Long noncoding RNA Sox2ot and transcription factor YY1 co-regulate the differentiation of cortical neural progenitors by repressing Sox2

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Abstract
Long noncoding RNAs (lncRNAs) are emerging as key regulators of crucial cellular processes. However, the molecular mechanisms of many lncRNA functions remain uncharacterized. Sox2ot is an evolutionarily conserved lncRNA that transcriptionally overlaps the pluripotency gene Sox2, which maintains the stemness of embryonic stem cells and tissue-specific stem cells. Here, we show that Sox2ot is expressed in the developing mouse cerebral cortex, where it represses neural progenitor (NP) proliferation and promotes neuronal differentiation. Sox2ot negatively regulates self-renewal of neural stem cells, and is predominately expressed in the nucleus and inhibits Sox2 levels. Sox2ot forms a physical interaction with a multifunctional transcriptional regulator YY1, which binds several CpG islands in the Sox2 locus in a Sox2ot-dependent manner. Similar to Sox2ot, YY1 represses NP expansion in vivo. These results demonstrate a regulatory role of Sox2ot in promoting cortical neurogenesis, possibly by repressing Sox2 expression in NPs, through interacting with YY1.

Introduction
Production of distinct types of neural progenitors and neurons is regulated by coding and noncoding RNAs in the mammalian cerebral cortex1–5. Long noncoding RNAs (lncRNAs), generally considered as transcripts of hundreds of nucleotides (nt) in length with little or no protein-coding potential, comprise a large proportion of the mammalian genome6–8. Though thousands of lncRNAs have been reported, relatively few have been mechanistically characterized. Those that have been characterized appear to work through a wide variety of mechanisms, including control of chromatin structure, transcription, mRNA processing, and translation9,10. Emerging studies have shown a specific and dynamic expression of lncRNAs in embryonic stem (ES) cells, tissue-specific stem cells, and progenitors in various species. However, the molecular mechanisms of lncRNA function in specific cells and tissues and their relationship to diseases remain unclear11–13.

Sox2 is a pluripotency gene that maintains the stemness of human and rodent ES cells14–16. However, the molecular regulation of Sox2 expression is incompletely understood. Several CpG islands are harbored in the Sox2 locus, which cover and flank the Sox2 gene. This suggests a possible role for CpG islands in Sox2 regulation by attracting transcription factors for transcriptional initiation, or propagating transcriptional silencing via DNA methylation17. For instance, the Sox2 promoter in ES cells is marked by permissive H3K4me3 histone marks, while the surrounding CpG islands are bivalently marked,
indicating that the locus may also be poised for repression\textsuperscript{18}. Sox2 is also highly expressed in neural stem cells (NSCs) and neural progenitors (NPs) in the mouse embryonic cerebral cortex, maintains the populations of NSCs and NPs, and promotes proliferation\textsuperscript{19,20}. Sox2 expression is downregulated in postmitotic, differentiated neurons; however, the regulatory mechanisms governing this transition are incompletely understood\textsuperscript{16,21–24}.

Interestingly, the lncRNA Sox2 overlapping transcript (Sox2ot) is highly conserved among species and overlaps the Sox2 gene in the genome\textsuperscript{25–27}. Sox2ot appears to be transcribed from several transcription start sites and can be differentially spliced, thus generating several Sox2ot isoforms\textsuperscript{25,28}. While the function of Sox2 is defined, the roles of Sox2ot in development, stem cell expansion, and differentiation are unknown.

In this study, we demonstrate that Sox2ot negatively regulates neural progenitor proliferation by interacting with the epigenetic regulator YY1 in the developing mouse cerebral cortex. Overexpression of Sox2ot causes a decrease in NPs with a concomitant increase in neurons, while Sox2ot knockdown results in increased NPs and a loss of differentiated neurons. There is a strong interaction between Sox2ot and YY1 in the nucleus of neuroectodermal cells. Furthermore, we found that YY1 binds to CpG islands at the Sox2 locus and represses NP proliferation, and that this binding is dependent on Sox2ot. Thus, we propose that Sox2ot exerts its effects in NP development with its RNA-binding partner YY1 through negative regulation of Sox2 in the developing cortex.

