Essential roles of the histone methyltransferase ESET in the epigenetic control of neural progenitor cells during development

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SUMMARY

In the developing brain, neural progenitor cells switch differentiation competency by changing gene expression profiles that are governed partly by epigenetic control, such as histone modification, although the precise mechanism is unknown. Here we found that ESET (Setdb1), a histone H3 Lys9 (H3K9) methyltransferase, is highly expressed at early stages of mouse brain development but downregulated over time, and that ablation of ESET leads to decreased H3K9 trimethylation and the misregulation of genes, resulting in severe brain defects and early lethality. In the mutant brain, endogenous retrotransposons were derepressed and non-neural gene expression was activated. Furthermore, early neurogenesis was severely impaired, whereas astrocyte formation was enhanced. We conclude that there is an epigenetic role of ESET in the temporal and tissue-specific gene expression that results in proper control of brain development.

KEY WORDS: ESET, Astrocyte, Epigenetic control, Histone methyltransferase, Neural progenitor

INTRODUCTION

During cortical development, neural progenitor cells (NPCs) give rise to various types of neurons – initially deep layer neurons and then upper layer neurons – and finally differentiate into astrocytes via changes in differentiation competency (Alvarez-Buylla et al., 2001; Fishell and Kriegstein, 2003; Fujita, 2003; Götz and Huttner, 2005; Miller and Gauthier, 2007; Kageyama et al., 2007). These changes in competency are highly ordered and unidirectional (Frantz and McConnell, 1996), suggesting that a ‘clock’ is integrated into the differentiation system of NPCs to ensure that the cell composition and final size of the brain are characteristically similar among members of a given species. However, the precise mechanism by which the differentiation competency of NPCs switches at the proper time remains largely unknown.

It has been shown that NPCs switch differentiation competency by changing gene expression profiles that are regulated by transcription factors. For example, the zinc-finger transcription factors Fez1f2 and Ctip2 (Bcl11b – Mouse Genome Informatics) and the basic helix-loop-helix (bHLH) transcription factors neurogenin 1 (Ngn1, Neurog1) and Ngn2 regulate the generation of deep layer neurons (Arlotta et al., 2005; Molyneaux et al., 2005; Chen, B. et al., 2005; Chen, J. G. et al., 2005; Chen et al., 2008), whereas the homeodomain transcription factor Pax6 is involved in the differentiation of upper layer neurons (Fode et al., 2000; Schuurmans et al., 2004). By contrast, the orphan nuclear receptors COUP-TFI and COUP-TFI (Nr2f1 and Nr2f2 – Mouse Genome Informatics) induce astrocytic differentiation (Naka et al., 2008). Recent reports suggest that chromatin remodeling and epigenetic regulation also play important roles in controlling cell differentiation (Seuntjens et al., 2009; Strobl-Mazulla et al., 2010). For example, neuron-specific chromatin remodeling complexes such as BAF53 regulate dendrite development (Lessard et al., 2007; Yoo et al., 2009), while polycomb complexes such as Polycomb repressive complex (PRC) 1 and 2, which regulate histone modification, facilitate the switching from neurogenesis to astrogensis by repressing the proneural gene Ngn1 (Hirabayashi et al., 2009). Histone modifications provide fast and reliable regulation of gene expression, but their significance in neural development has not been fully investigated.

The histone H3 lysine 9 (H3K9) methyltransferase ESET (also known as Setdb1 or KMT1E) represses gene expression in euchromatin by interacting with the co-repressor KAP1 (TrIM28) (Schultz et al., 2002). Loss of ESET accelerates differentiation of embryonic stem (ES) cells toward the trophectoderm lineage by derepressing the emergence of proviral particles in ES cells (Matsui et al., 2010; Rowe et al., 2010; Karimi et al., 2011). There are ~1000 copies of the endogenous retroviral-like sequences known as the intracisternal A-particle (IAP) and their expression is partly repressed by ESET in ES cells (Matsui et al., 2010; Rowe et al., 2010; Karimi et al., 2011). This regulatory mechanism is of particular importance because upregulation of IAP expression has been associated with retrotransposition and increased susceptibility to cancer (Heidmann and Heidmann, 1991; Howard et al., 2008). Because ESET ablation leads to embryonic lethality before implantation (Dodge et al., 2004), the functions of ESET in the development of postimplantation embryos, such as neural development, have not been examined. To circumvent this problem, we investigated the role of ESET in brain development using ESET conditional knockout (cKO) mice.
MATERIALS AND METHODS

Mouse lines
ESET flox (f) (Matsui et al., 2010), Emx2-Cre (Kimura et al., 2005) and Nes-Cre (Isaka et al., 1999) mice were previously described. Emx2-Cre;ESET(f/+) or Nes-Cre;ESET(f/+) male mice were crossed with ESET(f/f) female mice, and the day when a vaginal plug was observed was scored as embryonic day (E) 0.5.

