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Short Article

Cross-Regulation between an Alternative Splicing Activator and a Transcription Repressor Controls Neurogenesis

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SUMMARY

Neurogenesis requires the concerted action of numerous genes that are regulated at multiple levels. However, how different layers of gene regulation are coordinated to promote neurogenesis is not well understood. We show that the neural-specific Ser/Arg repeat-related protein of 100 kDa (nSR100/SRRM4) negatively regulates REST (NRSF), a transcriptional repressor of genes required for neurogenesis. nSR100 directly promotes alternative splicing of REST transcripts to produce a REST isoform (REST4) with greatly reduced repressive activity, thereby activating expression of REST targets in neural cells. Conversely, REST directly represses nSR100 in nonneural cells to prevent the activation of neural-specific splicing events. Consistent with a critical role for nSR100 in the inhibition of REST activity, blocking nSR100 expression in the developing mouse brain impairs neurogenesis. Our results thus reveal a fundamental role for direct regulatory interactions between a splicing activator and transcription repressor in the control of the multilayered regulatory programs required for neurogenesis.

INTRODUCTION

The transition from proliferating neural stem and progenitor cells (NPCs) to differentiated mature neurons is characterized by global changes in the transcriptome. The activation of neuronal-specific gene expression is controlled by critical factors including chromatin regulators, proneural bHLH transcription factors, components of the Wnt and Notch signaling pathways, and neuronal-specific miRNAs such as miR-124 and miR-9 (Suh et al., 2009). Neurogenesis also depends on the loss of inhibitory signals that prevent neuronal differentiation and promote NPC proliferation. For example, the repressor element 1 (RE-1) silencing transcription factor (REST/NRSF) is highly expressed in nonneuronal cells and in NPCs, where it acts as a key repressor of genes involved in neurogenesis (Chen et al., 1998; Chong et al., 1995; Schoenherr and Anderson, 1995). However, how REST is inactivated in concert with the various activating signals required for nervous system development is not well understood.

REST directly binds to RE-1 sites present on target genes and, together with interacting corepressors, silences expression of neural differentiation genes (Ooi and Wood, 2007). Loss of REST activity in vivo results in the derepression of REST target genes in nonneural tissues and NPCs (Chen et al., 1998), whereas REST overexpression inhibits the expression of target genes in neuronal cells (Paquette et al., 2000). Therefore, as NPCs differentiate into neurons, REST activity must be suppressed to allow expression of neuronal-specific genes. This suppression of activity is thought to arise in part from reduced *REST* transcript levels (Chong et al., 1995; Schoenherr and Anderson, 1995) and proteolytic degradation (Qureshi and Mehler, 2009). However, the extent to which these events affect REST activity and whether additional mechanisms exist to downregulate REST in differentiating NPCs is not known. In this study, we investigate the contribution of alternative splicing (AS) regulation to this process.

Transcripts from nearly all human multiexon genes undergo AS, with the brain possessing among the most complex repertoires of splice variants (Pan et al., 2008; Wang et al., 2008). This complexity reflects a growing list of AS events shown to have important roles in nervous system development and function. Neural-specific or enriched splicing factors such as Nova-1/2, Fox-1/2, and PTBP2 (nPTB, brPTB) function to establish AS networks that contribute to critical processes such as neuronal migration, synaptogenesis, and neurite outgrowth (Calarco et al., 2011; Li et al., 2007). We recently discovered the neural-specific Ser/Arg repeat-related protein of 100 kDa (nSR100/SRRM4), a splicing regulator that activates the inclusion of ~11% of brain-specific alternative exons (Calarco et al., 2009). These nSR100-dependent exons are