Results
Sox2ot and Sox2 are coexpressed in the ventricular zone in the developing mouse cortex

The main isoform of Sox2ot (GenBank Accession No. BC057611) contains five exons spanning >100 kilobases (kb) of the mouse genome, though the final mRNA is spliced to about 3 kb. Sox2, a single-exon transcript of about 2.5 kb, is located within one of the large introns of Sox2ot (Fig. 1a). None of the known isoforms of Sox2ot contain any overlapping exonic sequence with Sox2\textsuperscript{26}. Given the overlapping genomic orientation of Sox2 and Sox2ot, we performed in situ hybridization on embryonic mouse cerebral cortices to examine the expression patterns of each of these genes. Both Sox2 and Sox2ot were expressed in the ventricular zone (VZ) and subventricular zone (SVZ) of cortices at embryonic day 13.5 (E13.5) and E15.5, which correspond to the active stages of neural progenitor expansion (Fig. 1b–e). The expression level of Sox2ot was not as robust as Sox2. Expression of Sox2 and Sox2ot was reduced in postnatal day 0 (P0) cortices, corresponding with a reduction in the NP population (Supplementary Figure S1A). Moreover, sense probes for Sox2 and Sox2ot lacked specific staining in E13.5 cortices (Supplementary Figure S1B).

To establish a more quantitative picture of Sox2 and Sox2ot expression levels, we performed droplet digital quantitative PCR (ddPCR) to test their mRNA copy numbers in the dorsal cortex. From E12.5 to P14, the mRNA copy numbers of Sox2 and Sox2ot showed a reduced trend (Supplementary Figure S1C and D). Moreover, Sox2 was expressed at a higher level than Sox2ot at all developmental stages tested, and the ratio of Sox2ot/Sox2 expression was slightly increased, suggesting an elevated expression of Sox2ot through development (Fig. 1f).

To further verify the expression levels and sizes of Sox2 and Sox2ot transcripts, we performed Northern blot analyses on RNA extracted from E13.5 mouse dorsal cortices (Fig. 1g). As expected, Sox2 was expressed at a higher level than Sox2ot, which reflected the expression observed in the analyses of ddPCR (Fig. 1b–f). Both Sox2 and Sox2ot transcripts were detected at the expected sizes, 2.5 kb and 3 kb, respectively (Fig. 1g). Taken together, these experiments establish that Sox2ot is expressed in the VZ, where NPs reside in the developing cortex.

Sox2ot overexpression causes a reduction of neural progenitors

To explore the function of Sox2ot in NPs, we first overexpressed Sox2ot by in utero electroporation (IUE) at E13.5 and collected the brains at E14.5 for analysis. A bromodeoxyuridine (BrdU) pulse was given 1 h before tissue collection to label the dividing cells in the S-phase in a cell cycle. Electroporation in the mouse embryonic cortex will first label neural progenitors in the VZ. To avoid biased analyses, all quantification in this study was based on calculating the ratio of marker\textsuperscript{+/GFP\textsuperscript{+}} versus GFP\textsuperscript{+}. Measures of the general number of NPs with BrdU incorporation (BrdU\textsuperscript{+}/GFP\textsuperscript{+} cells versus GFP\textsuperscript{+} cells) and Sox2 staining (Sox2\textsuperscript{+}/GFP\textsuperscript{+} cells versus Sox2\textsuperscript{+} cells) were both decreased in Sox2ot overexpression (OE) cortices compared to controls (Ctrl) (Fig. 2a–d). The reduction in the numbers of Sox2\textsuperscript{+} cells in GFP\textsuperscript{+} cells suggests the potential for direct negative regulation of Sox2 by Sox2ot. Two specific NP populations, radial glia cells (RGCs) and intermediate progenitors (IPs) that can be labeled by Pax6 and Tbr2, respectively, also showed a decrease in the Sox2ot OE cortices compared to controls, by calculating the ratios of Pax6\textsuperscript{+}/GFP\textsuperscript{+} and Tbr2\textsuperscript{+}/GFP\textsuperscript{+} cells versus GFP\textsuperscript{+} cells (Supplementary Figure S2A–D).

