Enhancer SINEs Link Pol III to Pol II Transcription in Neurons

Highlights

- eSINEs share chromatin marks with other neuronal enhancers

- eSINEs are transcribed by Pol III in response to neuronal depolarization

- eSINEs provide a molecular link between Pol III and Pol II transcription

- The eSINE FosRSINE1 controls Fos expression during neuronal differentiation

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In Brief

Spatiotemporal regulation of gene expression requires the interaction between promoters and distally located enhancers. Policarpi et al. identify a subset of SINEs that functions as enhancers for activity-dependent neuronal genes. The enhancer SINE FosRSINE1 regulates Fos transcription and is necessary for both activity-dependent dendritogenesis and proper brain development.

Data and Software Availability

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Enhancer SINEs Link Pol III to Pol II Transcription in Neurons

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SUMMARY

Spatiotemporal regulation of gene expression depends on the cooperation of multiple mechanisms, including the functional interaction of promoters with distally located enhancers. Here, we show that, in cortical neurons, a subset of short interspersed nuclear elements (SINEs) located in the proximity of activity-regulated genes bears features of enhancers. Enhancer SINEs (eSINEs) recruit the Pol III cofactor complex TFIIIC in a stimulus-dependent manner and are transcribed by Pol III in response to neuronal depolarization. Characterization of an eSINE located in proximity to the Fos gene (FosRSINE1) indicated that the FosRSINE1-encoded transcript interacts with Pol II at the Fos promoter and mediates Fos relocation to Pol II factories, providing an unprecedented molecular link between Pol III and Pol II transcription. Strikingly, knockdown of the FosRSINE1 transcript induces defects of both cortical radial migration in vivo and activity-dependent dendritogenesis in vitro, demonstrating that FosRSINE1 acts as a strong enhancer of Fos expression in diverse physiological contexts.

INTRODUCTION

All organisms respond to environmental conditions by modifying gene expression in a manner that is strictly regulated both temporally and spatially. Although this adaptive response is of fundamental importance for any cell type, it is particularly relevant to neurons. Failure to rapidly adapt the transcriptional output to ever-changing conditions compromises most brain tasks, including learning and memory formation (Flavell and Greenberg, 2008; Sweatt, 2016; West and Greenberg, 2011).

In eukaryotic cells, three RNA polymerases regulate the transcription of largely non-overlapping sets of genes. RNA polymerase II (Pol II) transcribes protein-coding genes, and Pol I and Pol III transcribe rRNA and tRNA genes, respectively (Roeder, 1996). Pol III also transcribes the 5S rRNA, small RNAs, microRNAs, and RNAs derived from DNA-repetitive elements such as short interspersed nuclear elements (SINEs) (Dieci et al., 2007). Despite the fact that most genes transcribed by Pol III are highly conserved across species, the genomic occupancy of the Pol III complex varies greatly between organisms and between cell types within the organism (Barski et al., 2010; Moqtaderi and Struhl, 2004; Moqtaderi et al., 2010; Raha et al., 2010), implying that it may have additional, cell-specific functions. In human cells, Pol III complexes bind preferentially to genomic regions adjacent to Pol II transcriptional start sites (TSSs) (Moqtaderi et al., 2010; Oler et al., 2010), and expressed tRNA genes are predominantly located in the vicinity of active Pol II promoters (Oler et al., 2010). Thus, Pol III and Pol II transcription may be functionally linked.

Gene expression is regulated by multiple mechanisms, including the interaction of promoters with distal enhancers, which are short genomic elements (typically < 200 bp) often positioned several kilobases away from their target genes (Kolovos et al., 2012). Enhancers function in an orientation-independent manner and are characterized by distinctive features, such as an “open” chromatin and the presence of the histone modifications H3 lysine 4 monomethylation (H3K4me1) and lysine 27 acetylation (H3K27ac) (Heintzman et al., 2009; Kolovos et al., 2012; Zentner et al., 2011). Enhancers are often transcribed into non-coding RNAs known as enhancer RNAs (eRNAs), which stabilize the formation of DNA loops, possibly facilitating the interaction of enhancers with gene promoters (Kolovos et al., 2012). Recently, a distinct class of enhancers has been shown to provide spatiotemporal specificity to gene expression during neuronal development (Frank et al., 2015) and in mature neurons (Kim et al., 2010; Malik et al., 2014; Schaukowitch et al., 2014; Teles et al., 2015). However, the mechanisms by which they regulate transcription remain poorly understood.

We previously showed that, in neurons, a group of SINEs undergoes de novo histone acetylation and recruits the Pol III general transcription factor TFIIIC (Crepaldi et al., 2013). SINEs are an abundant class of retrotransposons often considered as non-functional DNA because of their non-coding, repetitive nature. They are short modular sequences that, similarly to other Pol III-transcribed genes, possess an internal Pol III promoter containing A and B boxes (Muotri et al., 2007; White, 2011). These elements bind the multi-subunit complex TFIIIC, which, in turn, recruits TFIIIB and Pol III. Most SINEs carry mutations that disrupt the promoter region, leaving only a few subtypes...
with transcriptional potential (Ichiyanagi, 2013). Recent studies indicated that SINEs often acquire novel functions in the host genome in a phenomenon known as exaptation (Huda et al., 2010; Rebollo et al., 2012). In mammalian fibroblasts exposed to heat shock, for example, SINE transcripts inhibited Pol II-dependent transcription by interacting with the Pol II enzyme (Allen et al., 2004; Mariner et al., 2008). Two members of the ancient SINE family Amnicta SINE1 (AmnSINE1) were shown to act as enhancers for the fibroblast growth factor 8 (Fgf8) and special AT-rich sequence-binding protein 2 (Satb2) genes in the developing mouse brain (Sasaki et al., 2008; Tashiro et al., 2011). Similarly, in humans, members of the Alu family are enriched for enhancer-like histone modifications in a tissue-specific manner and preferentially engage in long-distance interactions with gene promoters and other Alu elements (Su et al., 2014).

Here, we identified a class of SINEs that function as enhancers of activity-regulated neuronal genes. Genome-wide analyses revealed that enhancer SINEs (eSINEs) bear the epigenetic marks H3K4me1 and H3K27ac, recruit TFIIIC, and are transcribed by Pol III in response to depolarization. We discovered that an eSINE located in the proximity of the activity-dependent gene Fos (which we named FosRSINE1) functions as a Fos enhancer and is transcribed in depolarized neurons. FosRSINE1 eRNA interacts with Pol II at the Fos promoter and is necessary for the relocation of Fos to Pol II transcription factories upon depolarization. Strikingly, this mechanism is required for activity-dependent dendritogenesis and for cortical radial migration and neuronal differentiation of neural progenitors during embryonic development. Together, our findings demonstrate the profound effect of FosRSINE1 on Fos gene expression and reveal a functional link between Pol III and Pol II transcription.

RESULTS

Genome-wide Occupancy of the Pol III Machinery Identifies eSINEs

In response to neuronal depolarization, a group of SINEs located near activity-dependent genes undergoes de novo acetylation at H3K9/14 (Crepaldi et al., 2013). Because acetylated SINEs possess an internal Pol III promoter (Muotri et al., 2007; White, 2011), we reasoned that they may represent a class of Pol III-transcribed neuronal enhancers. To investigate this hypothesis, we first employed chromatin immunoprecipitation sequencing (ChIP-seq) to map the Pol III machinery genome-wide in resting or depolarized mouse primary cortical neurons. We employed paired-end sequencing to obtain reads that were significantly longer than SINEs and, therefore, easily mappable (Figure S1A). For each experimental condition, we produced over 32 million high-quality mapped reads, providing excellent depth (Figure S1B).

ChIP-seq was performed using antibodies that recognize the catalytic subunit of Pol III (Rpc155) or the DNA binding subunit of TFIIIC (Gtf3c1). As expected, both Rpc155 and Gtf3c1 were highly enriched at tRNA genes (Figures S1C and S1D). Gtf3c1 was widely distributed across the genome, with 66,662 and 58,090 peaks in control (Ctrl) and depolarized conditions, respectively (Table S1). Interestingly, a considerable fraction of Rpc155 and Gtf3c1 peaks overlapped at least one SINE (17.4% and 20.0% in resting and stimulated neurons, respectively, for Gtf3c1 and 20.7% and 21.1% for Rpc155; Table S1). Comparative analysis in untreated and depolarized neurons revealed that 7,700 genomic regions showed significant recruitment of Gtf3c1 in response to depolarization, of which 1,151 overlapped with SINES (Table S2). We named this group of 1,151 SINES characterized by activity-dependent recruitment of TFIIIC eSINES. The levels of Gtf3c1 binding to these elements were higher than for randomly selected SINES (rndSINES, \( p < 2.2e^{-16}, \) Mann-Whitney test for both Ctrl and KCl conditions; Figure 1A). Importantly, Gtf3c1 binding to eSINEs increased in response to stimulation (\( p < 2.2e^{-16}, \) Mann-Whitney test), whereas it was unchanged for rndSINES. Similarly, Pol III binding was higher on eSINEs compared with rndSINES (\( p < 2.2e^{-16}, \) Mann-Whitney test for both Ctrl and KCl conditions; Figure 1B), and recruitment to eSINES increased in KCl-treated neurons (\( p = 2.9e^{-11}, \) Mann-Whitney test). ChIP-seq tracks of representative eSINES are shown in Figure S1E.