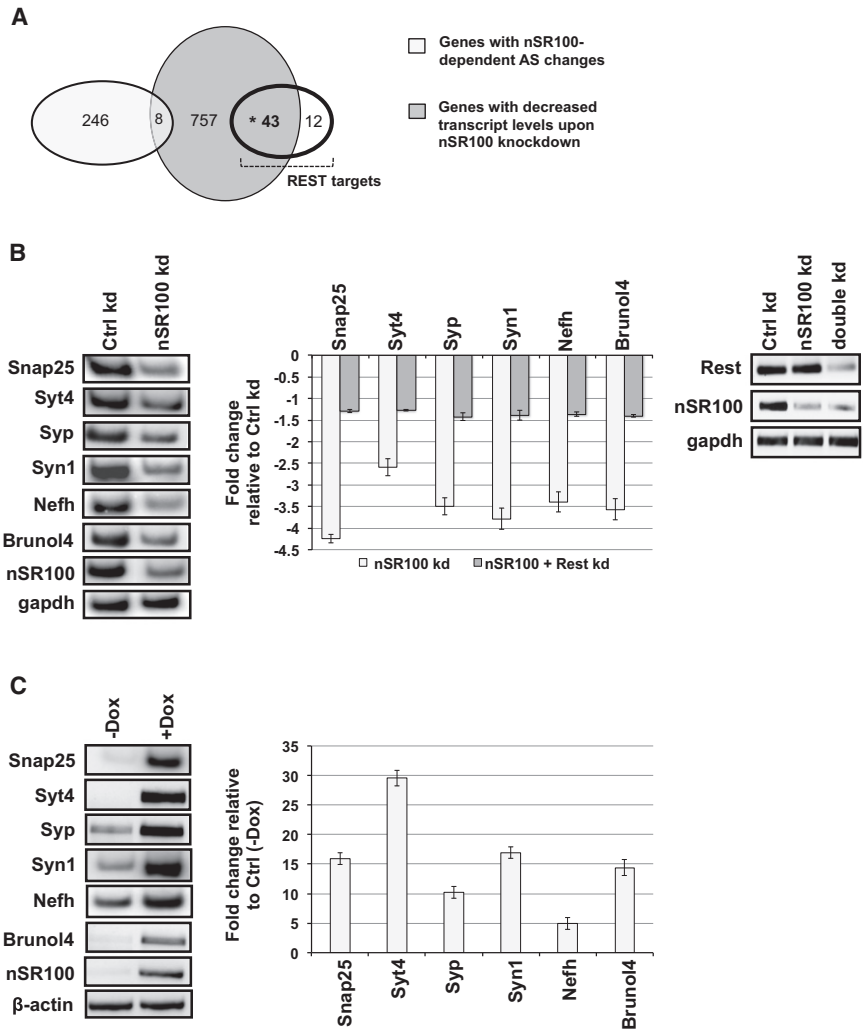

Figure 1. nSR100 Expression Affects mRNA Steady-State Levels of REST Target Genes

(A) Venn diagram of sets of genes regulated by nSR100 and REST in N2a cells. Genes with ≥ 15% AS and/or ≥ 1.5-fold transcript-level changes upon nSR100 knockdown are represented. *p = *9.4E-39*, hypergeometric test.

(B) (Left) RT-PCR assays monitoring expression of REST targets in control and nSR100 knockdown N2a cells. Bar graph represents qPCR analysis of fold gene expression changes relative to control when nSR100 is knocked down (light gray bars) and upon double knockdown of REST and nSR100 (dark gray bars). n = 4, error bars ± SD. (Right) RT-PCR reactions confirm efficient knockdown of nSR100 and/or REST. Gapdh mRNA levels were used as loading control.

(C) (Left) RT-PCR assays monitoring the expression of REST targets in uninduced and Dox-induced nSR100-expressing 293T cells. Bar graph represents qPCR analysis of fold gene expression changes relative to control when nSR100 is overexpressed. n = 4, error bars ± SD. β-actin mRNA levels were used as loading control.

significantly enriched in genes with critical roles in neural differentiation, and loss of nSR100 impairs development of the zebrafish nervous system (Calarco et al., 2009). However, the specific roles of nSR100 and its various target AS events in the development of the vertebrate nervous system have not been determined.

In this study, we show that nSR100 directly activates the inclusion of a neural-specific exon in REST transcripts leading to the production of a truncated isoform of REST (REST4) (Palm et al., 1998), which lacks critical domains required for REST-mediated transcriptional silencing of target genes. Furthermore, REST directly silences the expression of nSR100, thereby preventing the expression of REST4 and other neural-specific AS variants in nonneural cells. We also observe that blocking nSR100 expression in the developing mouse brain prevents neuronal differentiation and leads to the accumulation of NPCs. These results thus reveal direct regulatory interactions between a splicing activator and a transcription repressor that serve to coordinate different layers of gene regulation required for neurogenesis.

RESULTS

nSR100 Is Required for the Expression of REST Target Genes

Using splicing-sensitive microarrays, we previously identified a network of mouse brain-specific exons that are activated by nSR100 (Calarco et al., 2009). To further investigate the role of nSR100 in regulating the transcriptome of neural cells, we performed high-throughput RNA-sequencing (RNA-Seq) of mRNA from undifferentiated Neuro2A (N2a) cells stably expressing control or nSR100-targeting shRNAs. In addition to increased skipping of many alternative exons, knockdown of nSR100 resulted in changes in mRNA expression of a set of genes that were not detectably altered at the splicing level (Figure 1A and data not shown). Approximately 12% (1406/11629) of expressed genes in N2a cells displayed changes (1.5- to 30-fold) in steady-state mRNA levels upon depletion of nSR100. Literature searches were used to determine if subsets of these genes are controlled by common transcription factors, and REST (Bruce et al., 2004; Schoenherr and Anderson, 1995) was identified as one such regulator.