To confirm these results quantitatively, we dissected the GFP-positive area of the cortex 24 h after E13.5 electroporation and extracted RNA for real-time quantitative reverse transcription PCR (qRT-PCR) (Supplementary Figure S3). As expected, Sox2ot was upregulated in Sox2ot OE brains, while Sox2 was downregulated, reflecting the
immunostaining results (Supplementary Figure S3A). Pax6 and Tbr2 RNA levels also mirror protein-level changes upon Sox2ot OE (Supplementary Figure S3B).

Moreover, we hypothesized that the loss of NPs upon Sox2ot OE could be explained by cell death or by differentiation. We then examined cell death using activated Caspase3 as a marker. In E14.5 brains, activated Caspase3 expression was not significantly altered due to Sox2ot overexpression, indicating that cell death does not explain the loss of NPs (Supplementary Figure S2E and F). These results demonstrate that Sox2ot OE represses NP expansion in both RGCs and IPs, and that this function is not carried out through cell death.

**Sox2ot knockdown increases neural progenitors**

To further examine the effect of Sox2ot on NP expansion, we applied a knockdown approach using short hairpin RNAs (shRNAs) for Sox2ot, named as shSox2otA (shA) and shSox2otB (shB) (Supplementary Figure S4A). In cell culture testing, both shA and shB significantly reduced Sox2ot expression, though shB had a slightly stronger effect (Supplementary Figure S4B). We again used the IUE technique to knock down Sox2ot in the developing cortex. Both BrdU incorporation and Sox2 staining increased in shSox2ot brains compared to control brains (Fig. 2e–h). The specific progenitor population markers for RGCs and IPs also showed increases upon Sox2ot knockdown (Supplementary Figure S5). In each case, the increase in NPs is slightly larger for shB than shA, though both are significant, reflecting the strength of knockdown observed in cell culture experiments (Fig. 2; Supplementary Figure S4B). Our qRT-PCR of electroporated brains also confirmed these results, showing Sox2ot knockdown, Sox2 increase, and

**Fig. 1 Characterization of Sox2 and Sox2ot expression throughout cortical development.** a Diagram of mouse Sox2ot locus. Green, Sox2ot probe; orange, Sox2 probe. Chr, chromosome. Exon 1 (E1) to 5 (E5) are labeled. b–e Low-power (top panels) and high-power (bottom panels) images of in situ hybridization in the cerebral cortex of wild-type mice at E13.5 and E15.5. The ventricular zone expression of Sox2 (arrowheads) and Sox2ot (arrows) is highlighted. f Ratio of Sox2ot/Sox2 expression in cDNA from the dorsal cortex at various developmental stages detected by droplet digital quantitative PCR (ddPCR). n = 3 repeats. g Northern blot detecting Sox2 and Sox2ot in the RNA extracted from the E13.5 dorsal cortex (upper panels). Gapdh is shown as a loading control (lower panels).
upregulation of both Pax6 and Tbr2 upon electroporation of shSox2otA (Supplementary Figure S4C and D). These observations further support the functionality of Sox2ot as a negative regulator in NP expansion in the developing cortex.

**Sox2ot affects neural differentiation**

To determine the effects of Sox2ot OE on the differentiation of NPs, we electroporated the Sox2ot OE construct at E13.5 and collected brain tissues 4 days after electroporation at E17.5, thus allowing more time for Sox2ot to exert its effects on neurogenesis. Two markers for differentiated neurons, Tbr1 and Satb2, which label early-born and late-born neurons, respectively, each showed an increase with Sox2ot OE (Fig. 3a–d). This expansion of differentiated neurons accounts for the loss of NPs observed in 1-day electroporations, suggesting an early differentiation (Fig. 2a–d). These results were confirmed at the RNA level for Tbr1 by qRT-PCR of RNA extracted from electroporated cells in the cortex (Supplementary Figure S3B).

