Supporting Information

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SI Materials and Methods

Primary Cell Cultures. Uncommitted and more lineage-restricted progenitor species derived from embryonic day 14.5 (E14.5) ventral forebrain of CD1 mice were isolated and propagated in serum-free media (SFM) containing specific growth factors for various times and subsequently analyzed using immunofluorescence microscopy to identify neural lineage species, Western blot to examine REST and CoREST protein expression (1, 2) and ChIP-chip to define binding sites for REST and CoREST as previously outlined (3–5). To assess the homogeneity of NSCs and other neural species used for ChIP-chip analyses, we performed detailed immunofluorescence colabeling studies and confirmed the presence of the desired lineage-specific marker(s) and the absence of alternative markers for other neural species. Institutional Animal Care and Use Committee guidelines were followed for all experimental paradigms in which primary mouse tissue was used.

Neural Stem Cell Clones. These clones were elaborated by administration of basic fibroblast growth factor (bFGF, 10 ng/mL) for 7 d in vitro (DIV) in the presence of heparin (2 μg/mL) and clones were dissociated with 0.05% trypsin (GIBCO) for 15 min at 37 °C. Thereafter, individual cells were repropagated in the presence of bFGF plus heparin for an additional 2 DIV to generate secondary NSC clones as previously described and used for subsequent experiments (referred to herein as NSCs) (6–8). This experimental paradigm effectively removes intermediate progenitor species and other proliferating neural cell types present in primary NSC clones and generates effective yields exhibiting >98% homogeneity. NSCs were defined by the presence of nestin and the absence of RC2, Olig2, Mash1, and neuronal and glial lineage markers. These clonal populations were used for the assessment of NSC-associated properties [self-renewal, proliferation, lineage restriction, and multilineage potential (i.e., potential to give rise to neurons, oligodendrocytes, and astrocytes)] as previously described (9–12).

Lineage-Restricted Neuronal-OL Progenitor (N/OP) Clones. These clones were derived from dissociated ventral forebrain-derived NSC clones by addition of bFGF and the N-terminal active form of Shh (N-Shh, 100 ng/mL) for 2 DIV to obtain >98% homogeneity, as previously described (6–8). N/OPs were defined by the presence of nestin, Olig2, and Mash1 and the absence of RC2 and neuronal and glial markers.

Radial Glial (RG) Species. These clones were elaborated from dorsal forebrain-derived NSC clones that were plated on poly-D-lysine (PDL)-coated dishes in SFM supplemented with laminin (3 μg/mL) in the presence of bFGF (10 ng/mL) and leukemia inhibitory factor (LIF; 10 ng/mL) for 2 DIV to obtain >97% purity (7, 8). RG were defined by the presence of nestin and RC2 and the absence of coexpression of Olig2 and Mash1 as well as neuronal and glial markers.

Oligodendrocyte (OL) Lineage Species. As previously described, OL precursor cells (OLpres) were derived from N/OP clones by the addition of platelet-derived growth factor (PDGF-AA, 10 ng/mL) for 2 DIV. Using this culture paradigm, OLpres (NG2+/O4−) represented >98% of the cell population. Progressive stages of OL lineage maturation were generated by sequential propagation of OLpres in SFM containing laminin (3 μg/mL; BD Biosciences) on PDL-coated culture dishes at nonconfluent cellular densities. OL progenitors (NG2−/O4+) were subsequently elaborated by the addition of PDGF-AA (10 ng/mL) for 2 DIV to achieve >95% homogeneity. Postmitotic OLs (GC/O1+) and myelin expressing OLs (MBP+) were elaborated from OL progenitors following withdrawal of PDGF-AA for 2 and 4 DIV to achieve >95% homogeneity, respectively (8, 13, 14).

Astrocyte (AS) Species. These species were elaborated by dissociation of NSC clones with trypsin for 15 min at 37 °C and repropagation of individual cells using epidermal growth factor (EGF) for 7 DIV with subsequent addition of bone morphogenetic protein 2 (BMP2) for 5 DIV (15, 16). ASs (nestin−/GFAP+) were elaborated using this culture paradigm to achieve a purity of >98%.