Using multiple sources of published data (Johnson et al., 2008; Jørgensen et al., 2009), we defined a list of high confidence REST target genes that are expressed in N2a cells and determined what proportion of these are affected by knockdown of nSR100. A high confidence REST target was defined as a gene that has ChIP evidence of REST occupancy at one or more RE-1 binding sites, and which displays increased mRNA levels upon loss of REST activity. Fifty-five such targets had detectable expression in N2a cells (Table S1). This agrees with previous

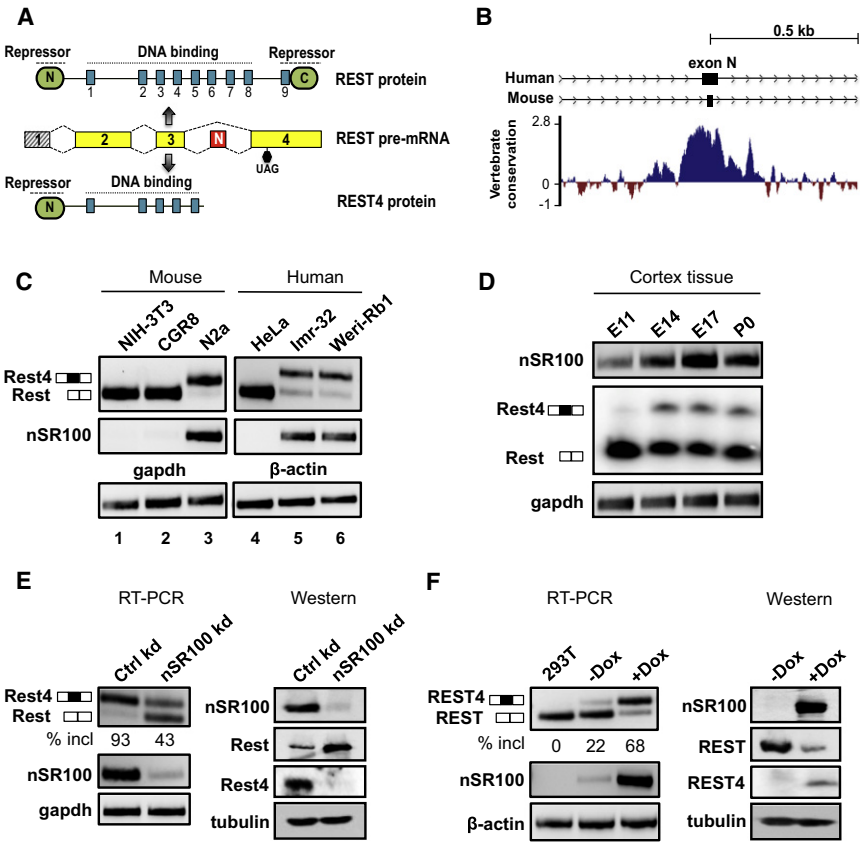

Figure 2. Expression of REST4 is nSR100-Dependent

(A) Alternative splicing of REST pre-mRNA. Red box indicates the neural-specific (N) exon.

(B–D) Conservation plot of the neural-specific (N) exon and flanking 500 nucleotides of intron sequence of the REST gene in 46 vertebrate species (B). The human and mouse N exons are 62 and 16 nucleotides long, respectively. The plot was generated using the *phyloP* conservation track from the UCSC genome browser (http://genome.ucsc.edu) (Kent et al., 2002). RT-PCR profiling of REST AS and nSR100 mRNA expression in (C) neural and nonneural mouse and human cell lines, and (D) the developing mouse embryonic cortex tissue.

(E) (Left) RT-PCR assays monitoring REST AS in control and nSR100 knockdown N2a cells. (Right) Western blots probed with anti-nSR100, anti-REST (C-terminal-specific), and anti-REST4 antibodies. Tubulin levels were used as a loading control.