We next examined the effect of Sox2ot knockdown on neuron populations and found that both Tbr1 and Satb2 expression was decreased, which is opposite to the effect
of Sox2ot OE (Fig. 3e–h). Decreased neurogenesis corresponds to the increase in the neural progenitor pool upon Sox2ot knockdown (Fig. 2e–h). The observed decrease in Tbr1 was confirmed by qRT-PCR on RNA purified from electroporated brain cells (Supplementary Figure S4D). Collectively, these results establish a role for Sox2ot in the NP choice between proliferation and differentiation.

Sox2ot is localized to the nucleus in neuroectodermal cells

Having established that Sox2ot plays a role in regulating cortical neurogenesis, we sought to characterize how Sox2ot exerts its function. Because there are a wide variety of cell types present in the developing cortex, we turned to cultured and relatively homogeneous mouse ES cells (mESCs) and their derived neuroectodermal cells. mESCs were cultured for 4 days and then treated for 24 h with...
either DMSO vehicle or the small molecule JQ1, a BET protein inhibitor shown to cause differentiation to a neuroectodermal fate\(^29\) (Fig. 4a). To confirm the identity of mESCs after treatment, we performed qRT-PCR for various stem cell and neuronal markers. mESCs treated only with DMSO showed enrichment for stem cell markers, including \(\text{Fgf4}, \text{Oct4}, \text{Nanog}\), while mESCs treated with JQ1 expressed higher levels of neuroectodermal markers, such as \(\text{Nestin}, \text{Pax6}, \text{and Foxa2}\) (Fig. 4b, c). Notably, markers of more mature neurons, \(\text{NeuroD1}\) and \(\text{Tubb1}\), were not enriched in the neuroectodermal cells, further validating their NP identity (Fig. 4c). Moreover, \(\text{Sox2}\) and \(\text{Sox2ot}\) were differentially expressed between differentiated stem cells and neuroectodermal cells, with \(\text{Sox2}\) decreased in neuroectodermal cells while \(\text{Sox2ot}\) increased, reflecting the changes in \(\text{Sox2ot}\) and \(\text{Sox2}\) expression observed in vivo (Fig. 1f), and indicating that \(\text{Sox2ot}\) may be specific to the differentiation of the neural lineage (Fig. 4c, d).

As lncRNAs can function in both the nucleus and the cytoplasm, we next determined the subcellular localization of \(\text{Sox2ot}\). Lysis of cells followed by low-speed centrifugation pellets the relatively large nuclei intact, while leaving the cytoplasm as the supernatant, allowing reliable separation of nuclear and cytoplasmic RNAs. Using RNA extracted from neuroectodermal cells differentiated from
female ES cells, we performed qRT-PCR for Sox2ot and determined that it is significantly enriched in the nuclear fraction (Fig. 4e). We also measured Gapdh and Xist as cytoplasmic and nuclear controls, respectively, and found that each of them was highly expressed in the expected fraction (Fig. 4f). Moreover, we examined Sox2ot expression in cultured mouse neural progenitors derived from E12.5 cortex using RNA fluorescent in situ hybridization (FISH). Sox2ot was mostly expressed in the nucleus (Fig. 4g).

These results demonstrate Sox2ot nuclear localization, suggesting that Sox2ot may play roles in transcriptional and epigenetic regulation of the switch in NP fate choice.