GABAergic Neurons (GABANs). These neurons were elaborated by addition of BMP2 (10 ng/mL) to N/OPs and propagated on PDL-coated culture dishes containing laminin (3 μg/mL; BD Biosciences) for 2 DIV (6, 7). To guarantee that GABAergic achieved >99% homogeneity for ChIP-chip experiments, 10 μM cytosine arabinoside (Sigma) was added to the culture media 24 h before harvesting, eliminating the presence of all cycling cells (17–19), and the homogeneity of the surviving cells was confirmed by immunofluorescence microscopy as previously noted.

Cholinergic Neurons (CHOLNs). These neurons were elaborated from ventral forebrain-derived NSC clones by modification of a previously described culture paradigm (20). Our modifications to the initial culture paradigm included, specifically, that (*i*) we isolated (murine) E12.5 ventral telencephalic NSCs that give rise to CHOLNs (21) and (*ii*) we expanded these NSCs in suspension in the presence of neurobasal medium (GIBCO) supplemented with N2, N-Shh (100 ng/mL), and nerve growth factor (NGF) (200 ng/mL) as well as bFGF (10 ng/mL) for 2 DIV. These NSC clones were subsequently transferred onto PDL-coated culture dishes and cultured in the presence of laminin (3 μg/mL) as described (20). To guarantee that CHOLNs achieved >99% homogeneity, 10 μM cytosine arabinoside (Sigma) was added to the culture media 24 h before harvesting, eliminating the presence of all cycling cells (17–19), and the homogeneity of the surviving cells was confirmed by immunofluorescence microscopy.

Medium Spiny Projection Neurons (MSNs). These neurons were elaborated from ventral forebrain-derived NSC clones by modification of a previously defined culture paradigm (22). Our modifications to the initial culture paradigm included, specifically, that (*i*) we isolated (murine) E12.5 ventral telencephalic NSCs that give rise to MSNs (23) and (*ii*) we expanded these NSCs in suspension in the presence of neurobasal media, B27, bFGF (10 ng/mL), N-Shh (50 ng/mL), and brain-derived neurotrophic factor (BDNF) (50 ng/mL) for 1 DIV. These NSC clones were subsequently transferred onto PDL-coated culture dishes and cultured in the presence of laminin (3 μg/mL) and 1% FBS as described (22). To guarantee that MSNs achieved >99% homogeneity, 10 μM cytosine arabinoside (Sigma) was added to the culture media 24 h before harvesting, eliminating the presence of all cycling cells (17–19), and the homogeneity of the surviving cells was confirmed by immunofluorescence microscopy.

Glutamatergic Neurons (GLUTNs). These neurons were elaborated from RG species, which were cultured for an additional 4 DIV in SFM following the withdrawal of LIF (7). To guarantee that GLUTNs achieved >99% homogeneity, 10 μM cytosine arabinoside (Sigma) was added to the culture media 24 h before harvesting, eliminating the presence of all cycling cells (17–19),

and the homogeneity of the surviving cells was confirmed by immunofluorescence microscopy.

Growth Factor Preparations. Briefly, the following growth factor preparations were used in our various clonal culture paradigms: recombinant bFGF (Collaborative Biomedical Products); recombinant EGF, N-Shh, and PDGF-AA (R&D Systems); and BMP2 (a gift from Genetics Institute). To generate comparative neuronal subtype species, the following additional growth factor preparations were used: recombinant mouse β-NGF (R&D Systems), human BDNF (BioVision), and recombinant LIF (Chemicon).