(F) (Left) RT-PCR assays monitoring REST AS in control 293T, uninduced, and Dox-induced nSR100-expressing 293T cells. (Right) Western blots probed with anti-nSR100, anti-REST, and anti-REST mAb12C11 (N-terminal-specific; recognizes REST and REST4).

findings indicating that REST can bind to hundreds of genes, with relatively few displaying significant expression changes when REST activity is reduced (Johnson et al., 2008; Jørgensen et al., 2009). Remarkably, 78% (43/55) of the REST targets were predicted by RNA-Seq analysis to be downregulated upon nSR100 knockdown (Figure 1A). qRT-PCR experiments validated 95% (19/20) of these RNA-Seq predictions (Figures 1B and S1). To confirm that these genes are targets of REST in N2a cells, double knockdown of REST and nSR100 was performed. As expected, steady-state mRNA levels of all (6/6) tested REST target genes were restored to levels detected in cells expressing control shRNA (Figure 1B). Next, using 293T cell lines expressing nSR100 under doxycycline (Dox)-inducible control, we observed that nSR100 overexpression is sufficient to derepress all (6/6) tested REST target genes in 293T cells (Figure 1C). These results indicate that the vast majority of experimentally supported REST target genes expressed in N2a cells require nSR100 for expression.

nSR100 Regulates the Alternative Splicing of REST Transcripts

Since nSR100 has no known direct role in transcription, we hypothesized that it might affect the expression of REST target genes by controlling the splicing of REST transcripts. The REST gene was reported to possess a neural-specific alternative exon located between its third and fourth exons (Palm et al., 1998; Palm et al., 1999). Skipping of this exon is required to generate full-length REST protein in nonneural cells, whereas its inclusion creates a frameshift that introduces a stop codon at the beginning of exon 4 (Palm et al., 1998; Palm et al., 1999) (Figure 2A). The resulting truncated protein isoform, referred to as "REST4," lacks four zinc fingers and a C-terminal repressor domain, both of which are required for full DNA-binding and repressive activity of REST (Palm et al., 1998; Shimojo et al., 2001; Tapia-Ramírez et al., 1997). However, the extent to which AS regulation contributes to the control of REST activity and the splicing factor(s) responsible for producing the REST4 isoform were not previously determined.

Consistent with an important regulatory role, the neural-specific exon in REST is surrounded by intronic sequence that is highly conserved across vertebrate species (Figure 2B). In agreement with previous findings (Palm et al., 1999), this exon displays a conserved pattern of regulation in mouse and human cells, with complete skipping in nonneural cell lines (Figure 2C, lanes 1, 2, and 4), and almost full inclusion in neural cell lines (Figure 2C, lanes 3, 5, and 6). Inclusion of the neural-specific exon and REST4 expression correlates with nSR100 expression (Figure 2C; see below). RT-PCR assays were used to compare the expression levels of both factors in mouse embryonic cortex tissue at different developmental time points. Neuronal differentiation in the cortex initiates at ~E12.5. Prior to this stage, the cortex consists mostly of undifferentiated NPCs. At E11, nSR100 expression is relatively low and REST expression is relatively abundant (Figure 2D). At subsequent stages however, nSR100 is expressed at higher levels while REST transcripts undergo AS to produce increased levels of REST4 and decreased levels of the REST isoform.

To investigate whether nSR100 mediates neural-specific AS of REST transcripts, we monitored the expression of REST and REST4 isoforms in the N2a cell lines expressing control or nSR100-targeting shRNAs. Relative to the control, knockdown of nSR100 resulted in increased skipping of the REST neural-specific exon (Figure 2E, left panel), along with an increase in REST and decrease in REST4 protein expression (Figure 2E, right panel). In contrast, in the nSR100-expressing 293T line, Dox induction of nSR100 resulted in near full inclusion of this exon (Figure 2F, left panel). This AS shift correlated with a switch in the relative expression levels of REST and REST4 (Figure 2F, right panel).

Previously, we found that most exons that depend on nSR100 for inclusion are also regulated by PTBP1 and its neural paralog, PTBP2 (Calarco et al., 2009). However, the intron sequences flanking the REST neural exon were not found to contain C/U-rich sequences that resemble PTBP1/PTBP2 binding sites. Furthermore, knockdown or overexpression of PTBP1 and/or PTBP2, did not result in an appreciable effect on the relative ratios of REST:REST4 isoforms (Figures S2A and S2B and data not shown). Collectively, these results provide evidence that nSR100 is specifically required for neural-specific AS of REST transcripts, resulting in expression of the REST4 isoform.

nSR100-Mediated Regulation of REST Activity Is Independent of the Proteasomal Degradation Pathway

Previous studies have shown that the E3 ubiquitin ligase βTRCP promotes reduced REST levels during neuronal differentiation through recognition of a phospho-degron located in the C-terminal domain (amino acids 1024–1032) of REST (Westbrook et al., 2008). REST4, which lacks this phospho-degron, is therefore not subject to βTRCP regulation. Our experiments reveal that nSR100 can regulate REST protein levels independently of βTRCP-mediated proteasomal degradation. Western blotting assays show that REST protein levels decrease to a similar extent when nSR100 is overexpressed, even when βTRCP is knocked down (Figure S2C) or when the proteasome is blocked with the inhibitor MG132 (Figure S2D). Therefore, we propose that an nSR100-dependent switch from REST to REST4 isoform expression serves as an independently acting mechanism to reduce the levels of REST protein and the repression of REST target genes as cells commit to a neuronal fate.

