Title: MeCP2 gene dosage-dependent neurodevelopmentally restricted defects arise by aberrant activation of cell fate-determining bivalent genes

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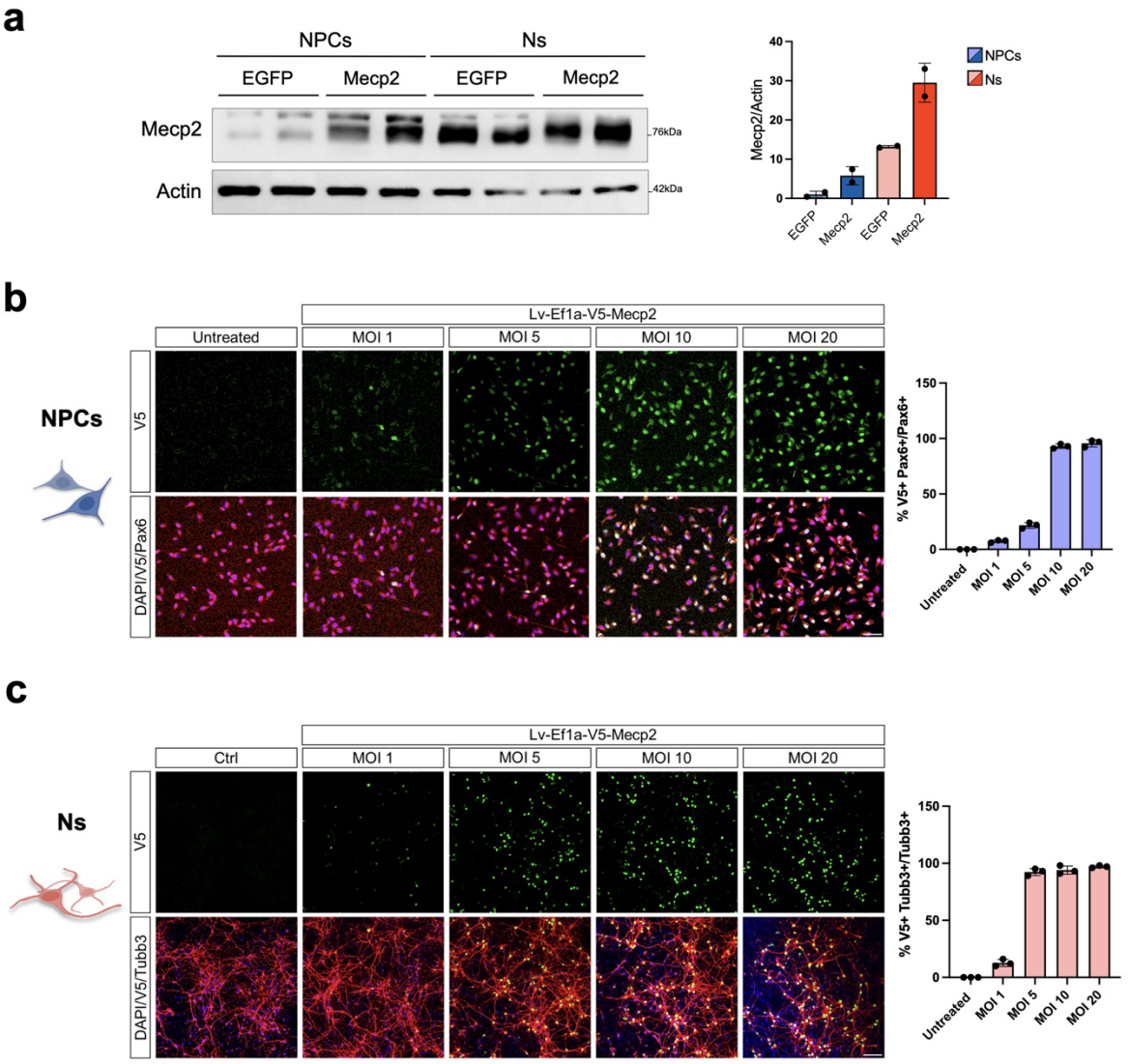
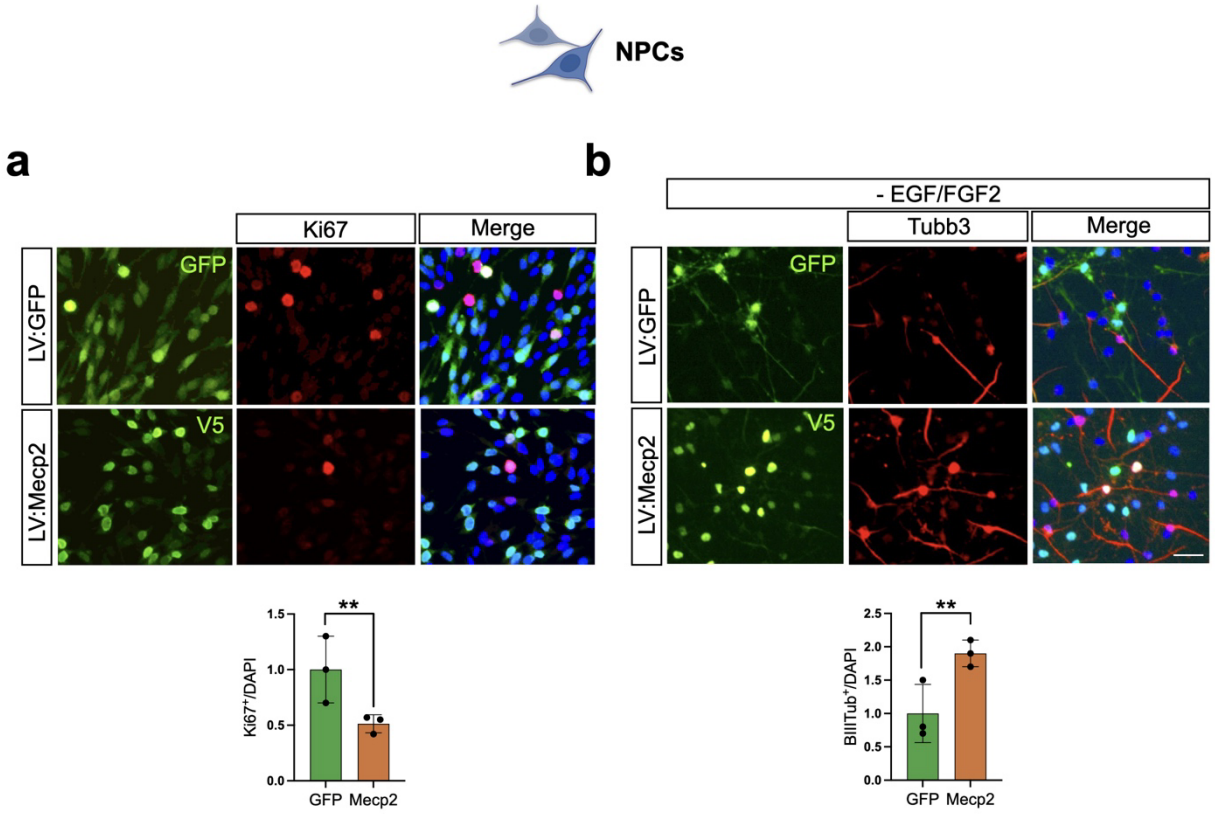
Supplementary Figures