Specific Antibody Preparations. All antibodies exhibited selective immunoreactivity for mouse cells and tissue sections, and each antibody exhibited a complete absence of alternate cross-reactivity. The following antibodies were used: CoREST, REST, and normal rabbit IgG (1:100; Upstate), the neuroepithelial marker (nestin, mIgG1, 1:200; Pharmingen), N/OP markers [Olig2, goat IgG, 1:300 (R&D); and Mash1, mIgG1, 1:100 (Pharmingen)], radial glial intermediate progenitor markers [RC2, mIgM, 1:10 (DSHB); and GLAST, guinea pig IgG, 1:000 (Millipore)] OLpre marker (NG2, rIg, 1:500; Chemicon), OL progenitor marker (O4, mIgM, 1:700; Sigma), postmitotic OL marker (GC/O1, mIgM, 1:350; Chemicon), myelin expressing OL marker (MBP, mIgG2b, 1:500; Sternberger Monoclonals), AS marker (GFAP, mIgG1, 1:400; Sigma), neuronal markers [β-tubulin, mIgG2b, 1:700 (Sigma); and doublecortin, rIg, 1:100 (Santa Cruz)], postmitotic neuronal marker (MAP2, mIgG1, 1:100; Sigma), GABAN marker (GABA, rIgG, 1:1,000; Sigma), GLUTN marker (Glutamate, rIgG, 1:1,000; Sigma), CHOLN marker (ChAT, goat IgG, 1:100; Millipore), and MSN marker (DARPP32, rIgG, 1:100; Santa Cruz). Isotype specific secondary antibodies were used at a 1:1,500 dilution according to the required fluorophore combinations (Invitrogen). Secondary antibodies used for Western blot analysis were HRP conjugated (GE Healthcare). The CoREST and REST antibodies were first validated using a peptide competition assay (Fig. S1 *C* and *D*).

Gene Ablation Paradigm. Individual depletion of REST and CoREST in NSC cultures was performed by the application of lentiviral constructs containing REST-shRNA and CoREST-shRNA as well as control (scrambled, Scr-shRNA) (Santa Cruz) according to the manufacturer's guidelines. Briefly, freshly isolated NSC preparations were infected with lentiviral particles in the presence polybrene (1 μg/mL; Santa Cruz) and were propagated in SFM containing bFGF (10 ng/mL) for 48 h. Thereafter, puromycin (0.5 μg/mL; Santa Cruz) was added to the culture medium for 24 h to eliminate noninfected cells. Virally infected NSCs were subsequently expanded for 4 DIV in SFM containing bFGF plus heparin, and these primary clones were, in turn, dissociated and repropagated to obtain secondary clones. The depletion of REST (>50%) and CoREST (>65%) was confirmed by QRT-PCR analysis. These clonal populations were used for the assessment of NSC-associated properties (self-renewal, proliferation, lineage restriction, and multilineage potential) as described above (9–12).

Western Blot Analysis. Cells were homogenized in 9 vol of buffer containing 0.32 M sucrose, 50 mM Tris-HCl, pH 8.0, EDTA-free protease inhibitor mixture (Roche), and 0.5 mM phenylmethylsulfonyl fluoride using a glass–Teflon homogenizer (10 strokes at 800 rpm) on ice; centrifuged at 900 × *g* for 10 min; and lysed in SDS sample-loading buffer (1, 2, 7, 8).

Chromatin Immunoprecipitation-on-Chip (ChIP-Chip) Assays. As previously described, ChIP-chip is an in vivo technique that can be used to identify transcription factor binding sites (3–5, 7, 8). Briefly, we performed a series of ChIP-chip experiments in NSCs and other neural cell types to build profiles of CoREST and REST target genes. ChIP was performed using 1×10^6 cells for each neural subtype and 10 μg of CoREST, REST, or control rabbit IgG antibodies (Upstate) as well as no antibody (input). Samples were analyzed by quantitative ChIP (QChIP) using known and previously validated CoREST and REST targets as positive controls (24, 25). Enrichment of fragments by ChIP was quantified by using 1 μL of ChIP product for real-time PCR, using the SYBR Green kit (Applied Biosystems) in a 7,000 Real Time PCR system (Applied Biosystems). The mouse promoter array was based on MM8/mouse genome build 36 from February 2006 (NimbleGen). The design was based only on normal RefSeq genes (17,355 genes) and included 2,000 bp upstream of the transcriptional start sites and 500 bp downstream. The probe sizes ranged from 50 to 75 bp and the spacing interval was 100 bp.

