respect to *Gapdh* expression.

Chromatin immunoprecipitation (ChIP)

All the buffers used for chromatin immunoprecipitation were supplemented with PI. ChIPs were performed as previously described (Skene et al., 2010), with minor changes. Briefly, terminally differentiated neurons were fixed with 1% formaldehyde for 10 min and chromatin was sonicated in ice-cold lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1), to obtain fragments of ~300 bp in length. For ChIP without or with RNase A treatment, the chromatin was incubated with 10 μg RNase A/PBS or with PBS (mock), for 30 min at 37 °C. For ChIP with the anti-MeCP2 and anti-H4K20me3 antibodies, the sheared chromatin was diluted 10-fold in dilution buffer A (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) and precleared with protein A-Sepharose (GE-Healthcare); then 5% of the

supernatant was saved as the input. For ChIP with the anti-H3K9me3 antibody, the sonicated chromatin was diluted in dilution buffer B (1% Triton X-100, 0.5 mM EGTA, 140 mM NaCl, 10 mM Tris-HCl, pH 8) and precleared with protein A-Sepharose; then 1% of the supernatant was saved as the input. Appropriate amounts of chromatin were incubated with the anti-MeCP2, anti-H4K20me3 or anti-H3K9me3 antibodies, or with 5 µg normal rabbit IgG antibodies (Millipore). The immunoprecipitated complexes were recovered with protein A-Sepharose and washed with ice-cold buffer, following the different protocols: for ChIP with the anti-MeCP2 and anti-H4K20me3 antibodies, one wash in buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1), four washes in buffer 2 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8.1), one wash in buffer 3 (250 mM LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1) and three washes in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA); for ChIP with the anti-H3K9me3 antibody, one wash in high-salt buffer (0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, in PBS) and four washes in wash buffer (500 mM LiCl, 1% NP-40, 1% sodium deoxycholate, 100 mM Tris-HCl, pH 8). The immuno-complexes were then reverse crosslinked, and the DNA was purified and used for qPCR, which was performed with SsoAdvance Universal SYBR Green supermix (Bio-Rad) on a Real Time PCR system (CFX96; Bio-Rad), according to the manufacturer's protocols. Major satellites (For)/(Rev) (Maison et al., 2011) and GAPDH prom F2/R2 were used to amplify the *MajSat* DNA and *Gapdh* promoter (Table S1). Data are expressed as fold-enrichment over background (IgG), which were calculated in terms of $2^{-\Delta\Delta Cq\_ChIP/IgG}$, according to Equations [1] and [2].

$$\Delta Cq = Cq_{IP\ sample\ or\ IgG} - (Cq_{Input} - Log_2\ [input\ dilution\ factor]) \quad [1],$$

$$\Delta\Delta Cq = \Delta Cq_{IP\ sample} - \Delta Cq_{IgG} \quad [2],$$

where Cq is the quantification cycle and the input dilution factor is the fraction of the input chromatin that was saved.
Details of the antibodies used are given in Table S3. The immunoprecipitation of MeCP2 has been performed using an anti-MeCP2 antibody that recognizes a portion of the protein downstream of the TRD.

RNA immunoprecipitation

Native RNA immunoprecipitation (RIP) assays were performed using the Magna RIP RNA Binding Protein Immunoprecipitation kits (Millipore). For the terminally differentiated neurons, RIP was performed according to the manufacturer's instructions. For mouse brain, fresh tissue was finely minced with scalpels and Dounce-homogenized in ice-cold RNase-free PBS, and the cell pellet was processed according to the manufacturer's instructions. Immunoprecipitations were performed with the anti-MeCP2 antibodies (Table S3) or with 5 µg normal rabbit IgG antibodies. The RNA purified from immuno-complexes was reverse transcribed using the SuperScript II First Strand Synthesis system (Life Technologies), and the cDNA was amplified by qPCR, performed with SsoAdvance Universal SYBR Green supermix (Bio-Rad) on a Real Time PCR system (CFX96; Bio-Rad), according to the manufacturer's protocols. *MajSat* and *MALAT1* cDNA were amplified using major satellites (For)/(Rev) (Maison et al., 2011) and MALAT1 mas1 Forward/Reverse (Maxwell et al., 2013) primers, respectively (Table S1). Data are expressed as fold-enrichment over background (IgG), which was calculated in terms of $2^{-\Delta\Delta Cq\_RIP/IgG}$, according to Equations [1] and [2], where input dilution factor is the fraction of the input sample saved.
The immunoprecipitation of MeCP2 has been performed using an anti-MeCP2 antibody that recognizes a portion of the protein downstream of the TRD.

Fluorescence Resonance Energy Transfer

WT-GFP neurons were fixed with 3% PFA for 10 min, washed with PBS and CSK buffer, permeabilized with 0.5% Triton X-100/CSK buffer supplemented with 2 mM VRC for 5 min on ice, washed with CSK buffer and PBS, and stored in 70% ethanol overnight at -20 °C. Following dehydration with 80%, 95% and 100% ethanol, the cells were incubated with 0.2 µM LNA fluorescent probe (Qiagen) (major 3; Table S2) in 50% formamide, 10% dextran sulphate, 2 mg/ml BSA, 10 mM VRC, 2× SSC, for 35 min at 37 °C. After three washes in 2× SSC/50% formamide, pH 7.2 for 5 min at 42 °C and three washes in 2× SSC for 5 min at 42 °C, the slides were mounted using Mowiol (Sigma-Aldrich).
The samples were analyzed with a confocal microscope (Leica SP8 STED3x) run with LasX 3.5 software (Leica), using a 63x (NA1.4) objective lens (Leica). After excitation of the sample with Laser Argon at λ=458 nm, fluorescence emission spectra were registered in xyλ modality in the range between 483 nm and 633 nm in 31 detection steps (bandwidth: 10 nm; stepsize: 5 nm).

Supplemental References

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