**Sox2ot binds YY1 in neuroectodermal cells**

In some cases, the simple act of transcription constitutes lncRNA functionality, while in other cases, the lncRNA interacts with protein partners to exert its function. After finding Sox2ot in the nucleus, we asked whether Sox2ot might interact with any proteins to repress Sox2 expression. Using a candidate approach with several proteins previously shown to bind lncRNAs and to regulate target gene expression, we performed RNA immunoprecipitation (RIP) on neuroectodermal cells (Fig. 5a). The candidates included YY1, which is important for tethering Xist lncRNA to the X chromosome during X inactivation; CTCF interacts with lncRNAs during imprinting and is a protein-interacting partner of YY1; and Pax6 interacts with lncRNA Paupar in neuroblastomal cells. We found that Sox2ot was highly enriched by YY1 pulldown, while CTCF and Pax6 showed no significant enrichment over IgG control (Fig. 5b, data not shown). To ensure that the RIP experiment was specific, we tested the pulldown of several neural mRNAs such as NeuroD1 and other lncRNAs (e.g., Paupar) by YY1 and found significant enrichment only of Sox2ot (Fig. 5c). As a positive control, we also tested the pulldown of the known Pax6-bound lncRNA Paupar, and found its significant enrichment with Pax6 (Fig. 5d). These results demonstrate a Sox2ot binding partner and give insight into the possible mechanisms of Sox2ot action through the multiple known functions of YY1.

**YY1 binds CpG islands in the Sox2 locus**

A subset of lncRNAs function by the cis-regulation of target genes near the same genomic locus. Due to the overlapping genomic organization of the Sox2/Sox2ot locus (Fig. 1a), the similar expression patterns of Sox2 and Sox2ot (Fig. 1b–e), the negative effects of Sox2ot on Sox2 expression (Fig. 2c, d, Supplementary Figure S3A), and the presence of favorable CpG island binding sites in the Sox2 locus (Fig. 6a), we hypothesized that Sox2ot and its binding partner YY1 might function through repression of Sox2. Thus, we performed chromatin immunoprecipitation (ChIP) on chromatin in neuroectodermal cells to determine if YY1 could be found at the Sox2 locus.
Indeed, YY1 was found to bind at each of the three CpG islands in the Sox2 locus (Fig. 6b). YY1 was not detected in the non-CpG island upstream region of the Sox2 transcription start site (Fig. 6c).

To address the dependence of YY1 binding on Sox2ot, we performed ChIP of YY1 binding at each Sox2 CpG island under Sox2ot overexpression or knockdown conditions. Upon overexpression of Sox2ot, YY1 binding at each CpG island was enriched, while knockdown of Sox2ot depleted YY1 binding (Fig. 6d and Supplementary Figure S6). As a control, we also tested YY1 binding at upstream region #6, and found no effect from Sox2ot manipulation (Fig. 6d). These results support a direct regulatory role for Sox2ot and YY1 in repression of Sox2 in NPs.

**YY1 knockdown causes an increase in neural progenitors**

To further validate the functional relationship between Sox2ot and YY1, we performed IUE to knock down YY1 in developing cortices using two different shRNAs, shYY1A and shYY1B (Fig. 7; Supplementary Figure S3C). We examined NPs at E14.5, 1 day after IUE, and found that the general progenitor cell markers BrdU and Sox2, both increased upon YY1 knockdown (Fig. 7a–d). The specific RGC and IP markers Pax6 and Tbr2 also showed increased expression in shYY1 cortices (Fig. 7e–h). We
Fig. 7 YY1 knockdown increases neural progenitors. a–h Electroporation of shRNAs against YY1 (shYY1 A and shYY1 B) at E13.5 for analysis at E14.5 significantly increased the number of BrdU-incorporating or Sox2+ Pax6+, or Tbr2+ cells co-labeled with GFP in the cortex, compared to the scrambled shRNA control (ShCtrl). i–l Electroporation of shRNAs against YY1 at E13.5 for analysis at E17.5 significantly decreased the number of Tbr1+ or Satb2+ cells co-labeled with GFP in the cortex. Yellow and white cells indicate co-labeled cells. Data are presented as mean±SD; n ≥ 5 sections from at least four different brains for all electroporations; p values in relation to the scrambled shRNA control (**p < 0.001). Scale bar=50 µm.
next examined the changes in cortical neurogenesis due to YY1 knockdown using differentiated neuronal markers Tbr1 and Satb2. We found that both early- and late-born neurons were decreased (Fig. 7i–l).