ChIP-Chip Data Analysis. Analysis was performed essentially as previously described (7, 8). Enrichment was calculated for each probe by computing the log-ratio value for the ChIP immunoprecipitated product in comparison with the input chromatin. For all ChIP-chip experiments, to find promoter peaks, a maximum log-ratio value for a window consisting of three consecutive probes was determined for both experimental data and a random permutation of the data. A positive threshold was then established to determine the probability for real enrichment. This positive threshold was determined by examining signals of known CoREST and REST binding sites, GluR2, and calbindin, respectively (24, 25). A 90% positive threshold was used. The gene target lists were generated on the basis of the intersection of genes across a minimum of two arrays for each experimental paradigm. We validated these ChIP-chip results in a representative sample of cell types with QChIP and found 83 and 94% correlation for CoREST and REST, respectively. These results indicate that the ChIP-chip technique and data analysis methods we used to characterize CoREST and REST target genes are effective approaches for identifying valid binding sites.

Gene Expression Analysis. The gene expression array was based on MM8/mouse genome build 36 from February 2006 (NimbleGen). The Robust Multi-Array Average algorithm was used and data were also analyzed by the significant analysis of microarray methods (SAM). Each progenitor cell type (N/OP and RG) was compared with the corresponding NSC species. Three biological replicates were performed for each cell type.

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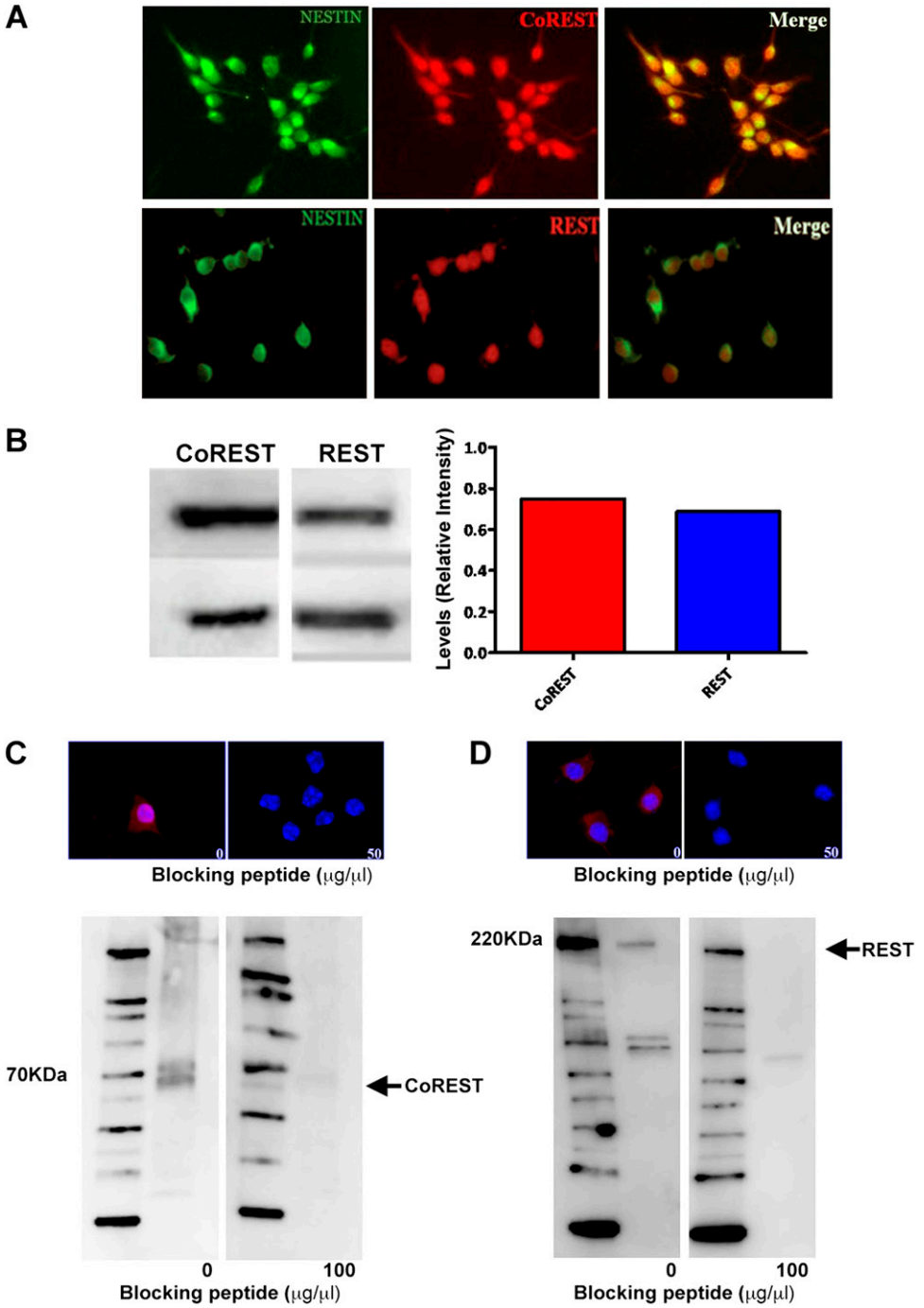