To investigate whether Gtf3c1 recruitment to eSINES correlated with activity-dependent transcription genome-wide, we analyzed RNA sequencing (RNA-seq) data performed under similar experimental conditions (Kim et al., 2010; Malik et al., 2014). We observed that eSINES were enriched in the proximity of inducible, but not housekeeping, genes (Figure 1C). Although...
alternative roles cannot be excluded, this genomic localization suggests that eSINEs may represent a new class of regulatory elements that coordinate activity-dependent transcription in neurons. Next, 120 genes that were induced at least 2-fold in response to depolarization were paired with the closest eSINE (distances ranging from < 1 kb to several hundred kilobases from the TSS; Table S3). This group of eSINEs showed recruitment of the Pol III machinery in response to depolarization (Figures 1D and 1E, right plots; p < 2.2e-16 and p = 2.1e-7 for Gtf3c1 and Rpc155 respectively, Mann-Whitney test) whereas 120 mdSINEs did not (Figures 1D and 1E, left plots).

In accordance with previous studies indicating that Pol II and Pol III co-localize on the proximity of expressed genes, the enrichment of Rpc155 and Gtf3c1 at the TSSs and gene bodies of the paired 120 genes increased in depolarized neurons (Figure 1F; Moqtaderi et al., 2010; Oler et al., 2010; Raha et al., 2010). As expected, no significant recruitment of the Pol III machinery was observed on housekeeping genes (HGs; Figure 1G); lower occupancy of Pol III and Gtf3c1 was observed at randomly selected genes (RGs) and silent genes (SGs), with no changes upon neuronal activity (Figure 1G). A positive correlation (r² = 0.5, p = 9.7e-8; Figure 1H) was found between the recruitment of Gtf3c1 at eSINEs following depolarization and the transcription of genes for which the closest eSINE was located at a distance of 100 bp or less from the TSS (45 of 120 genes). This subset of genes included Fos, Gadd45b, and other well-characterized activity-dependent genes, such as FosB, JunB, Egr4, Crem, Npas4, Nr4a1, and Nr4a3 (Figure 1H; Table S3).

**eSINEs Bear the Epigenetic Hallmarks of Enhancers and Are Transcribed**

Epigenetic marks commonly used to identify putative enhancers include H3K27ac and H3K4me1 (Kolovos et al., 2012; Zentner et al., 2011). ChIP-seq experiments performed on Ctrl and depolarized neurons revealed that both histone modifications were higher at the 1,151 eSINEs than at mdSINEs (p = 2.2e-16, Mann-Whitney test for both Ctrl and KCl conditions; Figures 2A and 2B). H3K4me1 and H3K27ac were present at eSINEs in resting neurons, indicating that they may be epigenetically primed prior to neuronal activation. H3K4me1 and H3K27ac were also enriched at the 120 eSINEs paired with activity-dependent genes (p = 2.5e-15 and p = 6.2e-12, respectively, under Ctrl conditions; Figures S2A and S2B). The recruitment of Gtf3c1 to a panel of eSINEs in response to depolarization and the presence of H3K4me1 and H3K27ac were confirmed using ChIP-qPCR (Figure S2C). It should be noted that eSINEs displayed levels of H3K4me1 and H3K27ac similar to previously identified enhancers for the activity-dependent genes Arc and Fos (Kim et al., 2010; Schaukowitch et al., 2014; Figure S2C). In contrast, SINEs located in the proximity of housekeeping (Actb and Hprt) and repressed (Prrt1 and Ndufb5) genes did not recruit Gtf3c1 and were devoid of the enhancer marks H3K4me1 and H3K27ac (Figure S2C).

Enhancers are active genomic regions that, similarly to promoters, are associated with an open, easily accessible state of the chromatin (Boyle et al., 2008; Frank et al., 2015). When the chromatin accessibility of eSINEs was tested using the DNaseI hypersensitivity assay, we observed increased sensitivity to DNaseI digestion after depolarization (Figure 2C), indicating that the chromatin surrounding eSINEs becomes depleted of nucleosomes and primed for transcription. In contrast, SINEs located in the proximity of either housekeeping (Actb and Hprt) or repressed (Prrt1 and Ndufb5) genes were not sensitive to DNaseI (Figure 2D). Notably, the hypersensitivity of eSINEs to DNaseI after neuronal stimulation was comparable with that observed at the TSS of the housekeeping gene Actb (actin beta; Figure 2E), whereas Fsh (follicle-stimulating hormone), a gene that is not expressed in neurons, did not show DNaseI hypersensitivity (Figure 2E).

Enhancers are transcribed in a number of organisms and cell types in response to external stimuli (Koikkonen et al., 2013; Koch et al., 2011; Wang et al., 2011), and the eRNAs produced are critical for enhancer function (Lam et al., 2013; Li et al., 2013; Melo et al., 2013; Schaukowitch et al., 2014; Telese et al., 2015). We therefore investigated whether eSINEs were expressed in response to neuronal depolarization using qRT-PCR and found that they were transcribed in activated neurons (Figure 2F). Importantly, eSINE RNA transcripts located in the proximity of canonical Pol III transcriptional terminators (Figure 3), indicating that they are bona fide Pol III transcripts. As expected, SINEs located proximal either to housekeeping (Actb and Hprt) or repressed (Prrt1 and Ndufb5) genes were not transcribed (Figure 2G). Thus, eSINEs represent a new class of neuronal enhancers that recruit TFIIIC and are transcribed by Pol III.

**Functional Characterization of Fos<sup>RSINE</sup> and Gadd45b<sup>B1F</sup> eSINEs**

To gain further insights into the biochemical and functional properties of eSINEs, two elements located in the proximity of Fos and Gadd45b (named Fos<sup>RSINE</sup> and Gadd45b<sup>B1F</sup>) were selected for further analysis. Fos and Gadd45b are activity-dependent genes with well known functions in the nervous system (Greenberg et al., 1986; Ma et al., 2009) that are transcribed in vivo in cortical neurons upon exposure to a novel enriched...
environment (NEE) and in response to neuronal depolarization in vitro (Crepaldi et al., 2013; Sheng et al., 1990; Sultan et al., 2012). Moreover, both eSINEs are de novo acetylated on H3K9K14 in the somatosensory cortex of mice exposed to NEE (Crepaldi et al., 2013). Despite the fact that SINE sequences are highly repetitive, FosRSINE1 and Gadd45bB1F have distinctive features that allow unequivocal identification (Figure S4A). We first confirmed that FosRSINE1 and Gadd45bB1F recruited Gtf3c1 and Rrpc155 in response to depolarization (Figures 3A and 3B) and displayed the H3K4me1 and H3K27ac marks (Figures 3C and 3D). FosRSINE1 and Gadd45bB1F also showed activity-dependent hypersensitivity to DNaseI comparable to Fos and Gadd45b (Figure 3E). qRT-PCR of FosRSINE1 and Gadd45bB1F RNA at different times after neuronal depolarization revealed that both eSINEs are robustly transcribed (Figure 3F).

Importantly, transcription was not detected at genomic regions located immediately upstream (USR) or downstream (DSR) of the two SINEs were analyzed by qRT-PCR. Importantly, transcription was not detected at genomic regions located immediately upstream (USR) or downstream (DSR) of the two SINEs were analyzed by qRT-PCR. Interestingly, expression of transcription (Figure 3F), suggesting that Fos RSINE1 and Gadd45bB1F eRNA may control the expression of these genes.

We next investigated whether FosRSINE1 enhanced activity-dependent transcription using luciferase reporter assays. Neurons were transfected with plasmids expressing the luciferase coding region under the control of three activity-response gene promoters (Fos, Gadd45b, and Arc) and either depolarized or left untreated. Under stimulated conditions, inclusion of FosRSINE1 at the 3’ end of the luciferase cassette markedly increased luciferase expression for all tested promoters (Figure 3G). FosRSINE1 did not enhance luciferase expression when cloned 3’ of either the SV40 promoter of the pGL3-promoter vector or the Gapdh promoter (Figures S4B and S4C). Importantly, in neurons transfected with equimolar amounts of a vector carrying FosRSINE1 and a separate luciferase construct encoding the Fos promoter, FosRSINE1 failed to enhance luciferase expression (Figure S4D), indicating that genomic contiguity is required for enhancer function.