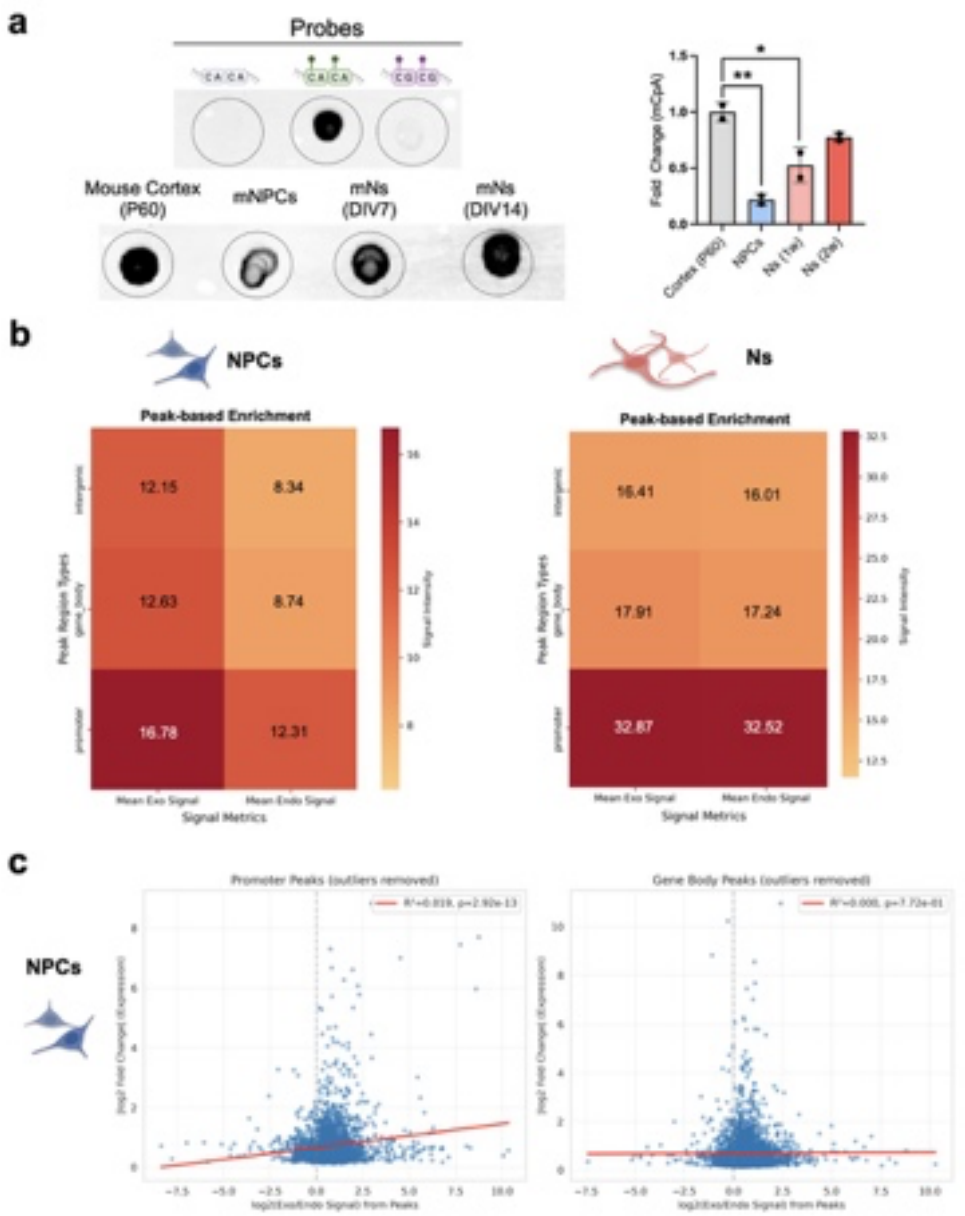


Supplementary Figure 1. Selection of the optimal MOI for efficient transduction of NPCs and Ns.

a) Left: western blot analysis of Mecp2 protein levels in NPCs and Ns transduced with the Mecp2 transgene or EGFP as control. Right: densitometric quantification of Mecp2 relative to Actin is shown as fold change normalized to EGFP-treated NPCs (n = 2 independent biological replicates per condition). Data are presented as mean values ± SD. **b,c**) anti-V5 and anti-Pax6 (NPC marker, **b**) or anti-Tubb3 (Ns marker, **c**) immunostaining in NPCs and Ns treated with increasing MOIs (1, 5, 10, 20) of Mecp2-expressing lentivirus. Left: bar graph showing the percentage of V5+ Pax6+ transduced cells over the total number of PAX6+ cells (**b**) and V5+ Tubb3+ transduced cells over the total number of Tubb3+ cells (**c**). Scale bar: 50um. n = 3 independent biological replicates. Data are presented as mean values ± SD.