Neural-Specific Alternative Splicing of REST Transcripts Is Directly Regulated by nSR100

We previously demonstrated that nSR100 binds directly to many of its regulated target transcripts (Calarco et al., 2009). To investigate whether nSR100 directly regulates AS of REST pre-mRNA, we performed in vivo UV crosslinking immunoprecipitation (CLIP) (Ule et al., 2003) assays using human Weri-Rb1 neural cells and anti-nSR100 or control (anti-IgG) antibodies (Figure 3A). Immunoprecipitated pre-mRNA was detected by RT-PCR reactions using primer pairs that amplify across intron-exon regions in target transcripts. Regions overlapping the neural-specific exon in REST and corresponding regions in previously defined direct targets of nSR100 (Calarco et al., 2009) showed specific and UV-dependent enrichment with the anti-nSR100 antibody (Figures 3A and S3A). In contrast, pre-mRNA regions overlapping alternative exons in transcripts that are not regulated by nSR100, are not enriched in the anti-nSR100 CLIP samples (Figures 3A and S3A).

To confirm direct regulation by nSR100, we next determined whether it is necessary and sufficient to promote neural-specific AS of REST transcripts in vitro. Incubation of an in vitro-synthesized pre-mRNA containing the REST neural-specific exon and surrounding sequences (Figure 3B) in extracts from Weri-Rb1 cells mock-depleted with a control antibody resulted in efficient inclusion of the neural-specific exon (Figure 3B, lane 1). In contrast, immunodepletion of ~90% of nSR100 protein (Calarco et al., 2009) resulted in pronounced skipping of this exon (Figure 3B, lane 2). Addition of recombinant nSR100 protein restored inclusion of the neural-specific exon to levels observed in the mock-depleted extract, whereas addition of an equivalent amount (corresponding to the highest concentration of nSR100) of another SR protein, SRp30c, did not appreciably affect inclusion levels of the neural-specific exon (Figure 3B, lanes 3–6; Figure S3B). Taken together with the data in Figures 1 and 2, these results show that nSR100 directly and specifically activates neural-specific AS of REST transcripts.

REST Directly Inhibits nSR100 Expression in Nonneural Cells

Chromatin immunoprecipitation experiments have shown that human and mouse REST occupy multiple RE-1 sites within the nSR100 gene (ENCODE Project Consortium et al., 2011; Johnson et al., 2008) (Figure 3C). Moreover, our data indicate that REST and nSR100 have reciprocal expression patterns (Figure 2). These observations suggest that nSR100 is a transcriptional target of REST. Indeed, knockdown of REST in CGR8 mouse embryonic stem cells resulted in the induction of nSR100 mRNA (Figure 3D). Furthermore, overexpression of FLAG-REST in N2a cells caused a decrease in nSR100 mRNA and protein levels and a consequent loss of inclusion of nSR100-dependent exons, including the REST neural-specific exon (Figures 3E and 3F). Taken together with the published ChIP data, these findings demonstrate that REST directly represses nSR100 expression in nonneural cells.

Loss of nSR100 in the Developing Mouse Brain Impairs Cortical Neurogenesis

Previous studies have indicated that regulation of REST is crucial for the differentiation of NPCs into neurons (Gopalakrishnan, 2009). Our observations of direct regulatory interactions between nSR100 and REST therefore suggested that loss of nSR100 expression in the developing mammalian brain would prevent neurogenesis. To investigate this possibility, we electroporated in utero NPCs in the ventricular/subventricular zone (VZ/SVZ) of E13/14 mice cortex with vectors expressing nuclear EGFP and control or nSR100-targeting shRNAs. Embryos were harvested three days posttransfection, sectioned and, visualized by confocal imaging. As the transfected ($EGFP^+$) precursor cells differentiate into neurons, they migrate away from the VZ/SVZ and eventually reside in the cortical mantle (CM) (Figures 4A and 4B, refer to control panels). Consistent with an important role for REST AS in the control of neuronal differentiation,