Fig. S1. CoREST and REST expression in neural stem cells (NSCs). (*A*) Immunofluorescence microscopy reveals that both CoREST and REST (TRITC) are expressed within the nucleus and the cytoplasm of NSCs (nestin, FITC). (*B*) Western blot analysis and graphic representation of the intensity of expression bands demonstrate the relative expression levels of CoREST or REST in comparison with the actin loading control. (*C* and *D*) Peptide competition assay for identifying CoREST and REST antibody specificity by immunocytochemistry and Western blot analysis for NSCs. (*C*) CoREST immunofluorescence microscopy (*Upper*) and Western blot analysis (*Lower*) in the presence or absence of blocking peptide. CoREST primary antibody staining is depicted with TRITC and Hoescht nuclear staining is depicted with DAPI. (*D*) REST immunofluorescence microscopy (*Upper*) and Western blot analysis (*Lower*) in the presence or absence of blocking peptide. REST primary antibody staining is depicted with TRITC and Hoescht nuclear staining is depicted with DAPI.

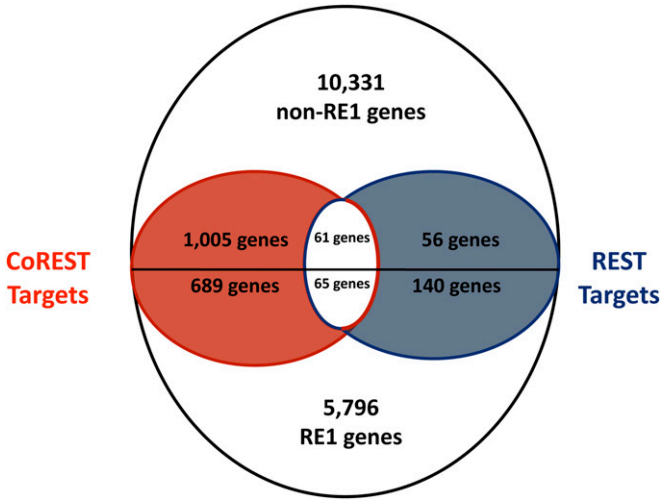

Fig. S2. Comparative distributions of repressor element-1 (RE1)-containing motifs in CoREST and REST neural stem cell target genes. CoREST targets (red) and REST targets (blue) were compared with a previously characterized set of RE1-containing genes (RE1 genes) (1, 2). Note the distribution of REST targets, which favor RE1 genes; CoREST targets, which favor non-RE1 genes; and targets of both CoREST and REST, which are more evenly distributed.

1. Bruce AW, et al. (2004) Genome-wide analysis of repressor element 1 silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) target genes. *Proc Natl Acad Sci USA* 101:10458–10463.
2. Otto SJ, et al. (2007) A new binding motif for the transcriptional repressor REST uncovers large gene networks devoted to neuronal functions. *J Neurosci* 27:6729–6739.