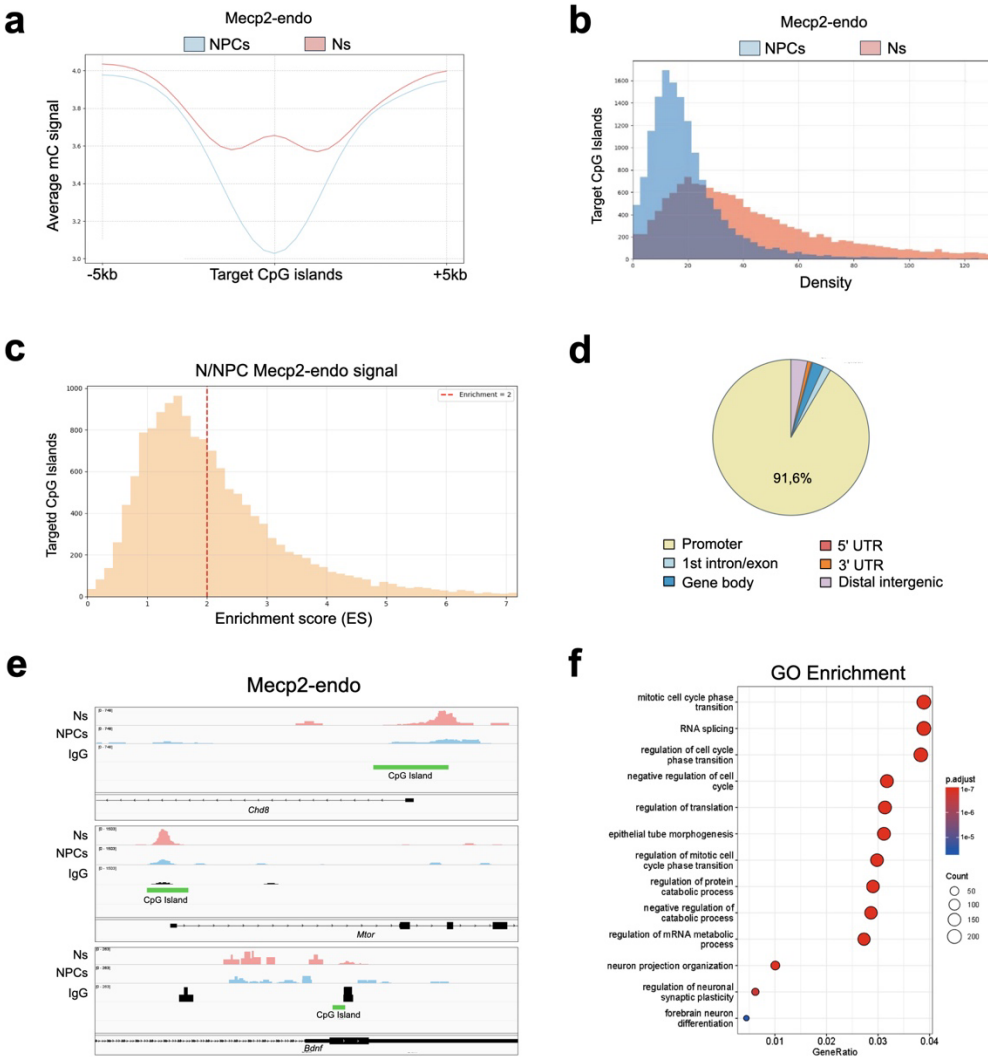


Supplementary Figure 2. Mecp2 overexpression in NPCs accelerates neurogenesis *in vitro*.
a) Top: anti-V5 and anti-Ki67 (proliferation marker) immunostaining in NPCs treated with EGFP or Mecp2. Bottom: bar graph showing the percentage of Ki67+ cells over total cells. Scale bar: 50um. n = 3 independent biological replicates. **b**) Top: anti-V5 and anti-βIII-Tubulin (neuronal marker) immunostaining in NPCs treated with EGFP or Mecp2 after removal of proliferative factors (EGF and bFGF2). Bottom: bar graph showing the percentage of Tubb3+ cells over total cells. Scale bar: 30um. n = 3 biological replicates. Data are presented as mean values ± SD. **p < 0.01, two-sided t-test. (Ki67 NPCs EGFP vs NPCs Mecp2 p = 0.008 (**); Tubb3 NPCs EGFP vs NPCs Mecp2 = 0.003 (**)).