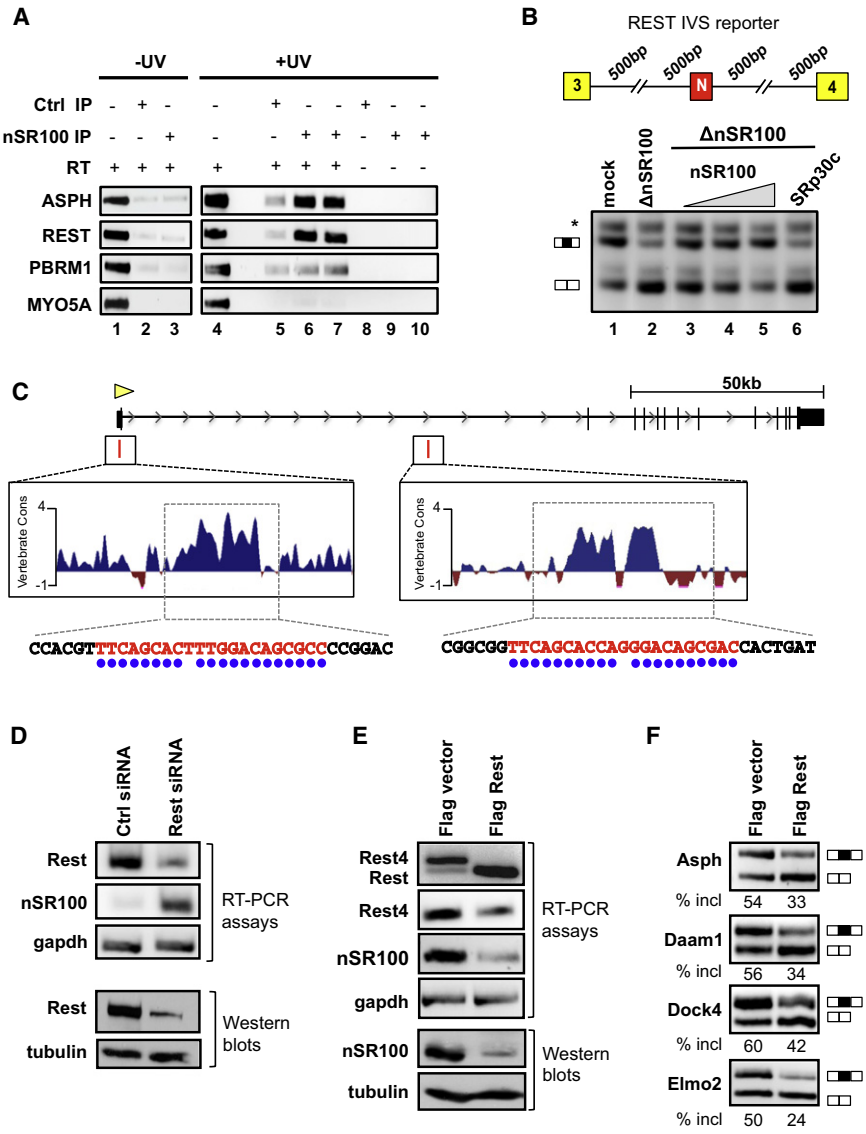

Figure 3. Direct Regulatory Relationships between nSR100 and REST in Neural and Nonneural Cells

(A) UV-CLIP assays showing that nSR100 directly interacts with endogenous REST pre-mRNA transcripts. Immunoprecipitations using anti-nSR100 (lanes 3, 6, 7, 9, and 10) or control (rabbit anti-mouse) antibodies (lanes 2, 5, and 8) are shown. Lane 1 represents input RNA. Reverse transcriptase (RT) was omitted in lanes 8–10 to control for possible genomic DNA contamination.

(B) Recombinant nSR100 protein specifically promotes the inclusion of REST exon N in vitro. A splicing substrate consisting of native REST sequences was incubated in mock or nSR100-immunodepleted Weri-Rb1 cell extracts supplemented with and without purified proteins. Splicing activity was monitored by RT-PCR assays with primers annealing to exons 3 and 4. Purified nSR100 (25, 50, and 100 ng) or purified SRp30c protein (100 ng) was used. *, nonspecific band.

(C) Schematic of the human nSR100 gene. Red vertical bars represent ChIP-Seq-identified high-scoring (cluster score = 1000/1000) (ENCODE Project Consortium et al., 2011) REST-occupied RE-1 sites in the nSR100 locus. Vertebrate conservation (46 species) of sequences within the ChIP peak-containing regions are shown. RE-1 sequences are indicated in red. Nucleotides that match the RE-1 consensus motif (Johnson et al., 2007) are represented by blue dots. Adapted from UCSC genome browser using the hg19 genome assembly.

(D) (Top) RT-PCR assays monitoring nSR100 mRNA levels in CGR8 cells transfected with control or REST-targeting siRNAs. (Bottom) Western blots probed with anti-REST antibody.