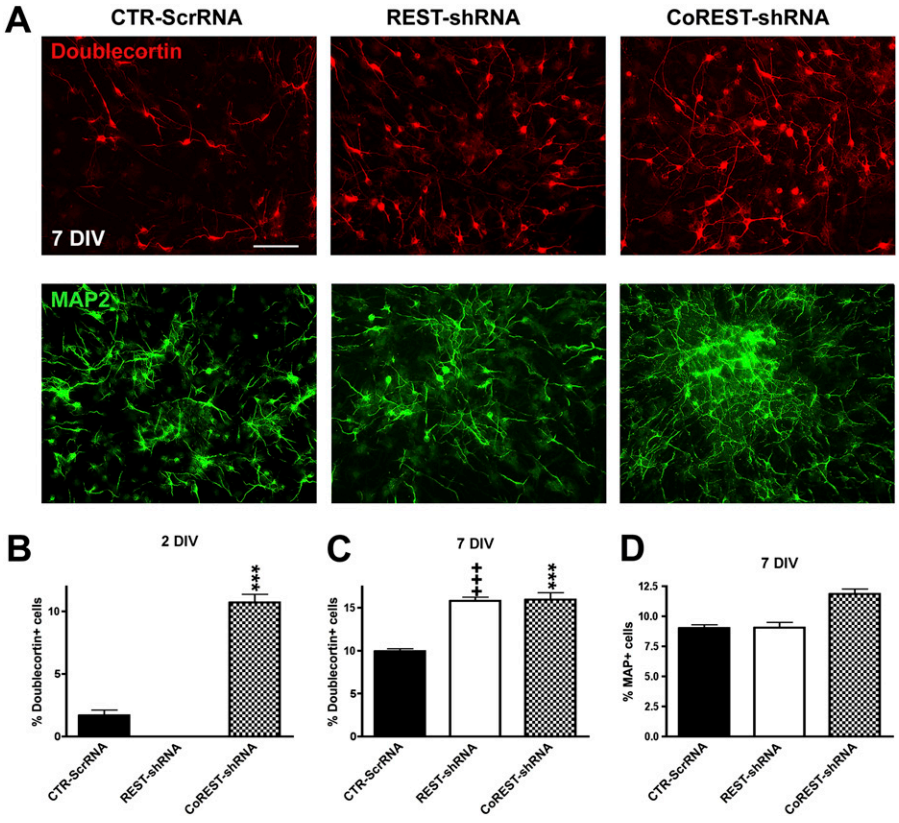

Fig. S3. Effects of selective REST and CoREST depletion on neuronal lineage maturation. (*A*) Immunofluorescence microscopic analysis illustrates the clonal composition of early (doublecortin, TRITC) and more mature (MAP2, FITC) neuronal species at 7 DIV. (Scale bar, 200 μm.) (*B–D*) The elaboration of early neuronal species at cell cycle exit seems to depend on the presence of REST, whereas the elaboration of early and more mature neuronal species seems to depend on the presence of CoREST. Bars in *B–D* represent the mean ± SEM of three independent biological replicates. *** and +++$P < 0.0001$.