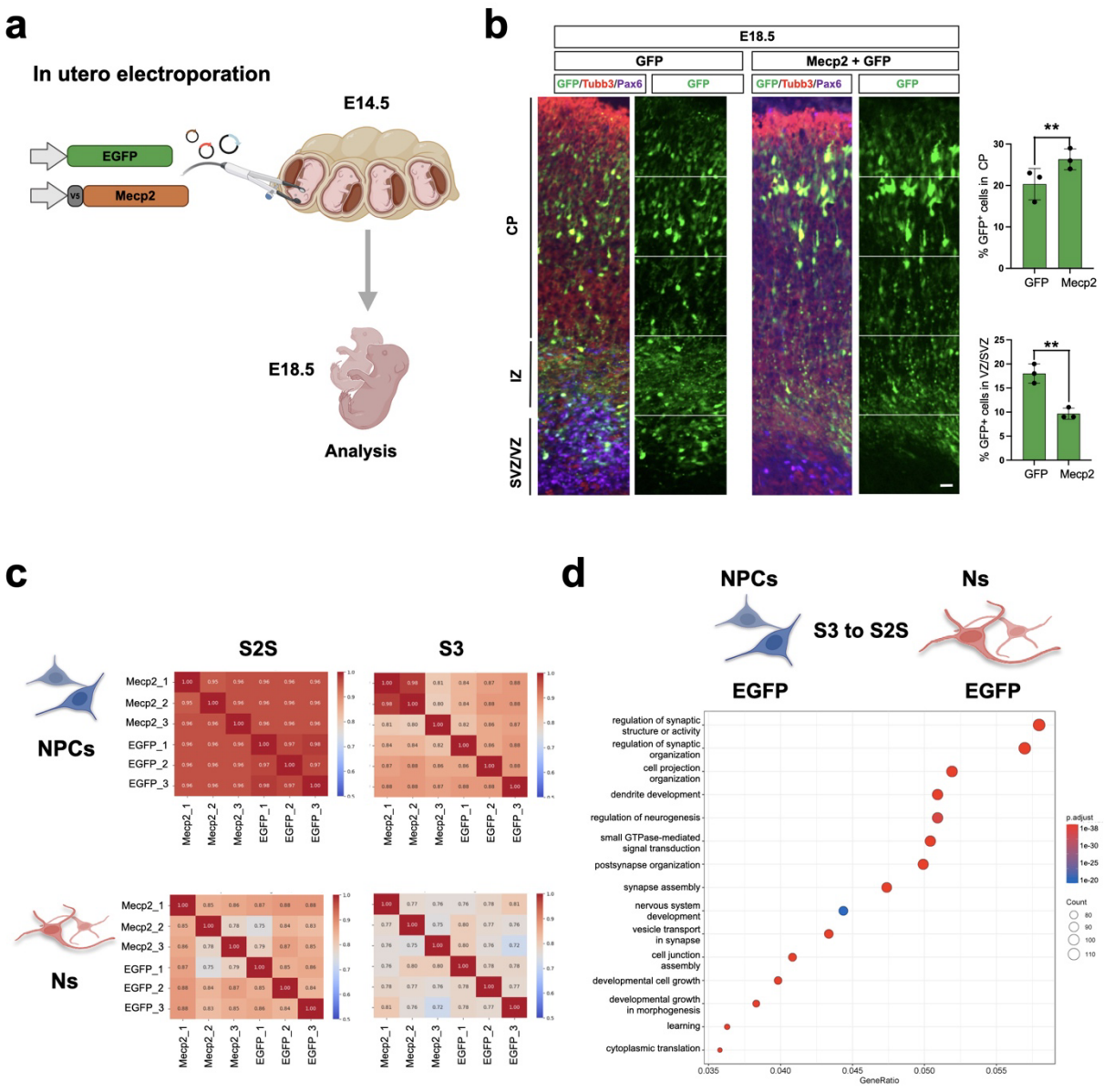
Supplementary Figure 3. Integration of Mecp2 binding, CpA methylation, and gene expression in NPCs and neurons.

a) Representative dot blot showing CpA methylation status. Left top panel: specificity control of the antibody using three probes: unmethylated proble, CpA-methylated probe, and CpG-methylated probe. Left bottom panel: dot blot assessing CpA methylation levels in DNA extracted from adult C57BL/6 WT mouse cortex (P60, positive control), NPCs and Ns after 7 and 14 days in vitro (DIV). Right: bar graph showing densitometric quantification of meCpA dot-blot signals, normalized to total DNA and reported as fold change relative to P60 cortex control (n=2 biological replicates). Data are presented as mean values ± SD. *p < 0.05; **p < 0.01, ANOVA-one way with Tukey's post hoc test. (Cortex P60 vs NPCs = 0.003 (**); Cortex P60 vs Ns 1wk p = 0.018 (*)). **b**) Peak-based enrichment analysis of Mecp2-endo and -exo binding profiles in NPCs and Ns across promoters, gene bodies, and intergenic regions, with signal intensities normalized to region length to allow comparison across genomic features. **c**) Correlation of Mecp2-exo enrichment with DEGs in NPCs, at promoters (left) or within gene bodies (right).