(E) RT-PCR assays and western blots monitoring nSR100 and/or REST4 expression in N2a cells transfected with FLAG-REST overexpression plasmid or an empty FLAG vector. REST4 isoform (panel 2) was specifically detected using primers annealing to the exon 3-exon N splice junction and exon 4.

(F) RT-PCR reactions monitoring % inclusion of nSR100-regulated exons upon REST overexpression in N2a cells.

REST4 was detected only in the CM but not in the VZ/SVZ regions where NPCs accumulate (Figure S4B).

Knockdown of nSR100 increased the percentage of EGFP$^+$ cells remaining in the VZ/SVZ, such that only ~3% of cells populated the CM compared to ~30% in the control (Figures 4A, 4B, and S4A). As expected, the cells that fail to migrate also do not express REST4 (Figure S4B). To test if the observed migration defect is due to a failure of NPCs to undergo neuronal differentiation, we stained brain sections using an antibody specific for the neural radial precursor marker Pax6. Upon loss of nSR100, the fraction of Pax6$^+$EGFP$^+$ cells doubled compared to the control (Figure 4C). In addition, neuronal βIII-tubulin immunostaining revealed a 3-fold reduction in the number of βIII-tubulin$^+$ EGFP$^+$ neurons upon nSR100 knockdown (Figure 4C). These phenotypic effects were a specific consequence of knockdown of nSR100, since they could be rescued by coelectroporation of an nSR100 expression plasmid that is resistant to silencing by the shRNA used to target endogenous nSR100 transcripts (Figures 4D, S4C, and S4D). These results thus provide evidence that blocking nSR100 expression in the developing mouse brain causes an accumulation of NPCs that do not commit to a neuronal fate and that this defect is due, at least in part, to a block in neural-specific AS of REST transcripts.

DISCUSSION

Since nSR100 and REST control specific AS and transcriptional networks, respectively, their direct negative regulatory interactions invariably impact both networks (Figure 4E). In neural cells, nSR100 promotes the inclusion of a network of neural-specific alternative exons in functionally related genes, one of which is the neural-specific exon in REST4 transcripts.

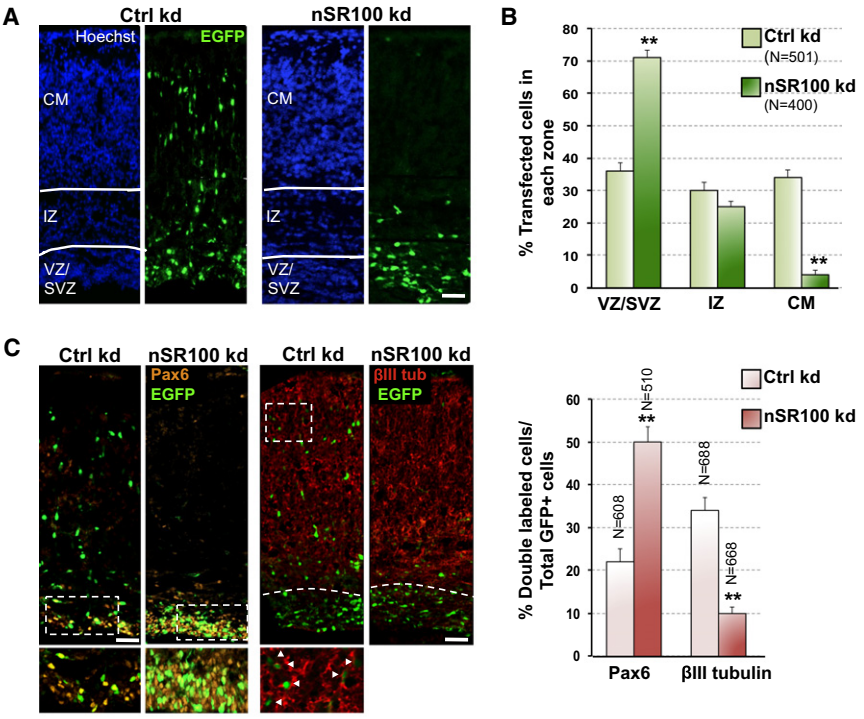

Figure 4. Loss of nSR100 Disrupts Cortical Neurogenesis In Vivo

(A) Photomicrographs of cortical sections immunostained for EGFP to show transfected cells (green) and counterstained with Hoechst to show nuclei (blue).

(B) Quantification of EGFP$^+$ cells located in the VZ/SVZ, IZ, and CM of brains transfected with control or nSR100 shRNAs.