Furthermore, changes in both the neural progenitor and mature neuronal populations caused by YY1 knockdown were confirmed by RT-PCR of RNA from electroporated brains (Supplementary Figure S4C and D). These results are very similar to those observed upon Sox2ot knockdown in vivo (Figs. 2 and 3, Supplementary Figure S4C and D). We also performed RT-PCR for YY1 in RNA extracted from brains electroporated with Sox2ot OE and shSox2ot to ensure that the observed phenotypes are not due to changes in YY1 expression upon Sox2ot manipulation (Supplementary Figure S3C). Altogether, these results further support the functional relationship between Sox2ot and YY1.

Discussion

As IncRNAs are continually annotated and validated, it has become clear that they are potent regulators of many cell processes. However, the mechanistic explorations of IncRNA functions are lacking. Here, we characterized the function of IncRNA Sox2ot in the developing mouse cerebral cortex through in vivo methods, determining that it promotes the differentiation of NPs into neurons, and suppresses the expansion of NPs. Using a mouse ES cell culture system, we also investigated the mechanism of Sox2ot regulation in neuroectodermal cells and found that it is active in the nucleus and interacts with the multifunctional transcription factor YY1. We demonstrated YY1 binding to CpG islands in the Sox2 locus and provided evidence that this binding is mediated by Sox2ot and provides direct cis-regulation of Sox2 expression in NPs (Supplementary Figure S7). Our results characterize Sox2ot function and give insight into the mechanism of a specific IncRNA, while providing support for the general archetype of IncRNA function as a scaffold for protein recruitment.

In this study, we demonstrated Sox2ot expression in NPs, and identified a novel role for Sox2ot in repression of NP expansion. Our result is distinct from studies of Sox2ot in cancers, where it is found to positively regulate Sox2 expression. Though the cancer studies did not explore the mechanism of Sox2ot, one possible explanation for the opposing effects on Sox2 is that Sox2 must be reactivated, often by amplification, in cancers, whereas Sox2 is already highly expressed in NPs in the cortex. Thus, the transcriptional and epigenetic landscape surrounding the Sox2 locus may look very different and requires different regulation in cancer cells versus NPs.

IncRNAs have been shown to work through a variety of mechanisms, including as scaffolds for ribonucleoprotein complexes, activators of distinct transcriptional programs, and recruiters of proteins to specific loci. Our results support Sox2ot action through epigenetic regulation by recruiting the binding partner YY1 to the Sox2 locus. YY1 is a ubiquitously expressed, multifunctional polycomb group transcription factor, can be activating or repressive depending on the context, and often works by binding CpG islands and recruiting cofactors to perform epigenetic modifications. Studies of YY1 in early nervous system development have demonstrated its importance for neurulation and proper patterning, but its role in cortical development has not been characterized. Here, we demonstrate a new prodifferentiative role of YY1 in NPs, as its knockdown results in increased NPs and decreased neurons.

We propose that the function of the Sox2ot–YY1 complex is to modulate Sox2 expression in NPs, helping to balance progenitor pool maintenance with neurogen-esis, such that low levels of Sox2ot allow high Sox2 expression, thus promoting proliferation (Supplementary Figure S7A), while higher Sox2ot expression represses Sox2, resulting in differentiation of neurons (Supplementary Figure S7B). There are several proposed models of YY1-mediated repression, including passively blocking activation by covering DNA recognition sites or interfering with activation factor binding, or actively repressing transcription through co-repressor recruitment. Mechanistically, we propose that low Sox2ot expression allows transcription from the Sox2 locus (Supplementary Figure S7C), while higher expression of Sox2ot allows recruitment of YY1, likely with cofactors, to the CpG islands at the Sox2 locus (Supplementary Figure S7D). Since YY1 binding sites are pervasive throughout mammalian genomes, Sox2ot is potentially important for preferentially recruiting YY1 to the Sox2 locus through cis-regulation of Sox2ot to its overlapping gene Sox2. Moreover, the Sox2ot–YY1 complex perhaps also binds to promoters of other genes beside of Sox2. Identification of other YY1 cofactors in the IncRNA-YY1 system would
give further insight into the method of YY1-mediated repression occurring in brain development.