To establish whether transcription of FosRSINE1 is necessary for enhancer function, we generated a mutated version of the eSINE bearing a six-nucleotide mismatch that disrupts Gtf3c1 and Pol III recruitment (Orioli et al., 2012). Mouse neuroblastoma cells (Neuro-2a) were transfected with a vector carrying either wild-type (pBS_RSINE1WT) or mutant FosRSINE1 (pBS_RSINE1mut) and stimulated with forskolin. FosRSINE1 expression levels were significantly lower in cells transfected with the mutant compared with the wild-type vector (Figure S4E). Strikingly, this mutated version of FosRSINE1 failed to enhance activity driven by Fos, Gadd45b, and Arc promoters (Figure 3G). Thus, Pol III binding to FosRSINE1 is required for enhancer activity.

**Fos**<sub>RSINE1</sub> **eRNA Is Required for Enhancer Function**

To investigate the mechanisms by which eSINEs regulate activity-dependent transcription, the FosRSINE1 genomic sequence was disrupted using a high-fidelity variant of Cas9 (SpCas9-HF1; Kleinstiver et al., 2016). A guide RNA targeting FosRSINE1 was expressed in Neuro-2a cells together with a vector encoding SpCas9-HF1. As a control, we transfected the SpCas9-HF1-encoding plasmid and an empty vector (no guide). Efficient editing of the FosRSINE1 locus was assessed by Surveyor assay (Figure S5A) and confirmed by DNA sequencing of individual clones (Figure S5B). In cells transfected with FosRSINE1 guide RNA, the induction of FosRSINE1 eRNA upon forskolin stimulation was drastically reduced compared with empty vector (Figure 4A), whereas induction of a different eSINE (Dusp1MIRc) was not affected by (Figure 4A). Strikingly, disruption of the FosRSINE1 genomic locus was sufficient to abolish forskolin-dependent transcription of Fos (Figure 4A), indicating that the genomic integrity of FosRSINE1 is necessary for Fos expression upon neuronal stimulation.

Experiments that knockdown eRNAs have recently shown that enhancer transcripts regulate the expression of nearby genes (Lam et al., 2013; Li et al., 2013; Melo et al., 2013; Mousavi et al., 2013; Schaukowitch et al., 2014). To investigate whether FosRSINE1 enhancer activity is mediated by Pol III-dependent eRNA synthesis, we employed several complementary approaches. First, cortical neurons were treated with the specific Pol III inhibitor ML60218 (50 μM, 15 min; Wu et al., 2003) and depolarized. ML60218 treatment decreased transcription of the Pol III-transcribed gene tRNA<sub>Leu</sub> (Figure 5A), indicating efficient inhibition of Pol III. Importantly, inhibition of Pol III markedly reduced transcription of both FosRSINE1 and the Fos gene in response to neuronal activation (Figures 4B and 4C). The effect of ML60218 on Fos transcription was not due to non-specific inhibition of Pol II because the expression levels of the Pol II-transcribed β-Actin were unchanged (Figure 5B). Conversely, treatment of cortical neurons with the specific Pol II inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (Yankulov et al., 1995) led to a strong reduction in Fos induction and β-Actin transcription but had no effect on either FosRSINE1 or tRNA<sub>Leu</sub> transcription (Figures 4B and 4C; Figures S6A and S6B).
Similar results were obtained for two additional eSINEs (Gadd45bB1F and Dusp1MIRc) and the paired activity-dependent genes (Gadd45b and Dusp1) (Figures S6C–S6F). Next we used locked nucleic acid (LNA) oligonucleotides to specifically target FosRSINE1 eRNA for degradation. In Neuro-2a cells, an LNA targeting FosRSINE1 eRNA (LNARSINE1) reduced FosRSINE1 levels without affecting Dusp1MIRc expression (Figures S6G and S6H). Cortical neurons were transfected with either LNARSINE1 or a control LNA (LNACTR) and depolarized, and the Fos transcript was measured using RNA fluorescence in situ hybridization (FISH). As expected, in LNACTRL-treated neurons depolarization induced the appearance of clearly detectable ribonucleoparticles containing Fos mRNA (Figure 4D, top right, white arrowheads). Remarkably, LNA RSINE1 caused a 70% reduction of Fos mRNA expression compared with LNACTR (Figures 4D and 4E). Similarly, an LNA targeting Dusp1MIRc eRNA (LNAMIRc) strongly reduced Dusp1 mRNA levels following KCl stimulation (Figures S7A and S7B). The specificity of LNARSINE1 was tested by performing luciferase assays on neurons expressing vectors encoding luciferase under the control of either the Fos promoter and FosRSINE1 (prom_RSINE1) or the Fos promoter alone (prom). In neurons transfected with prom_RSINE1, LNARSINE1 completely abolished FosRSINE1 enhancer activity but was ineffective in decreasing luciferase expression in neurons transfected with a vector containing Fos promoter only (Figure 4F). Together, these data show that Pol III-dependent transcription of FosRSINE1 is necessary for enhancing Fos expression in depolarized neurons.
FosRSINE1 eRNA Controls Fos Gene Relocation to Transcription Factories

Transcription factories (TFs) are discrete nuclear foci formed by clusters of Pol II and transcription factors that create a permissive environment for transcription (Rieder et al., 2012) where enhancers and promoters may physically interact (Cook, 2010). Because activity-dependent genes relocate to TFs upon depolarization (Crepaldi et al., 2013), we investigated whether eSINE eRNA regulates this process by performing immuno-DNA FISH on cortical neurons transfected with LNAs (Figure 5A). TFs were labeled using a Pol II antibody, and the distance between the center of the DNA-FISH signal and the nearest TF was measured. In neurons transfected with LNA^CTR, neuronal stimulation decreased the distance between the Fos locus and the nearest TF (Figure 5B) and led to higher co-localization of FISH signals with TFs (Figure 5C). Strikingly, transfection with LNA^RSINE1 inhibited depolarization-dependent relocation of Fos to TFs (Figures 5A–5C), whereas the nuclear position of Csn2, a gene that is not expressed in neurons, was not affected (Figures 5A–5C). Our findings indicate that FosRSINE1 eRNA promotes Fos relocation to TFs in response to stimulation and may contribute to the spatial organization of chromatin within neuronal nuclei.

FosRSINE1 eRNA Interacts with Pol II to Mediate Transcriptional Initiation and Elongation

It has been proposed that eRNAs may contribute to Pol II loading onto target promoters (Mousavi et al., 2013). Moreover,
transcripts originating from SINEs have been shown to bind Pol II in mouse fibroblasts (Allen et al., 2004; Mariner et al., 2008). To investigate whether FosRSINE1 eRNA interacted with Pol II, neurons were subjected to RNA immunoprecipitation (RIP) using a Pol II antibody. We observed that, upon depolarization, the Pol II antibody immunoprecipitated FosRSINE1 eRNA at high levels (Figure 6A), indicating binding. Additional evidence of the interaction was obtained by performing electrophoretic mobility shift assays (EMSAs) using in vitro-transcribed α-32P-labeled FosRSINE1 eRNA. The radioactive probe was loaded on a non-denaturing polyacrylamide gel either alone (probe only) or in the presence of neuronal nuclear proteins. At least three distinct protein complexes were bound to FosRSINE1 eRNA (Figure 6B, lane 3, white arrowheads). Antibodies that recognize the pre-initiating, initiating, or elongating forms of Pol II (8WG16, 4H8, and H5, respectively; Sutherland and Bickmore, 2009) displaced or super-shifted at least one band, confirming that FosRSINE1 eRNA was associated with Pol II and indicating that the interaction can occur at different stages of transcription (Figure 6B, lanes 4–6).

We next asked whether FosRSINE1 eRNA influenced Pol II recruitment to the endogenous Fos promoter using Pol II ChIP. In untransfected or LNA CTR-transfected Neuro-2a cells, Pol II binding to the Fos gene significantly increased upon stimulation at both the promoter (8WG16 and 4H8 antibodies) and at the coding region (4H8 and H5 antibodies) (Figures 6C–6F). In cells transfected with LNA STARSINE1, stimulus-dependent binding of pre-initiating Pol II to the Fos promoter was conserved (Figure 6C), whereas recruitment of both the initiating and elongating forms of Pol II at the Fos promoter and coding region was prevented (Figures 6D–6F). These results indicate that, in the absence of the FosRSINE1 eRNA, Pol II is unable to progress into active transcription and provide the first evidence of a functional link between a Pol III-dependent transcript and Pol II.