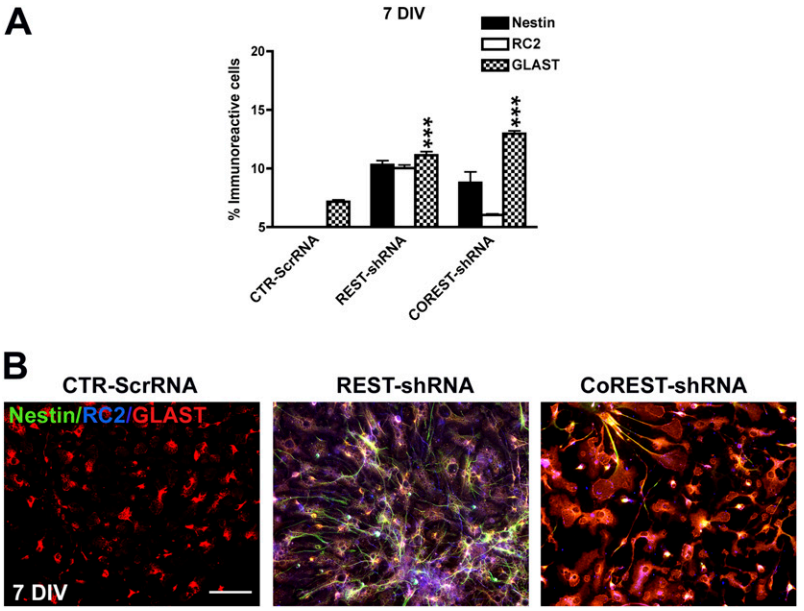

Fig. S4. Effects of selective REST and CoREST depletion on the generation of intermediate progenitor species. (*A*) The immunofluorescence microscopic analysis of nestin+ (FITC), RC2+ (DAPI), and GLAST+ (TRITC) clones at 7 DIV revealed that both CoREST- and REST-depleted clones display significant increases in the maintenance of intermediate progenitor species, compared with controls. However, the profiles of intermediate progenitor species are distinct. CoREST-depleted clones exhibit differential profiles of nestin, RC2, and GLAST staining, whereas REST-depleted clones exhibit coexpression of these markers. Bars in *A* represent the mean ± SEM of three independent biological replicates. ***$P < 0.0001$. (*B*) Immunofluorescence microscopic analysis demonstrating the lineage composition of secondary clones derived from NSCs following selective ablation of REST or CoREST, including the comparative profiles of neural stem cells (nestin, FITC) and radial glial (RC2, DAPI; GLAST, TRITC) species. (Scale bar, 200 μm.)

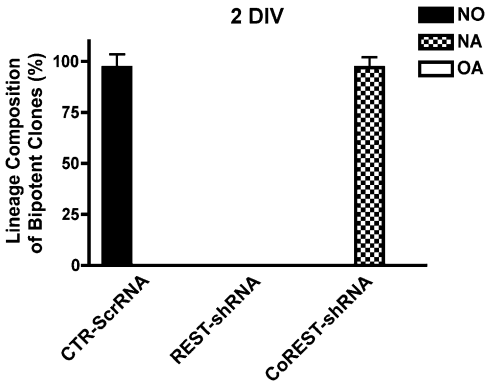

Fig. S5. Effects of selective REST and CoREST depletion on neural lineage composition for bipotent clones generated in the neural fate decision assays (Fig. 2*C*). Neuronal-oligodendrocyte (NO) clones were exclusively generated in the control condition at 2 DIV, whereas neuronal-astrocyte (NA) clones were exclusively generated in CoREST-depleted condition. Note that oligodendrocyte-astrocyte (OA) clones were not observed under any clonal culture condition. In addition, the number of bipotent clones in each culture condition represents only a subset of the total clonal population. Bars represent the mean ± SEM of three independent biological replicates.

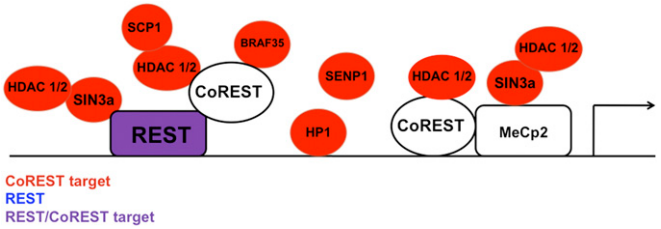

Fig. S6. CoREST is a key transcriptional and epigenetic regulator of the REST/CoREST repressor complex. Schematic of REST/CoREST regulatory complexes with factors encoded by CoREST NSC target genes is depicted in red and that of dual CoREST/REST target genes is depicted in purple. Note that no members of the REST/CoREST regulatory complexes represent REST NSC target genes. The schematic is based on a previously described model of the REST/CoREST regulatory complexes (1).