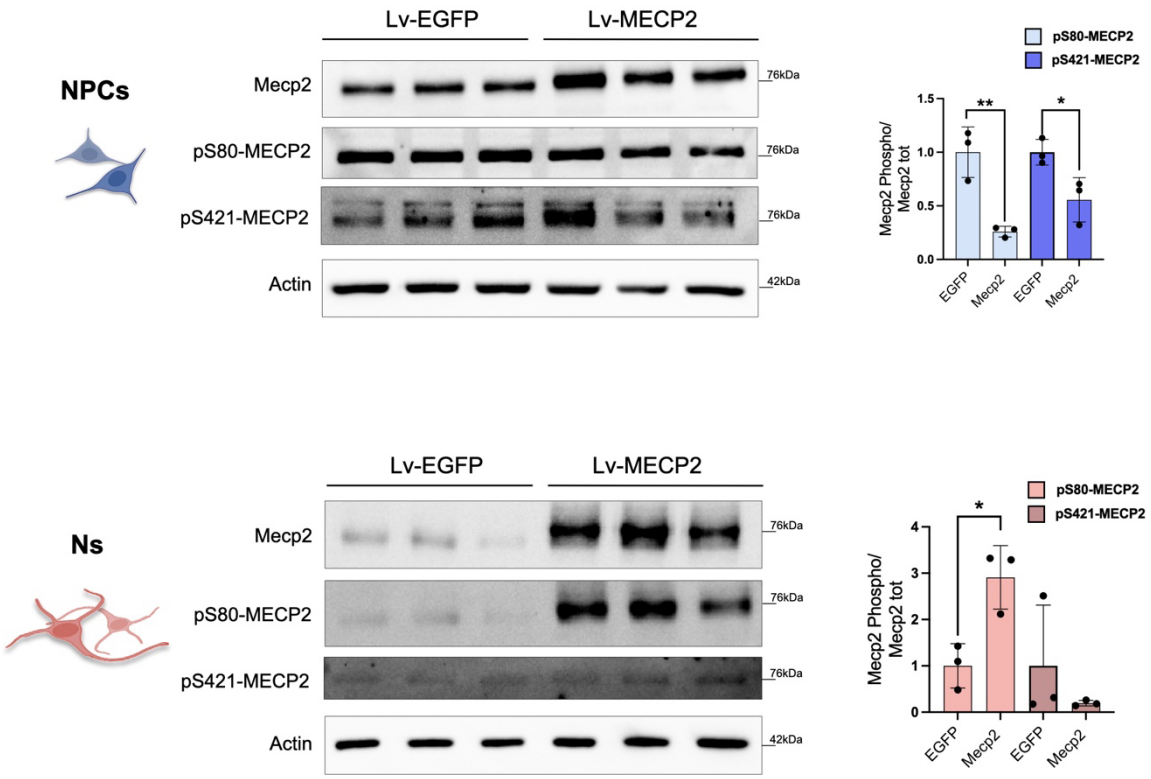
Supplementary Figure 4. Differential enrichment of endogenous Mecp2 at CpG islands during NPC to neuron differentiation.

a) Line plots showing the average distribution of the average methyl-C levels across CpG islands targeted by Mecp2 in NPCs and Ns. **b**) Graph showing the density of Mecp2-endo signal at CpG islands commonly targeted in both NPCs (light blue) and Ns (light red). **c**) Quantification of Mecp2-endo enrichment at CpG islands co-targeted in Ns vs NPCs. In brief, for each condition, we calculated the signal intensity of both Mecp2-endo at shared CpG island targets and computed the enrichment score (ES: Mecp2 endo/endo NPCs). CpG islands with ES >2 (dash red line) were considered significantly enriched by exogenous Mecp2. **d**) Pie charts showing genomic annotations of CpG islands significantly enriched by Mecp2-endo in Ns vs NPCs. **e**) Representative GO categories significantly enriched among genes associated with CpG islands differentially bound by Mecp2 in Ns vs NPCs. **f**) Genome browser snapshots showing CUT&Tag coverage at selected loci with differential Mecp2 enrichment: *Chd8*, *Mtor*, and *Bdnf*. Color legend: light blue = Mecp2-endo NPCs; light red = Mecp2-endo Ns; black = IgG control. Adjusted p values were calculated using Fisher's exact test for overrepresentation, followed by Benjamini–Hochberg correction.