FosRSINE1 eRNA Regulates Neuronal Differentiation and Cortical Development

The biological significance of FosRSINE1-dependent regulation of Fos transcription was investigated by studying dendritogenesis of primary cortical neurons. Dendritic growth in response to external cues requires the transcription of activity-regulated
Figure 7. FosRSINE1 Enhances Fos Expression in Diverse Physiological Contexts

(A) Representative images of cortical neurons transfected with a GFP-expressing vector in combination with either LNACTRL or LNARSINE1 and maintained under basal or depolarizing (KCl, 50 mM) conditions for 48 hr, followed by GFP immunostaining. Scale bar, 50 µm.

(B) Quantification of the total length of the dendritic processes of 29–31 neurons (n = 3). Shown are means ± SEM. **p < 0.01, two-way ANOVA.

(C) Sholl analysis of neurons analyzed in (B). For each distance point, the average number of intersections ± SEM is shown. *p < 0.05, **p < 0.01, ****p < 0.0001, two-way ANOVA.

(D) qRT-PCR of mouse neural progenitor cells (NPCs) and post mitotic-neurons (PMNs). Data show mean ± SEM of fold induction of Hprt SINE RNA, FosRSINE1 eRNA, and Fos mRNA (n = 6) normalized to expression levels in NPCs. **p < 0.005, ****p < 0.0001, paired t test.

(legend continued on next page)
by Fos (Greenberg et al., 1986; Malik et al., 2014). Thus, early and robust activation of Fos may be necessary for dendritic arborization. Neurons were transfected with a GFP-expressing vector and either LNA^{CTRL} or LNA^{RSINE1} and maintained under basal or depolarized conditions for 48 hr. As expected, neurons transfected with LNA^{CTRL} showed an increase in both dendritic length and complexity (Figures 7A–7C). Strikingly, activity-dependent dendritogenesis was completely abrogated in neurons transfected with LNA^{RSINE1} (Figures 7A–7C).

Because Fos has been shown to regulate mouse brain development (Velazquez et al., 2015), we reasoned that Fos^{RSINE1} may also enhance Fos expression in this physiological context. First, we assessed whether Fos^{RSINE1} was expressed in embryonic cortical neurons. Mouse neural progenitor cells (NPCs) were dissected at embryonic day 12.5 (E12.5) and differentiated in vitro into post-mitotic neurons (PMNs) (Nitaraka et al., 2016). A remarkable increase in both Fos^{RSINE1} and Fos expression levels was observed following differentiation of NPCs to PMNs (Figure 7D). Conversely, transcription of the non-enhancer SINE Hprt^{RSINE1} was unchanged (Figure 7D). Next, we asked whether Fos^{RSINE1} regulated the expression of Fos during neural development in vivo by performing in utero electroporation experiments. LNA^{CTRL} or LNA^{RSINE1} were electroporated together with a GFP expression plasmid into E13.5 mouse brains, and, after 2 days, neural migration and expression of differentiation markers were assessed. As observed previously (Nitaraka et al., 2016) after 48 hr, 33% of the progeny of neural progenitors electroporated with LNA^{CTRL} in the ventricular zone (VZ) had reached the cortical plate (CP) (Figures 7E and 7F). Inhibition of Fos^{RSINE1} expression resulted in an accumulation of cells within the VZ and a reduction of neurons migrating toward the CP (Figures 7E and 7F). Neural progenitors electroporated with LNA^{RSINE1} failed to differentiate and express T-Box Brain 1 (Tbr1) (Figures 7E and 7G), a transcription factor found in early-born neurons (Hevner et al., 2001). Consistent with the phenotype observed in Fos^{−/−} mice (Velazquez et al., 2015), depletion of Fos^{RSINE1} eRNA also resulted in increased proliferation of neural progenitors, as indicated by the elevated number of Ki67- and Sox2-positive cells (Figures 7H–7K). Co-electroporation of neuronal progenitors with a vector expressing Fos under the control of a cytomegalovirus (CMV) early enhancer/chicken β-actin (CAG) promoter fully rescued the defects induced by the inhibition of Fos^{RSINE1} (Figures 7E–7K), ruling out potential off-target effects of LNA^{RSINE1}.

Thus, during the early stages of cortical development, Fos^{RSINE1} regulates radial migration and neuronal differentiation of NPCs, whereas at the onset of neuronal maturation, Fos^{RSINE1} eRNA is required for activity-dependent dendritogenesis.

**DISCUSSION**

**eSINEs Are a Class of Neuronal Enhancers**

Repetitive elements are extremely abundant in the mammalian genome, however their function remains largely obscure. In recent years, SINE transcripts have been linked to protein synthesis, mRNA turnover, and RNA editing in both human and mouse cell lines (Athanasiadis et al., 2004; Kim et al., 2004; Levanon et al., 2004; Ohnishi et al., 2012). SINEs may also have regulatory functions independent of their transcription, providing for example, sites of alternative splicing (Levanon et al., 2004), generating new TSSs and polyadenylation signals (Chen et al., 2009; Faulkner et al., 2009), and offering an accessible chromatin environment for transcription factor binding (Gomez et al., 2016). SINEs also act as insulators, transcriptional repressors, or developmentally regulated enhancers (Allen et al., 2004; Lunyak et al., 2007; Nakanishi et al., 2012; Tashiro et al., 2011). We have identified a new class of enhancer SINEs that are transcribed by Pol III and likely regulate the expression of many Pol II-dependent genes in response to neuronal depolarization. eSINEs share biochemical and functional properties with previously identified neuronal enhancers, including the epigenetic marks H3K4me1 and H3K27ac (Figures 2A and 2B, and 3C and 3D) and hypersensitivity to DNaseI (Figures 2C and 3E), which is associated with open, nucleosome-depleted chromatin. Importantly, although we were not able to perform a genome-wide analysis of eSINE transcripts because of technical issues related to their repetitive nature, we showed that many eSINEs are transcribed in response to neuronal depolarization (Figures 2F and 3F) and that transcription is critical for target gene induction (Figure 4; Figure 5).

**A Functional Link between Pol III and Pol II Transcription**

The Pol II and Pol III transcriptional machineries have been traditionally considered independent entities that regulate the expression of largely non-overlapping genes. However, recent studies have demonstrated that they often co-localize on genomic loci in both murine and human cells and that co-localization of the two machineries correlates with transcription (Barski et al., 2010; Moqtaderi et al., 2010; Oler et al., 2010; Raha et al., 2010). Whether the two machineries are functionally linked and the mechanisms of such regulation is still unknown.

Transcription of enhancer elements was first observed more than 20 years ago (Collis et al., 1990), and, more recently, RNA
synthesis has been demonstrated at active enhancers of most cell types across many species (Kaikkonen et al., 2013; Koch et al., 2011; Wang et al., 2011). All neuronal enhancers identified to date are transcribed by Pol II and bind transcription factors that are also enriched on the promoter of their target genes (Kim et al., 2010; Malik et al., 2014; Telese et al., 2015). It has been postulated that recruitment of transcription factors to both enhancers and promoters helps to create a molecular bridge that physically connects the two elements, conferring specificity to enhancer-promoter pairings (Lam et al., 2014). In contrast, eSINEs are transcribed by Pol III and bind the general transcription factor complex TFIIIC (Figures 1A and 1B and 3A and 3B). Interestingly, eSINEs do not possess consensus sequences for any transcription factor associated with Pol II-transcribed neuronal enhancers (L.C. and A.R., unpublished data). Because TFIIIC and Pol III are recruited to both eSINEs and TSSs of activity-regulated genes (Figures 1A, 1B, and 1F), they may play a role in pairing eSINEs with their gene promoters in response to depolarization.

Binding of the Pol III machinery to eSINEs correlates with transcription of the closest genes in response to neuronal activation (Figure 1H), suggesting that contiguity of eSINEs with their target genes is necessary for enhancer functions. Consistent with this finding, FosRSINE1 eRNA failed to enhance luciferase expression driven by the minimal Fos promoter when encoded by a separate vector (Figure S4D). However, the higher-order structure of chromatin within the nucleus enables enhancers to contact promoters even when they are located far apart on the chromosome. We discovered that FosRSINE1 eRNA binds Pol II (Figures 6A and 6B) and is necessary for relocation of the Fos genomic region to transcription factories (Figure 5), perhaps inducing changes of chromatin structure that favor enhancer-promoter interactions. The finding that both Pol III and TFIIIC are recruited to Pol II-regulated genes (Figure 1F) further indicates that the two machineries may come into close proximity within transcription factories.