In recent years, IncRNAs as a group have been shown to be expressed in specific and regulated patterns in the brain, important for neural fate specification, and critical for the regulation of neurogenesis\(^5^5\). Specific IncRNAs have also demonstrated functions in neural development, including RMST, which is required for Sox2 binding to neurogenic transcription factors in neural stem cells, thus promoting neurogenesis, and TUNA, which is required for pluripotency and neural lineage commitment of mouse ES cells\(^5^2,5^3\). In the embryonic mouse cortex, the IncRNA Pinky interacts with RNA-spooling protein PTBP1 to regulate neurogenesis\(^5^4\). Finally, Paupar induces neural differentiation through both Pax6-dependent and Pax6-independent processes\(^3^5\).

In this study, we have characterized the expression of Sox2ot in NPs in the developing mouse cortex and established its role in repression of NP expansion. We also demonstrated a novel interaction of Sox2ot with the multifunctional transcription factor YY1 and showed that YY1 binds to CpG islands at the Sox2 locus in neuroectodermal cells. Through this work, we have provided a further example of one IncRNA important in neural development, gained insight into the modes of NP regulation, and expanded the understanding of IncRNA regulatory mechanisms.

**Materials and methods**

**In situ hybridization**

In situ hybridization was performed according to previously published methods\(^5^6\). Briefly, brain sections were hybridized with DIG-labeled RNA probes at 65 °C overnight. After washing with preheated wash solution (1×SSC, 50% formamide, 0.1% Tween-20) and MABT, sections were blocked with blocking buffer (1×MABT, 2% blocking reagent, 20% heat-inactivated sheep serum) and incubated with anti-DIG antibody (1:1,500, Roche) at 4 °C overnight. Brain sections were washed with 1×MABT and staining buffer (0.1 M NaCl, 50 mM MgCl\(_2\), and 0.1 M Tris–HCl, pH 9.5), and stained with BM Purple (Roche) at room temperature until ideal intensity. The probes were labeled with DIG-ndUTP using the DIG RNA labeling kit (Roche). The images of in situ hybridization were collected using a Leica digital camera under a dissection microscope (Leica, MZ16F).

**In utero electroperoration**

In utero electroperoration was performed as described previously\(^5^6\). Briefly, electroperoration was conducted at E13.5, and the brain tissues were collected either 24 h later at E14.5 or 96 h later at E17.5. Bromodeoxyuridine (BrdU, 50 µg/g body weight) was administered intraperitoneally as a single pulse 23 h after electroperoration, 1 h before sacrifice. Plasmid DNA was prepared using the EndoFree Plasmid Maxi Kit (Qiagen) and diluted to 2 µg/µL. DNA solution was injected into the lateral ventricle of the cerebral cortex and electroporated with five 50-ms pulses at 35 V using an ECM830 electroporator (BTX).

**Tissue preparation and immunohistochemistry**

Mouse brains were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight, incubated in 30% sucrose in PBS, embedded in OCT, and stored at –80 °C until use. Brains were sectioned (14 µm) using a cryostat. For antigen recovery, sections were incubated in heated (95–100 °C) antigen recovery solution (1 mM EDTA and 5 mM Tris, pH 8.0) for 20 min and cooled for 30 min. Before applying antibodies, sections were blocked in normal goat serum (NGS) in PBS with 0.1% Tween-20 for 1 h at room temperature. Sections were incubated with primary antibodies overnight at 4 °C and visualized using goat anti-rabbit IgG-Alexa-Fluor-488 or goat anti-chicken IgG-Alexa-Fluor-488 and goat anti-rabbit IgG Alexa-Fluor-546 or goat anti-mouse IgG-Alexa-Fluor-546 (1:1,000, Molecular Probes) for 1 h at room temperature. For sections stained with Sox2 primary antibody, normal donkey serum was substituted for NGS and donkey anti-rabbit IgG-Alexa-Fluor-488 and donkey anti-goat IgG-Alexa-Fluor-546 were used for visualization (1:1000, Molecular Probes).