(C) Confocal images of sections from control and nSR100 knockdown brains costained for EGFP (green) and Pax6 (orange) or β-III tubulin (red). Bottom panels are high magnification images of regions inside corresponding hatched boxes. Bar graph shows the quantification of the percentage of total EGFP$^+$ cells that costain for Pax6 or β-III tubulin.

(D) Quantification of the percentage of total EGFP$^+$ cells that costain for Pax6 or β-III tubulin in a separate set of control knockdown, nSR100 knockdown, and rescue (coinjected with nSR100 shRNA and nSR100 cDNA) brains.

(E) Schematic of the regulatory interactions between REST and nSR100 in nonneural and neural cells. Scale bars, 50 μm. n = 3 embryos, 2–3 mothers. N = total cells counted. Error bars, SEM. **p < 0.0001, Student's t test. VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CM, cortical mantle.

This nSR100-mediated AS switch from REST to REST4 splice isoforms is important for the neural-specific expression of REST-repressed target genes. In contrast, in nonneural cells, REST maintains nonneural transcription and AS profiles in part by inhibiting nSR100 activity. Direct opposing interactions between an AS regulator and a transcription repressor thus play a pivotal role in vertebrate nervous system development.

Previously reported mechanisms contributing to REST inactivation during NPC differentiation include downregulation of REST at the transcriptional level and proteolytic degradation via the F box protein βTRCP (Qureshi and Mehler, 2009). Our results indicate that nSR100-dependent regulation of REST AS provides an additional critical regulatory layer that contributes to the reduction in REST activity in differentiating NPCs. In particular, we detect an nSR100-dependent switch in expression between REST and REST4 isoforms in developing mouse embryos around the time NPCs begin to differentiate into neurons. This switch ensures that full-length REST protein is efficiently depleted such that neural-specific genes negatively regulated by REST can be expressed.

Finally, our results further reveal a mechanism by which largely nonoverlapping sets of genes regulated at the levels of AS and transcription during neurogenesis are coordinated with one another. These findings are reminiscent of pivotal roles for the SR-related proteins Tra and Tra2 in the control of AS events that alter the activity of transcription factors required for sex determination and courtship behavior in *Drosophila* (Demir and Dickson, 2005; Förch and Valcárcel, 2003), and also a recently discovered AS event that controls transcriptional regulatory networks required for embryonic stem cell pluripotency and reprogramming (Gabut et al., 2011). It will be of considerable interest in future studies to determine whether other splicing regulators play similarly important roles in controlling the multilayered regulatory networks required for development.

EXPERIMENTAL PROCEDURES

Cell Lines

Generation of N2a cell lines expressing nSR100- or GFP-targeting shRNAs has been previously described (Calarco et al., 2009). To establish inducible-nSR100 HEK293T cell lines, the full-length human nSR100 ORF was cloned into the pLD lentiviral vector (gift from Jason Moffat, University of Toronto). Lentiviruses

were produced and used to generate stable lines as described previously (Mak et al., 2010). nSR100 expression was induced using 2 mg/ml Dox for 48 h.

RNA-Seq Analysis

Illumina RNA-Seq datasets generated from N2a cell lines expressing control or nSR100-targeting shRNAs consisted of ~100 million × 34-mer reads and were analyzed to generate estimates for AS and mRNA expression levels as described previously (McIlwain et al., 2010).

In Vitro Splicing Assays

Weri-Rb1 cell extract mock- or nSR100-immunodepleted, recombinant nSR100 protein, and conditions for in vitro splicing assays have been described previously (Calarco et al., 2009).

In Utero Electroporation

E13/E14 CD1 mice were injected with 1 μg of nuclear EGFP and 3 μg of shRNA constructs with 0.05% trypan blue as a tracer, and brains were harvested as described before (Gauthier-Fisher et al., 2009). For rescue experiments, mice were injected with 1.33 μg each of nuclear EGFP, control or nSR100-targeting shRNAs, and nSR100-expressing or empty plasmids. Immunostaining was performed as previously described (Gauthier-Fisher et al., 2009). The Animal Care Committee of the Toronto Hospital for Sick Children approved all animal use in accordance with the policies established by the Canadian Council on Animal Care.

Imaging and Quantification

Images were obtained on a WaveFX confocal microscope (Quorum Technologies) and analyzed using Volocity and ImageJ software programs. For each condition tested, quantifications were obtained by averaging the results from three cortical sections per brain, for three brains in total, and counting at least 40 cells per section. Student's t test was used to determine statistical significance.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, Supplemental Experimental Procedures, Supplemental References and one table and can be found with this article online at doi:10.1016/j.molcel.2011.08.014.

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