**FosRSINE1 Enhances Fos Expression in Diverse Physiological Contexts**

The biological relevance of FosRSINE1 was demonstrated by the fact that FosRSINE1 eRNA is required for activity-dependent dendritic growth and branching (Figures 7A–7C). We previously showed that silencing of TFIIIC in cortical neurons increased dendritic length (Crepaldi et al., 2013). These findings may be explained by the fact that more than 80% of Gtf3c1 binding identified by ChiP-seq is located in genomic regions other than eSINEs and independently of Pol III (< 20% of co-localization on SINEs and < 15% genome-wide). Therefore, global silencing of TFIIIC is likely to affect genome organization and transcription in ways distinct from the inhibition of FosRSINE1 and Fos expression alone. Strikingly, the role of FosRSINE1 was not limited to regulating Fos expression in response to neuronal activity but extended to early brain development. Both Fos and FosRSINE1 levels increased during neuronal differentiation in vitro (Figure 7D), and FosRSINE1 eRNA was required for embryonic cortical development in vivo (Figures 7E–7K). Thus, in very different physiological contexts, Pol II–dependent transcription of the Fos gene is regulated by the Pol III–mediated transcription of its nearby eSINE.

In 1969 Britten and Davidson, formulated the hypothesis that transposable elements such as SINEs participate in gene-regulatory networks, contributing to speciation novelty (Britten and Davidson, 1969). More recently, repetitive elements have been shown to represent key determinants of macroevolution and clade-specific phenotypes (Tashiro et al., 2011). The considerable number of eSINEs identified in neurons suggests that they may have an unexpected and complex role that goes beyond controlling the expression of isolated genes and extend to globally linking Pol III with Pol II transcription.

**EXPERIMENTAL PROCEDURES**

**CRISPR-Cas9**

Single guide RNAs were designed toward the Fos eSINE using http://crispr.mit.edu/. The backbones for the vectors expressing the guide RNA (U6-BamBICassette-Sp-sgRNA, Addgene 65777) and the high-fidelity Cas9 (CMV-T7-humanSpCas9-HF1[N497A, R661A, Q926A]-NLS-3xFLAG, Addgene 72247; Kleinstiver et al., 2016) were obtained from Addgene. Annealed oligonucleotides composing the guide RNAs were cloned into the BsmBI site of U6-BamBICassette-Sp-sgRNA. Neuro-2a cells were plated into a 12-well plate and transfected 48 hr later with 250 ng U6-BamBICassette-Sp-sgRNA (either empty vector or containing FosRSINE1 guide RNA) and 750 ng CMV-T7-humanSpCas9-HF1 using Lipofectamine 2000. The medium was changed 2–3 hr after transfection, and cells were harvested 48 hr later. For RNA analysis, cells were starved 16 hr before adding forskolin (50 μM, 45 min). The guide RNA sequence was GGTGATGCACTTGAGGTCATGG (the last 3 letters are protospacer adjacent motif [PAM]).

**Immuno- and FISH**

Immuno- and DNA FISH experiments were performed as described previously (Crepaldi et al., 2013) with some modifications. Briefly, cells were fixed for 10 min in 3% paraformaldehyde (PFA) in PBS, followed by permeabilization for 10 min in 0.5% Triton X-100 in PBS. After blocking with PBS+ (PBS plus 0.1% casein, 1% BSA, 0.2% fish skin gelatin) for 1 hr, cover slips were incubated overnight with Pol II Ser5p (1:500, 4H8, Millipore 06-623) and GFP (1:200, Abcam ab13970) antibodies in PBS+. For detection, cover slips were incubated with anti-mouse Alexa Fluor 568 (1:1,000, Life Technologies) and anti-chicken Alexa Fluor 488 (1:1,000, Life Technologies) for 1 hr in PBS+. Post-fixation in 3% PFA in PBS (10 min) was followed by permeabilization in 0.1 M HCl and 0.7% Triton X-100 (0.1 M, 45 min) and by denaturation in 70% formamide in 2x saline sodium citrate (SSC) (80°C, 30 min). FISH hybridization with digoxigenin-labeled probes was carried out overnight at 42°C. The probes (bacterial artificial chromosome/clones [BACs] Fos RP24-233K8 and Cas2 RP23-110B6) were labeled with digoxigenin-deoxyuridine triphosphate (dUTP) using a nick translation kit (Roche, denuitratotyC, 5 min, and pre-annealed (37°C, 45 min) with Cot-1 DNA in hybridization buffer (50% formamide, 20% dextran sulfate, 2x SSC, and 1 mg/mL BSA) immediately before hybridization. FISH signals were amplified using sheep anti-digoxigenin fluoroscein Fab fragments (1:50, Roche 11207741910) and fluoroscein rabbit anti-sheep antibodies (1:100, Vector Laboratories FI-6000); DNA was counterstained with DAPI. Confocal images of neuronal nuclei were acquired using a Leica SPE3 confocal microscope. The co-localization threshold was set to 225 nm, corresponding to the distance at which the two smallest detectable objects overlap.

**In Utero Electroporation**

E13.5 pregnant mice were anesthetized with isoflurane in oxygen carrier (Abbott Laboratories), and the uterine horns were exposed through a small incision in the ventral peritoneum. Plasmid DNA solution (0.5–1.5 μg/μL), prepared using the EndoFree plasmid purification kit (Qiagen), was mixed with 50 μM antisense LNA GapmeR (in vivo-ready, Exiqon) and 0.05% Fast Green (Sigma) and injected through the uterine wall into the lateral ventricles of the embryos using pulled borosilicate needles and a Femtojet microinjector (Eppendorf). Five electrical pulses were applied at 35 V (50-ms duration) across the uterine neural nuclei.
Radial Neural Migration Analysis

Embryos were electroporated in utero with the indicated GFP vectors, and analysis of radial migration was performed as described using ImageJ and an Excel macro (Nitaraska et al., 2016). Images were acquired on an SP8 confocal microscope with Leica Application Suite Advanced Fluorescence (LAS AF) software using a 20× objective at 1,024 × 1,024 pixel resolution. Confocal images were run through a band-pass filter to segment and isolate cell-sized shapes, thresholded and segmented into 10 radial regions between the ventricle and the pial surface. Individual cell position along the radial axis was recorded and imported into Excel along with the coordinates of top (pial) and bottom (ventricle) boundaries obtained using ImageJ’s Path Writer plugin. The distance and percentage of migrating cells in each area were calculated using an Excel macro.

DATA AND SOFTWARE AVAILABILITY

The accession number for the ChIP-seq data reported in this study is GEO: GSE75191.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.11.019.

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Supplemental Information

Enhancer SINEs Link Pol III to Pol II Transcription in Neurons

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Figure S1. Quality control of ChIP-seq analysis, related to Figure 1.