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Table S1. Comparative analysis of pathways enriched in unique CoREST and REST NSC target genes identified through Ingenuity pathway analysis

Pathways enriched in unique CoREST target genes	−Log (*P* value)	Pathways enriched in unique REST target genes	−Log (*P* value)
Hypoxia signaling in the cardiovascular system	4.30	Glutamate receptor signaling	2.71
Wnt/β-catenin signaling	3.57	Circadian rhythm signaling	2.61
NRF2-mediated oxidative stress response	3.00	Serotonin receptor signaling	2.41
Protein ubiquitination pathway	2.97	Nitrogen metabolism	2.08
Role of BRCA1 in DNA damage response	2.97	cAMP-mediated signaling	1.22
p53 signaling	2.86	GABA receptor signaling	1.14
Cell cycle: G1/S checkpoint regulation	2.59	β-Alanine metabolism	1.04
Biosynthesis of steroids	2.54		
Axonal guidance signaling	2.26		
B cell receptor signaling	2.26		
Huntington's disease signaling	2.22		
Ephrin receptor signaling	2.22		
Tight junction signaling	2.10		
PDGF signaling	1.82		
IGF1 signaling	1.75		
PTEN signaling	1.71		
Synaptic long-term potentiation	1.71		
Neurotrophin/TRK signaling	1.63		
Clatrin-mediated endocytosis	1.56		
Phenylalanine, tyrosine, and tryptophan biosynthesis	1.53		
EGF signaling	1.49		
GM-CSF signaling	1.49		
PI3K/AKT signaling	1.41		
Activation of IRF by cytosolic pattern recognition receptors	1.32		
Integrin signaling	1.31		
Regulation of actin-based motility by Rho	1.22		
Erythropoietin signaling	1.20		
Glucocorticoid receptor signaling	1.20		
Calcium signaling	1.19		
JAK/Stat signaling	1.18		
Neuregulin signaling	1.16		
Nucleotide excision repair pathway	1.08		
Purine metabolism	1.06		
ERK/MAPK signaling	1.06		
SAPK/JNK signaling	1.04		
Chemokine signaling	1.03		
VEGF signaling	1.01		

Functional classification of unique CoREST and REST NSC target genes is shown. Target genes were classified by using the Ingenuity pathway analysis program (Ingenuity Systems). Only categories that were statistically significant are included.

Table S2. Comparative gene expression profiles associated with differential CoREST and REST promoter occupancy during neural stem cell-mediated lineage restriction

ChIP-chip target		Total differentially expressed		RE1 containing		PluriNet		Targets of Oct4/Sox2/Nanog	
		Up	Down	Up	Down	Up	Down	Up	Down
CoREST									
In NSC	In RG								
No	Yes	40	100	12	42	1	1	1	1
Yes	No	224	757	81	303	1	25	10	34
Yes	Yes	10	46	4	16	0	1	0	0
In NSC	In N/OP								
No	Yes	25	69	9	18	1	0	2	3
Yes	No	509	271	225	108	31	5	27	11
Yes	Yes	16	10	7	6	0	1	3	0
REST									
In NSC	In RG								
No	Yes	51	85	23	40	0	2	2	2
Yes	No	40	102	24	53	1	2	1	1
Yes	Yes	9	21	8	18	0	0	0	0
In NSC	In N/OP								
No	Yes	30	19	8	10	1	0	0	4
Yes	No	44	67	26	46	2	2	1	2
Yes	Yes	0	2	0	1	0	0	0	1

We performed comparative analyses of the numbers of up- and down-regulated genes associated with differential CoREST and REST promoter occupancy during transitions from NSCs into less and more lineage-restricted radial glia (RG) and neuronal-oligodendrocyte progenitors (N/OPs), respectively. The absence or presence of REST and CoREST promoter occupancy for target genes in each cell type is indicated by "No" and "Yes," respectively. We show subsets of genes that are RE1 motif containing (1, 2) and those involved in pluripotency networks (3, 4). We observed that these genes are associated with distinct expression profiles depending on the degree of NSC-mediated lineage restriction.

1. Bruce AW, et al. (2004) Genome-wide analysis of repressor element 1 silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) target genes. *Proc Natl Acad Sci USA* 101:10458–10463.
2. Otto SJ, et al. (2007) A new binding motif for the transcriptional repressor REST uncovers large gene networks devoted to neuronal functions. *J Neurosci* 27:6729–6739.
3. Muller FJ, et al. (2008) Regulatory networks define phenotypic classes of human stem cell lines. *Nature* 455:401–405.
4. Boyer LA, et al. (2005) Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 122:947–956.

Dataset S1. Composite profiles of REST and CoREST target genes in neural stem cells.

Dataset S1