Primary antibodies against the following antigens were used: Sox2 (1:200, Santa Cruz), Pax6 (1:500, Covance), Tbr2 (1:500, Abcam), BrdU (1:50, DSHB), Caspase3 (1:1000, R&D Systems), Tbr1 (1:500, Abcam), Satb2 (1:500, Abcam), GFP (1:1000, Rockland, rabbit), GFP (1:1000, Abcam, chicken), and Yy1 (1:200, Santa Cruz).

Images were captured using a Zeiss confocal microscope.

**Quantitation of immunostained tissue**

Coronal sections were collected in the medial cortical region. At least four sections from each brain and three electroporated brains from the same litter were chosen for antibody labeling. For neural progenitor markers (Sox2, BrdU, Pax6, and Tbr2), positive cells were quantified in fixed areas of 100 × 100 µm\(^2\), starting at the ventricular surface. For mature neuronal markers (Tbr1 and Satb2), cells were quantified in columns with a width of 200 µm and height from the lowest point of marker staining to the pial surface.

Cell counting in the mouse brain sections was performed on a fixed width (200-µm bin) of a representative column in the cortical wall. All sections analyzed were selected from a similar medial point on the anterior–posterior axis. Cell counting was performed in minimal three chosen areas in each brain, and at least
three brains were analyzed in each group. Cell counting in each chosen group was repeated at least three times and a mean was obtained. All data are presented as mean±SEM. *P* values were calculated using an unpaired Student’s *t* test.

**Embryonic stem cell culture**

Stem cell culture and JQ1 treatment were performed, as described previously. Briefly, R1 and LF2 ES cells were maintained for 4 days in DME and 15% FCS. Cells were treated for 24 h with JQ1 (BPS Biosciences) diluted to 2 μM in DMSO.

**RNA immunoprecipitation**

RNA immunoprecipitation was performed following the Abcam RIP protocol. Briefly, cells were cross-linked with a final concentration of 0.75% formaldehyde, harvested by trypsinization, and resuspended in PBS. The nuclei were pelleted by centrifugation and resuspended in RIP buffer (150 mM KCl, 25 mM Tris, pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5% NP40, and 100 U/mL RNase inhibitor). Chromatin was sheared by sonication, and nuclear membranes were pelleted by centrifugation. For each IP, 4 μg of antibody (YY1, Santa Cruz; CTCF, Santa Cruz; Pax6, Covance) was added, and the samples were incubated overnight at 4 °C with rotation. Protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) were added, and the samples were incubated at 4 °C for 2 h with rotation. The beads were pelleted by centrifugation, resuspended, and washed in RIP buffer. RNA was eluted in RIP elution buffer (50 mM Tris, pH 8, 10 mM EDTA, and 1% SDS) supplemented with proteinase K and RNase inhibitor overnight at 65 °C. Samples were treated with DNase and precipitated overnight.

**Statistics**

For immunostaining, at least four sections from each brain and at least three different brains from the same litter were chosen for antibody labeling and quantification. For qRT-PCR, triplicated samples were tested. Statistical comparisons were made by analysis of variance (unpaired Student’s *t* test).

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**Author contributions**

J.L.K. and T.S. conceived the study and designed the experiments; J.L.K. performed all immunohistochemistry; J.L.K., N.M., H.B.P., and M.E.D. designed and performed fractionation, RIP and ChIP experiments in mouse ES cells, and ddPCR and FISH experiments; S.K. performed in utero electroporations; Y.S. performed bioinformatic analyses; Y.N. extracted RNA samples and cell cultures; T.W. and M.E.D. designed and validated shRNA constructs against YY1; J.L.K. and T.S. wrote the paper; and T.S. supervised the project.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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