(A) Box and whiskers plots summarizing the size distribution of SINEs and of uniquely aligned ChIP-seq reads for each set of libraries. For each experimental condition, libraries from three separate biological replicates were sequenced. Lower and upper whiskers indicate the minimum and the maximum number of reads, respectively. Lower and upper box limits indicate the 25th and 75th percentile. The solid line denotes the median. (B) Histograms depicting the absolute read numbers for each experimental condition analyzed by ChIP-seq. Shown are uniquely aligned high-quality reads (MAPQ >= 10), low-quality reads, duplicates and not aligned reads. (C) Binding density profiles of Gtf3c1 and Rpc155 at the TSS of annotated tRNA genes, in cortical neurons under control (dashed line) or depolarized (solid line) conditions. Read density profiles of input samples are shown in grey. (D) Box and whiskers plots summarizing the distribution ofRpc155. Shown is ChIP-seq tag density at randomly selected (rnd) SINEs, eSINEs and tRNA genes. The solid line denotes the median. Lower and upper box limits indicate the 25th and 75th percentile respectively. Whiskers indicate 1.5 times the interquartile distance, measured from the median. (E) Representative genomic regions showing Gtf3c1, Rpc155, H3K4me1 and H3K27ac ChIP-seq signal at three eSINEs in both resting and depolarized neurons.
Figure S2. eSINEs share features of enhancer elements, related to Figure 2.
(A-B) Box and whiskers plots summarizing tag density of H3K4me1 (A) and H3K27ac (B) at a group of 120 eSINEs or at randomly selected SINEs of comparable size. The solid line denotes the median. Lower and upper box limits indicate the 25th and 75th percentile respectively. Whiskers indicate 1.5 times the interquartile distance, measured from the median. (C) Cortical neurons were either left untreated or exposed to KCl for 45 min and subjected to Gtf3c1, H3K4me1 and H3K27ac ChIP-qPCR. 7 eSINEs, 4 non-enhancer SINEs and two previously identified neuronal enhancers (known enhancers) are analyzed. Histograms show ChIP efficiency expressed as percentage over total chromatin input. Shown are mean ±SEM from 3 biological replicates. *, p<0.05, **, p<0.01, Student’s t test.
Figure S3. eSINE transcripts end in the proximity of canonical RNAPIII terminator sequences, related to Figure 2. Sanger sequencing of the indicated eSINE eRNAs. For each transcript the RNAPIII transcriptional terminator is highlighted.
Figure S4. Functional characterization of eSINEs, related to Figure 3.
(A) Agarose gel image and Sanger sequencing of PCR products corresponding to FosRSINE1 and Gadd45bR1F eSINEs, to demonstrate primer specificity. (-) no template, (+) genomic template. (B,C) Luciferase assay of cortical neurons transfected with the indicated vectors and stimulated with KCl (50 mM, 6 hours). Histograms show mean ±SEM of at least 5 biological replicates. ns, not significant by two-way ANOVA. (D) Luciferase assay of cortical neurons transfected with a vector encoding the luciferase coding sequence under the control of Fos promoter (prom), either alone or with vectors expressing FosRSINE1 (RSINE1) or FosRSINE1 mutated (RSINE1m) only. Histograms show mean ±SEM of luciferase activity for 8 biological replicates. **, p<0.01, ***, p<0.001, ****, p<0.0001, two-way ANOVA. (E) RNA levels of FosRSINE1 in Neuro-2a cells transfected with pBlueScript_FosRSINE1 or pBluescript_FosRSINE1mut, and stimulated with forskolin (Fsk, 50μM for 15 minutes). Histograms show mean ±SEM of 3 biological replicates. *, p<0.05, **, p<0.01, ***, p<0.001, ****, p<0.0001, two-way ANOVA.
Figure S5. CRISPR-Cas9 targeting of FosRSINE1 results in genomic mutations, related to Figure 4.

(A) Following CRISPR-Cas9 targeting of Neuro-2A cells, the genomic region (442 bp) containing the targeting FosRSINE1 region was amplified using high fidelity PCR. Following PCR product denaturation and re-annealing, sites of mismatch were identified by comparing untreated (-) with T7 endonuclease I treated (+) DNA. Sites of cleavage (arrowheads) indicate successful CRISPR-Cas9 targeting of the region. The control is a hybrid product with mismatches that produce cleavage products of 225 and 291 bp. (B) Sanger sequences of wild-type FosRSINE1 and of selected clones mutated by CRISPR-Cas9 technology. Conserved regions are highlighted in yellow. Single base substitutions are shown in purple.
Figure S6. eSINE eRNAs mediate enhancer functions, related to Figure 4.

(A-F) qRT-PCR of cortical neurons treated with either ML60218 (50μM, 15 minutes) or DRB (25μM, 15 minutes) and exposed to KCl for 45 minutes. Histograms show mean fold induction ±SEM over DMSO control for 3 biological replicates. *, p<0.05, **, p<0.01, *** p<0.001, two-way ANOVA. (G,H) Neuro-2a cells were transfected with LNA\textsuperscript{CTR} or LNA\textsuperscript{RSINE1} and either left untreated or treated with forskolin (50μM, 15 minutes). Histograms show fold induction of Fos\textsuperscript{RSINE1} and Dusp1\textsuperscript{MIRRC} eRNA over untransfected control. Data are shown as mean ±SEM for 3 biological replicates. *, p<0.05 **, p<0.01, two-way ANOVA.
Figure S7. Dusp1\textsuperscript{MIRc} eRNA is required for Dusp1 expression in response to stimulation, related to Figure 4.

(A) RNA FISH analysis of Dusp1 mRNA in cortical neurons transfected with GFP and either LNA\textsuperscript{CTR} or LNA\textsuperscript{MIRc} and exposed to KCl for 45 min or left untreated. Shown are maximal projections of confocal scans; reconstructed cell edges are in green, DAPI-counterstained nuclei are in blue, Dusp1 mRNA ribonucleoparticles are in red. (B) Quantitative analysis of RNA FISH experiments. Shown are mean ±SEM of at least 36 cells per condition, from 3 biological replicates. *, p<0.05, ***, p<0.001, two-way ANOVA.
Table S1. Genomic distribution of Gtf3c1, related to Figure 1.

|                              | Control       | Depolarised  |
|------------------------------|---------------|--------------|
| Gtf3c1 peaks                 | 66,662        | 58,090       |
| Extragenic                   | 19,007        | 11,699       |
| On SINEs                     | 2,961         | 1,961        |
| Intragenic                   | 34,012        | 32,385       |
| On SINEs                     | 6,684         | 7,114        |
| ±1 kbp from TSS              | 13,603        | 14,006       |
| On SINEs                     | 1,944         | 2,569        |
| tDNAs                        | 222           | 225          |
| 0.33%                        | 0.39%         |
| 5S rDNAs                     | 21            | 27           |
| 0.03%                        | 0.05%         |
| SINEs                        | 11,589        | 11,644       |
| 17.4%                        | 20.0%         |
| RNAPIII+                     | 9,841         | 3,556        |
| 14.8%                        | 6.1%          |
| RNAPIII- (ETCs)              | 56,821        | 54,534       |
| 85.2%                        | 93.9%         |
SUPPLEMENTAL TABLES

Table S1. Genomic distribution of Gtf3c1, related to Figure 1. The table reports the absolute count and the percentage of Gtf3c1 binding sites, in either control or depolarized neurons, at the indicated genomic regions.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Primary neuronal culture

All experiments performed in this study were approved by the UK Home Office and were performed under the project license 7008314 held by AR. All animal studies were approved by the Institutional Animal Care and Use Committees at University College London.

Cortical neurons were dissected from E15.5 C57BL/6J mouse embryos and dissociated as previously described (Crepaldi et al., 2013). Neurons were cultured on Nunc dishes (Thermo Scientific) or glass coverslips coated with 40µg/ml poly-D-lysine (Sigma) and 2µg/ml Laminin (BD Bioscience) and plated in MEM supplemented with 10% FBS and 5% Horse serum. After 6-24 hours, culture medium was replaced with Neurobasal medium supplemented with B27, 1mM glutamine, 1x penicillin-streptomycin and 10µM 5-Fluoro-2'-deoxyuridine (FdU, Merck). Cells were cultured at 37°C, 5% CO2 and one day before the experiment, 2/3 plating medium was replaced with medium without B27 (serum starve conditions). When indicated, 50µM of the RNAPIII inhibitor ML60218 (Cayman Chemical) or 25µM of the RNAPII inhibitor DRB (Sigma) was added to culture medium 15 minutes prior to KCl stimulation.

Neural progenitor culture

Mouse neural progenitor cells were dissected from E12.5 C57BL/6J mouse embryos and dissociated as previously described (Nitarska et al., 2016). After dissociation, cells were plated on Nunc dishes (Thermo Scientific) coated with 40µg/ml poly-D-lysine (Sigma) and 2 µg/ml Laminin (BD Bioscience) in DMEM/F12 medium supplemented with 1x B27, 1x N2, 1mM glutamine and 1mM NaHCO3. Plating medium was initially supplemented with 10ng/ml
of bFGF (Life technologies). After 2 days in vitro, half of the medium was changed into Neurobasal medium with 1x B27, 1mM glutamine and supplemented with 100ng/ml NT3 (Alomone labs). NT3 was supplemented every 3 days. After 5 days cells were supplemented with 10µM FdU. Cells were maintained in 37°C, 5% CO2 incubators for up to 7 days.

**Neuro-2a cells culture**

Neuro-2a cells were cultured in DMEM supplemented with 10% FBS and 1:100 penicillin-streptomycin.

**LNA inhibition of eSINE transcripts**

Control Antisense LNA GapmeR (LNA<sup>CTR</sup>) or specific Antisense LNA GapmeRs targeting the FosRSINE1 (LNAR<sup>SINE1</sup>) or Dusp1MIRc (LNA<sup>MIRc</sup>) were designed and synthesized by Exiqon. The sequences are shown below:

LNA<sup>CTR</sup> AACACGTCTATACG
LNA<sup>RSINE1</sup> TCTCAAGAACACGAC
LNA<sup>MIRc</sup> TGAGGCCAAATTCTGG

**RNA isolation and reverse transcription**

RNA was isolated from cortical neurons cultured for 6 days using TRIzol (Thermo Scientific) as previously described (Crepaldi et al., 2013). 1–2 µg of total RNA were reversed-transcribed in a 20µl reaction volume containing random hexamer mix and Superscript III reverse transcriptase (50U, Invitrogen) at 50°C for 1h. A no-RT control sample was used as an internal control to exclude genomic contamination. cDNAs were diluted 1:10 in ultra pure water and analyzed by qPCR.