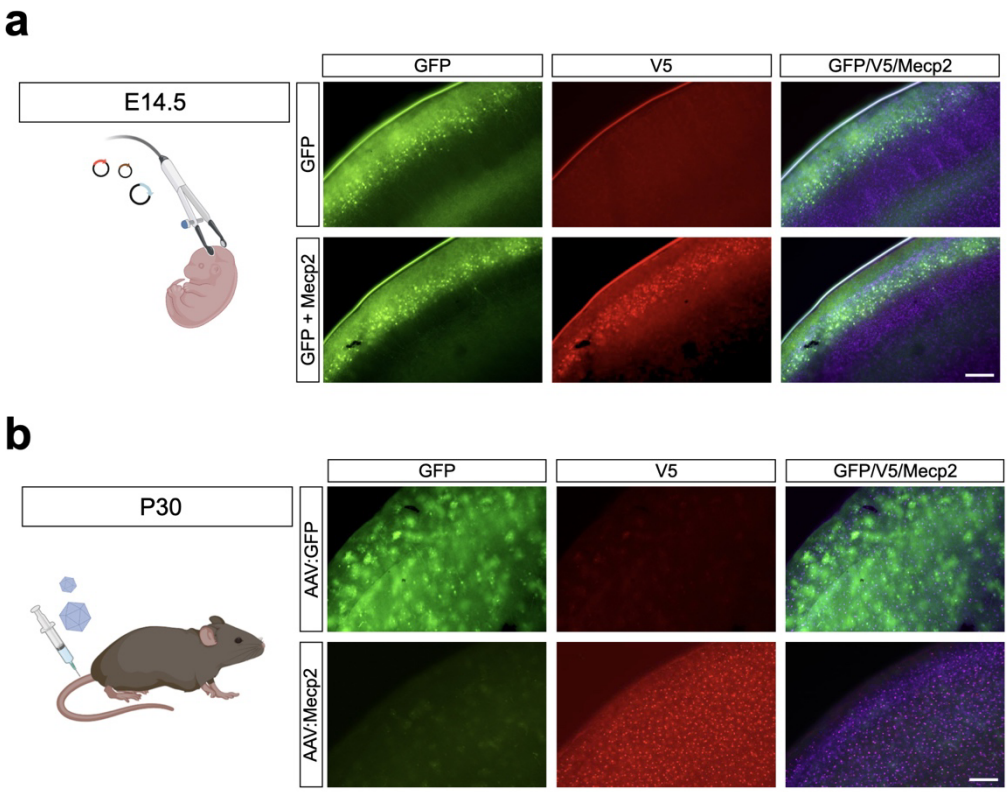
Supplementary Figure 5. 4f-SAMMY-seq analysis reveals major chromatin changes during NPC-to-neuron transition.

a) Schematic of the *in vivo* protocol for Mecp2 overexpression in embryonic NPCs. Briefly, wild-type embryos were electroporated *in utero* at E14.5 with a plasmid expressing EGFP alone (control group) or in combination with Mecp2 overexpressing plasmid. **b**) anti-GFP, anti-Pax6 (progenitor marker), and anti-Tubb3 (neuronal marker) immunostaining in control and Mecp2-electroporated cortices at E18.5. Right: bar graphs showing the percentage of EGFP+ cells in the Tubb3+ cortical plate (top) and in the Pax6+ ventricular/subventricular zones (bottom). Scale bar:100um. Data are presented as mean values ± SD. **p < 0.05, two-sided *t*-test. (CP EGFP vs Mecp2 p = 0.009 (**); VZ/SVZ EGFP vs Mecp2 p = 0.007 (**)). **c**) Spearman correlation plots of biological replicates for S2S and S3 fractions in EGFP- and Mecp2-treated NPCs and neurons. **d**) Representative GO categories significantly enriched among genes located in regions switching from S3 to S2S during NPC to neuron transition. Adjusted p values were calculated using Fisher's exact test for overrepresentation, followed by Benjamini–Hochberg correction. Images were created in BioRender.com. Vania Broccoli (2026).