**Cloning**

Luciferase reporter vectors were generated using the pGL3-Basic plasmid (Promega) as the backbone vector. To generate pGL3_fos reporter, an 800bp region upstream of the Fos coding sequence was amplified by PCR from C57BL/6 mouse genomic DNA using primers that carried overhangs with XhoI restriction sites. The resulting DNA sequence was inserted into a StrataClone PCR cloning vector (Agilent Technologies), cut out using XhoI restriction enzyme and subsequently cloned into a pGL3-Basic vector previously linearized by XhoI.
digestion. Ligation was performed using T4 DNA ligase (New England BioLabs) overnight at 16°C. A similar strategy was used to generate pGL3_Arc, pGL3_Gadd45b and pGL3_Gapdh luciferase plasmids. To generate pGL3_fos_RSINE1 sequence was amplified by PCR from mouse genomic DNA and inserted downstream of the reporter gene in the pGL3_fos vector. pGL3_fos plasmid was digested with BamHI restriction enzyme and the linearized vector was blunt ended using T4 DNA Polymerase (New England BioLabs). Ligation of the blunt ended vector with RSINE1 was performed overnight at 16°C using T4 DNA Ligase. The same strategy was used to clone RSINE1 into pGL3_Arc, pGL3_Gadd45b and pGL3_Gapdh luciferase plasmids. pGL3_fos_RSINE1m, pGL3_Arc_RSINE1m, pGL3_Gadd45b_RSINE1m and pGL3_Gapdh_RSINE1m and prom_RSINE1m vectors were generated by site-directed mutagenesis using the corresponding wild type plasmids as PCR template. RSINE1 wild type and RSINE1 mutated sequences were cloned into the cloning site of a pBlueScript vector (Agilent Technologies) to generate pBS_RSINE1 and pBS_RSINE1m, respectively.

**Luciferase reporter assay**

Cortical neurons were cultured in 12 well plates (7x105 cells per well) for 2 days and transfected with 0.8µg of total plasmidic DNA (Firefly and Renilla luciferase reporter plasmids at a 4:1 ratio), 100nM of either LNA^CTR^ or LNA^SINE1^, and 1.6µl Lipofectamine2000 in Optimem medium. One day after transfection, culture medium was replaced with medium containing low serum (0.33X B27) and after 24 hours, neurons were either left untreated or stimulated with 50mM KCl for 6 hours. Firefly and Renilla expression were analyzed using the Dual-Glo® Luciferase Assay System (Promega) according to the manufacturer’s instructions. Reading of the luminescence signals was performed using the Tecan InfiniteF200 plate reading machine.

**Chromatin immunoprecipitation and ChIP sequencing**

1x10^6 (ChIP) or 3x10^7 (ChIP-seq) cortical neurons were cultured in vitro for 6 days and either stimulated with 50 mM KCl for 45 minutes or left untreated. To crosslink proteins with
DNA, the medium was removed from neuronal cultures, and crosslinking buffer (0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA and 25 mM HEPES-KOH, pH 8.0) containing 1% formaldehyde was added for 10 min at room temperature. Cross-linking was stopped by adding glycine to a final concentration of 125 mM. Cells were rinsed three times with ice-cold PBS containing protease inhibitor cocktail and 1mM PMSF, collected by scraping and centrifuged (3000 rpm, 4°C, 5 minutes). Cell pellets were transferred to 1.5 ml tubes and lysed with 20 cell pellet volumes (CPVs) of buffer 1 (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, pH 8.0, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100 and complete protease inhibitor cocktail) for 10 min at 4°C. Nuclei were pelleted by centrifugation (3000 rpm, 10 min, 4°C), incubated with 20 CPVs of buffer 2 (200 mM NaCl, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, 10 mM Tris-HCl, pH 8.0, and complete protease inhibitor cocktail) for 10 min at RT and re-pelleted. 4 CPVs of buffer 3 (1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, 10 mM Tris-HCl, pH 8.0, and complete protease inhibitor cocktail) were added to the nuclei, and sonication was carried out by applying 30 pulses, 30 seconds each, at 30 seconds intervals. Insoluble materials were removed by centrifugation at (14000 rpm, 10 min, 4°C), the supernatant was transferred to a new tube and the final volume of the nuclear lysate was adjusted to 1 mL by adding buffer 3 supplemented with 0.3 M NaCl, 1% Triton X-100 and 0.1% deoxycholate. The lysates were pre-cleared with 70µL of Protein A-Sepharose beads (GE Healthcare, 1 hour, 4°C) and subjected to immunoprecipitation. One tenth of the chromatin was saved for input control, while the remaining fraction was incubated with 3-5µg of antibody and rotated overnight at 4°C. The following primary antibodies were used: rabbit anti-monomethyl-Histone H3 (Lys4) (Millipore 07-436), rabbit anti-Histone H3 (acetyl K27) (Abcam ab4729), rabbit anti- GTF3C1 (Bethyl A301-291A), anti-RNA polymerase III Rpc155 (homemade, Robert White, University of York, York, UK), normal mouse IgG (Santa Cruz sc2025) and normal rabbit IgG (Santa Cruz sc2027). Immune complexes were purified by incubating the lysates with 80µl of Protein A-Sepharose beads for 1 hour at 4°C. Beads were pelleted and washed twice with each of the following buffers: low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 150 mM NaCl), high-salt buffer
(0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 500 mM NaCl) and LiCl buffer (0.25 M LiCl, 1% IGEPAL CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA and 10 mM Tris, pH 8.1). For each wash, the beads were incubated for 10 min at 4 °C while rotating. Beads were rinsed once with 1× TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and the immunoprecipitated material was eluted by vortexing the beads twice for 15 min at RT in elution buffer (0.1 M NaHCO3 pH 8.0, 1% SDS). Crosslinking was reversed by adding 10µl 5M NaCl and incubating the samples at 65°C over night. DNA was purified using PCR purification columns (Qiagen) according to the manufacturer’s instructions and subjected to qPCR. Samples with significant enrichment over negative regions were selected to generate ChIP-seq libraries.

Libraries were prepared for sequencing using NEBNext ChIP-seq Library Prep Master Mix (NEB) or Microplex Library preparation kit v2 (Diagenode) according to the manufacturer’s instructions, and sequenced using Illumina HiSeq platform (GATC Biotech AG, Germany; Eurofins Genomics, UK). For each experimental condition three libraries from different biological replicates were prepared, and 32 to 47 million unique high-quality 100 bp paired-end reads were sequenced. Reads were aligned to the mouse genome (GRCm38/mm10) using Bowtie2 and filtered for alignment quality (MAPQ > 10). MACS2 was employed to identify regions of ChIPseq enrichment.

**Immuno-RNA Fluorescence In Situ Hybridization (RNA FISH)**

Cortical neurons cultured on glass coverslips for 2 days were transfected using Optimem medium and 0.8µl Lipofectamine2000 (Invitrogen). For Fos RNA FISH, 200ng of GFP expression vector was transfected along with 200nM of either LNA^{CTR} or LNAR^{SINE1}. For Dusp1 RNA FISH, 200ng of GFP expression vector was transfected along with 50 nM of LNA^{CTR} or LNA^{MIRc}. Twenty minutes before adding the DNA/Lipofectamine mix, culture medium was removed and replaced with warmed Optimem medium. After 3 hours, transfection medium was replaced with culture medium containing low serum (0.33X B27) and after 24 hours, neurons were stimulated with 50mM KCl for 45 minutes. Immuno-RNA FISH was performed as previously described (Crepaldi et al., 2013). The plasmidic DNA
template encoding Dusp1 coding sequence (IRAVp968E0114D) was obtained from Source Bioscience.

**DNaseI hypersensitivity assay**

12x10^6 neurons were harvested in lysis buffer (0.1% SDS, 0.5% Triton X-100, 20mM Tris- HCl pH 8.1 and 150mM NaCl) and incubated on ice for 15 minutes. Cell pellets were resuspended in 200µl of water and 10X DNasel buffer (Roche) and split between two 1.5 ml tubes. One sample was treated with 3 units of DNasel enzyme (Roche) and incubated for 20 minutes at 25°C while the other was left untreated and incubated for 20 minutes at 25°C. The enzyme was heat-inactivated at 95°C for 20 minutes and proteinase K was added to a concentration of 200µg/ml. Samples were incubated at 50°C overnight and proteinase K was heat-inactivated by incubation at 95°C for 1 hour. DNA was extracted and sensitivity to DNasel digestion was assessed at the desired genomic regions by qPCR.

**In vitro transcription**

10µg of a pBluescript plasmid containing genomic RSINE1 were digested at 50°C for 3h with 2µl of BssHII restriction enzyme and 5µl of SmartCut buffer (New England BioLabs) in a total volume of 50µl. The linearized product was purified and eluted in 40µl of H2O. 5µl of linearized plasmid were incubated over night at 37°C with 0.5mM NTPs, 1µl of RNase OUT enzyme (Invitrogen), 2µl of 10X transcription buffer (Roche) and 20u of either T3 (sense eRNA) or T7 (antisense eRNA) RNA polymerase enzymes (Roche) in a 20µl final volume reaction. The following day the reaction was stopped by adding EDTA to 10mM final and incubation at 37°C for 10 minutes. 1µl of DNasel (Roche) was added to the mix and incubated at 37°C for 1h and at 75°C for 10 minutes. In vitro transcribed RNA was purified using ProbeQuant™ G-50 Micro Columns (GE Healthcare) according to the manufacturer's instructions. In vitro transcription of the radioactive- labeled RSINE1 sense eRNA for EMSA was performed essentially as described above but [α-32P] CTP (800Ci/mmol) was also added to the reaction mix and incubated for 2h at 37°C.

**Electrophoretic mobility shift assay**
**Nuclear protein extraction:** 3.5x10^6 cortical neurons were either left untreated or stimulated with KCl for 15 minutes. Cells were washed once with cold PBS, scraped in cold PBS supplemented with protease inhibitor cocktail (1:100), phosphatase inhibitor cocktail II (1:1000), phosphatase inhibitor cocktail III (1:1000) and 1mM PMSF and transferred into 1.5 ml tubes. Cells were pelleted by centrifugation, resuspended in buffer A (20mM Hepes pH 7.9, 20mM KCl, 3mM MgCl2, 1mM EDTA, 1mM EGTA, 1.2% Igepal CA630) plus inhibitors and incubated on ice for 5 minutes. Nuclei were pelleted by centrifugation at 800g for 10 minutes at 4°C, resuspended in buffer C (40mM Hepes pH 7.9, 840mM KCl, 3mM MgCl2, 1mM EDTA, 20% Glycerol) plus inhibitors and incubated on ice for 30 minutes. The nuclei were centrifuged at 13000rpm for 15 minutes at 4°C and snap froze in liquid nitrogen to facilitate protein release. Nuclear protein concentration was determined using BCA Protein Assay (Thermo Scientific) according to manufacturer's instructions.

**Electrophoresis of RNA-Protein Complexes:** Non-denaturing, 4% polyacrylamide gel was cast in advance and pre-run in 0.5X TBE buffer for 90 minutes at 100V. RNA-protein complexes were prepared by mixing the reagents in the following order: mQ water to 25µl final volume, 5X loading buffer, 1µl of RNase OUT (Invitrogen), 750ng of non-radiolabeled competing nucleic acids, 1.5µg of nuclear protein extracts and 1mg of antibody. The mixtures were incubated at room temperature for 15 minutes, radiolabeled probe was added (2µl, 5x10^4 cpm) and incubation was carried out at room temperature for 15 minutes. The gel was run at room temperature in 0.5X TBE buffer at 200V for 2 hours, placed on a sheet of Whatman® 3MM filter paper, dried at 80°C for 1 hour and exposed to Amersham X-ray film (GE Healthcare Life Science) at -80°C.

**RNA Immunoprecipitation**

100µl of protein A Dynabeads were washed twice with RIP lysis buffer (50mM Tris- HCl, pH 7.4, 100mM NaCl, 1% Igepal CA-630, 0.1% SDS, 0.5% sodium deoxycholate, 1:100 protease inhibitor cocktail), resuspended in 100µl of the same buffer with 5µg of anti RNAPII 8WG16 antibody (Covance MMS-126R) or control mouse IgG (Santa Cruz sc2025) and rotated for 1 hour at room temperature. The beads were washed three times with RIP lysis
buffer and left in the last wash until ready to proceed. Cortical neurons (5x10^6) were stimulated with KCl for 15 minutes or left untreated, scraped in cold PBS, transferred to 1.5 ml tubes and centrifuged at 514g for 1 minute at 4°C. Cell pellets were resuspended in 1ml of RIP lysis buffer and sonicated using a Bioruptor (low amplitude, 5x30 sec on and 30 sec off). After sonication, 1ml of T20 IP buffer (50mM Tris-HCl, pH 7.4, 150 nM NaCl, 0.5% Tween 20, 0.1 mM EDTA, 10µl of protease inhibitors, 1µl of anti-RNase) was added to dilute the lysis buffer. The lysates were centrifuged at 21800g for 20 minutes at 4°C and supernatants were collected into new tubes. 50µl of lysate from each tube was saved for total RNA extraction and mixed with 190µl of elution buffer (100mM Tris-HCl, pH 7.4, 10mM EDTA, 1% SDS, 200mM NaCl). Remaining lysates were mixed with agarose beads and rotated for 1 hour at 4°C. Beads were washed once with high salt buffer (50mM Tris-HCl, pH 7.4, 1M NaCl, 1mM EDTA, 1% Igepal CA-630, 0.1% SDS, 0.5% sodium deoxycholate) and twice with PNK buffer (20mM Tris-HCl, pH 7.4, 10mM MgCl2, 0.2% Tween-20) and resuspended in 240µl of elution buffer. Cross-linking was reversed by adding 10µl of Proteinase K at 42°C for 1 hour and at 70°C for 45 minutes. Beads were centrifuged and eluted samples were brought to a volume of 500µl by adding RNase-free water. RNA was purified using the PureLink RNA Micro Scale Kit (Thermo Scientific) according to the manufacturer’s instructions. RNA was eluted in 15µl of RNase-free water. DNA digestion was performed using the Ambion Turbo DNA-free Kit (14µl RNA, 1.7µl 10xTurbo buffer, 1.5µl Turbo DNase) at 37°C for 30 minutes. The reaction was blocked by adding 2µl of Inactivating Reagent at room temperature for 5 minutes. After centrifugation for 2 minutes at 10,000g at room temperature, the supernatants were collected and residual genomic DNA was assessed by PCR. RNA were reverse-transcribed in a 20µl reaction volume containing random hexamer mix and Superscript III reverse transcriptase (50U, Thermo Scientific) at 50°C for 1h. cDNAs were diluted 1:2 in ultra pure water and analyzed by qPCR.

**Dendritogenesis assay**

Assays were carried out as described in Crepaldi et al., 2013. Briefly, 2-3 hours after plating, mouse cortical neurons were transfected using Optimem containing 200ng of GFP
expression vector, 200nM of LNA (LNA$^{CTR}$ or LNA$^{RSINE1}$) and 0.8µl Lipofectamine2000 (Thermo Scientific). After 3 hours, the medium was replaced with culture media containing 0.33X B27 (serum starve conditions) with or without 50 mM KCl. Cells were cultured for 48 hours followed by immunostaining with anti-GFP (Abcam ab13970, 1:2000). Images of GFP-transfected non-overlapping neurons were obtained using a Zeiss Axio Imager microscope and analyzed in Fiji. For quantification of total dendritic length and Sholl analysis we used the Simple Neurite tracer plugin.

**Tissue preparation and immunostaining**

Embryonic brains were fixed using 4% paraformaldehyde (PFA) in PBS overnight at 4oC. Fixed samples were cryoprotected using 30% sucrose overnight at 4oC. Brains were frozen in Optimal Cutting Temperature (O.C.T, Sakura) and 10µm coronal sections were cut using a Leica cryostat. Tissue sections were permeabilized using 0.3% Triton X100 and 10% normal goat serum in PBS at room temperature for 1h and incubated with primary antibodies overnight at 4oC. The following primary antibodies were used: rabbit anti-Ki67 (Abcam ab16667, 1:200), chicken anti-GFP (Abcam ab13970, 1:2000), mouse anti-Sox2 (Abcam ab79351, 1:500) and rabbit anti-Tbr1 (Abcam ab31940, 1:400). After three sequential washes with PBS, sections were incubated with Alexa Fluor conjugated secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI) for 90 min at RT. Sections were washed with PBS and mounted using Fluoromount-G (SouthernBiotechnology).

**Statistical analysis**

Data are presented as average and ± SEM. Statistics for multiple comparisons was performed using either t test or one- or two-way Anova as indicated in the figure legends. All analysis was performed using GraphPad Prism version 6.0 for Macintosh (GraphPad Software, San Diego, CA). * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 for all statistical analysis.