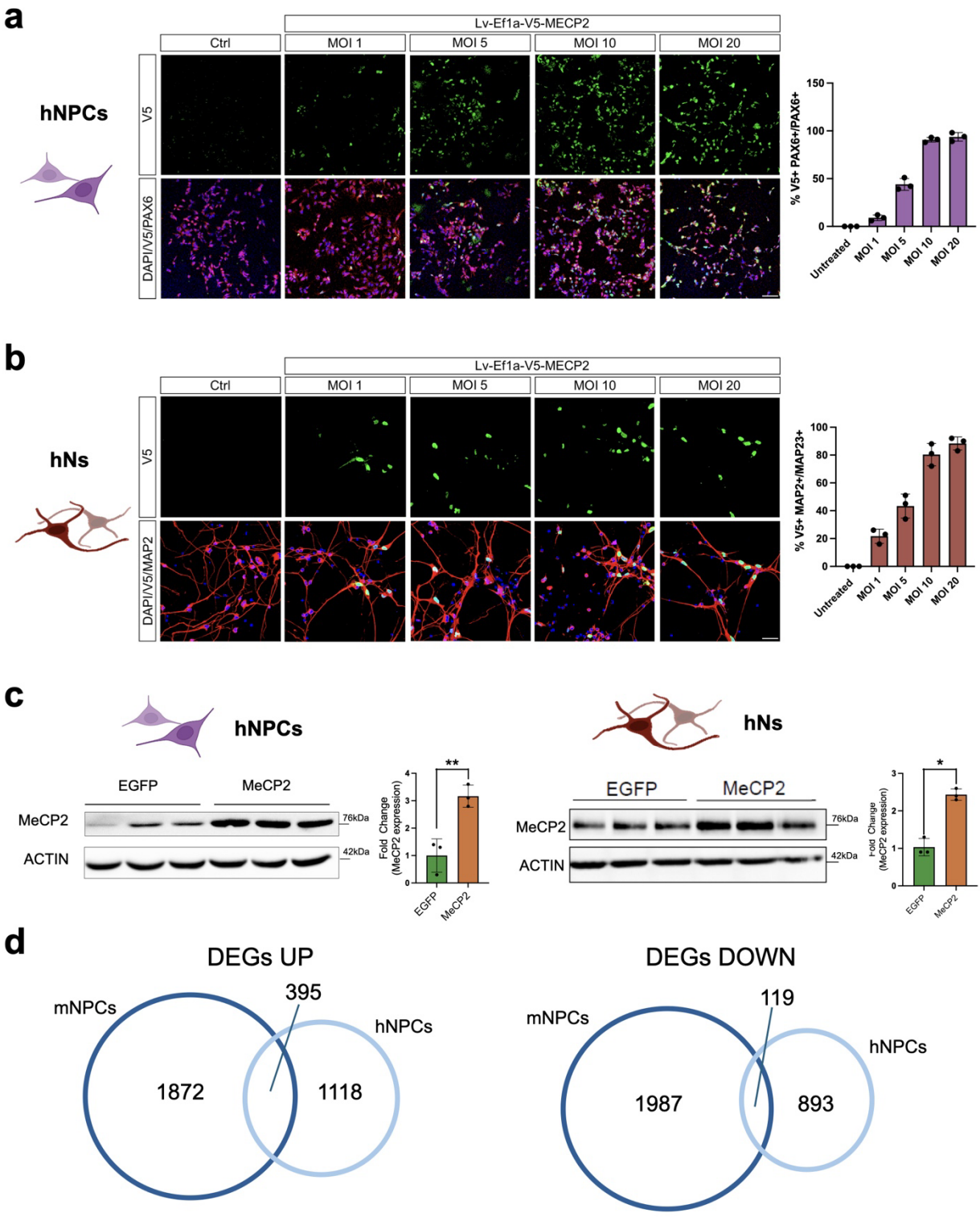
Supplementary Figure 6. Analysis of Mecp2 phosphorylation in NPCs and Ns overexpressing Mecp2.

Western blot analysis of Mecp2 phosphorylation at S80 and S421. Phosphorylation levels were normalized to total Mecp2 and subsequently to actin, in NPCs (top) and Ns (bottom) treated with EGFP or Mecp2. Data are reported as fold change relative to the EGFP control condition (n = 3 independent biological replicates per condition). Data are presented as mean values ± SD. *p < 0.05, **p < 0.01, two-sided *t*-test comparing Mecp2 versus EGFP treatment for each phosphorylation site. (NPCs Mecp2- pS80 EGFP vs Mecp2 p = 0.006 (**); pValue NPCs Mecp2- pS421 EGFP vs Mecp2 = 0.032; Ns Mecp2- pS80 EGFP vs Mecp2 = 0.018 (**)).



Supplementary Figure 7. *In vivo* analysis of Mecp2 overexpression in the mouse cortex.

a,b) anti-EGFP, anti-V5, and anti-Mecp2 immunostaining showing Mecp2 overexpression and distribution in the somatosensory cortex of mice treated at the embryonic level (**a**) or during adulthood (**b**). Scale bar: 200um.



Supplementary Figure 8. ***In vitro*** **characterization of Mecp2/MeCP2 overexpression in hNPCs and hNs.**

a,b) anti-V5 and anti-Pax6 (NPC marker, **A**) or anti-Tubb3 (Ns marker, **b**) immunostaining in human NPCs and Ns treated with increasing MOIs (1, 5, 10, 20) of Mecp2-expressing lentivirus. Left: bar graph showing the percentage of V5+ Pax6+ transduced cells over the total number of PAX6+ cells (**a**) and V5+ Tubb3+ transduced cells over the total number of Tubb3+ cells (**b**). Scale bar: 50um. n = 3 independent biological replicates. Error bars represent SD. **c**) Western blot analysis of Mecp2 protein levels in human NPCs and Ns transduced with the MeCP2 transgene or EGFP. Densitometric

quantification of MeCP2 relative to Actin is shown as fold change compared to the EGFP control (n = 3 independent biological replicates per condition). Data are presented as mean values ± SD. *p < 0.05, **p<0.01 two-sided *t*-test. (hNPCs EGFP vs Mecp2 p = 0.007 (**); hNs EGFP vs Mecp2 p = 0.03 (*)). **d**) Intersection plots showing the number of up-regulated and down-regulated genes shared between mouse and human NPCs upon Mecp2 overexpression.