In situ hybridization, immunohistochemistry and antibodies
Embryos were dissected, fixed in 4% paraformaldehyde (PFA)/PBS overnight and cryoprotected with 20% sucrose for at least 10 hours. On the following day, embryos were embedded in OCT compound (Tissue-Tek) and frozen at −80°C. Fixed sections were sectioned at 16 μm with a cryostat and dried for 2 hours. In situ hybridization was then performed as previously described (Imayoshi et al., 2008). Primers used to produce in situ hybridization probes are listed in supplementary material Table S1. For immunohistochemistry, embryos at different ages were fixed in 4% PFA for 2 hours, equilibrated and sectioned as described above. Sections were blocked with 5% normal goat serum or donkey serum in 0.1% Triton X-100/PBS and incubated with primary antibodies at 4°C overnight. The following primary antibodies were used: BrdU (1:100, BD Biosciences), Brn1 (Pou5f1) (1:100, Santa Cruz), Brn2 (Pou5f2) (1:250, Santa Cruz), cleaved Casp3 (1:500, Cell Signalling), Ctip2 (1:500, Abcam), Cux1 (1:100, Santa Cruz), ESET (1:1000, Cell Applications), Foxp2 (1:100, 1:100, Santa Cruz), Gfap (1:1000, Sigma), IAP (1:500, a gift from Dr Bryan R. Cullen, Duke University, Durham, USA), Ki67 (1:100, BD Pharmingen), nestin (1:500, BD Pharmingen), Pax6 (1:500, Abcam), Sox9 (1:500, a gift from Dr Michael Wegner, Friedrich-Alexander Universität Erlangen-Nürnberg, Germany), Tbr1 (1:500, Millipore), Tbr2 (Eomes) (1:500, Abcam) and Tuj1 (Tubb3) (1:500, Babco). The following secondary antibodies were used: Alexa 488-conjugated mouse, rabbit, rat or goat and Alexa 594-conjugated mouse, rabbit or rat (1:250, Molecular Probes). Immunofluorescence images were obtained with a Zeiss Axioptl confocal microscope.

Acquisition and analysis of microarray data
The dorsal telencephalon of E14.5 wild-type (WT) and Emx2-Cre;ESET(f/) (ESET cKO) mouse embryos were subjected to RNA extraction and expression profiling using Affymetrix GeneChip Mouse Genome Array (n=3) as previously described (Shimoto et al., 2008). Analysis of microarray data was performed using GeneSpring GX version 11. Microarray data are available at Gene Expression Omnibus under the following accession numbers: GSM990998, GSM990999, GSM991000, GSM991001, GSM991002 and GSM991003 (for telencephalon WT 1-3 and cKO 1-3, respectively).

RNA preparation and quantitative (q) RT-PCR
RNA was extracted from the dorsal telencephalon at different stages. Tissues were dissociated with trypsin and lysed with Trizol (Invitrogen); RNA was then extracted with phenol-chloroform and precipitated according to the manufacturer’s protocol. RNA samples were subjected to reverse transcription as previously described (Shimoto et al., 2008). qRT-PCR was performed with Thunderbird SYBR Green PCR Mix (TOYOBO) using the primers listed in supplementary material Table S1.

RNA deep sequencing
Total RNA was isolated from E14.5 WT and ESET cKO embryonic telencephalon (male), and an RNA integrity number (RIN) was confirmed to be greater than 8.0 using a 2100 Bioanalyzer (Agilent Technologies). Total RNA (1 μg) was used for generation of poly(A)+ RNA Seq libraries. The sequencing libraries were prepared using the Illumina TrueSeq RNA Sample Preparation Kit following the manufacturer’s protocol with the slight modification that fragmentation time was reduced to 2 minutes. After amplification for 15 cycles, PCR products were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter), and their size distribution and concentration were evaluated using a DNA 1000 Series II Assay on the 2100 Bioanalyzer. cDNA at 4.2 pmol/lane was applied to the flow cell and paired-end 75 nt reads were sequenced on an Illumina GAIIx following the manufacturer’s instructions (Wang et al., 2011). RNA sequencing results were presented using Integrative Genomics Viewer (Broad Institute) version 2.0.

Bromodeoxyuridine (Brdu) labeling
Brdu (2.5 mg; 5 mg/ml) was given to each pregnant mouse by intraperitoneal injection every 4 hours for a total of 12 hours at the indicated embryonic stage. Brains were fixed with 4% PFA, and sections were incubated in 2 M HCl for 30 minutes at 37°C, followed by neutralization in 0.1 M sodium tetraborate buffer for 10 minutes. Sections were incubated with mouse anti-Brdu antibody overnight at 4°C and then with Alexa 488-conjugated goat anti-mouse IgG.

Cell death assay
TUNEL assays were performed with the ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit (Chemicon International) according to the manufacturer’s protocol.

Neurosphere assay
Neurosphere assays were performed as described previously (Ohtsuka et al., 2011). The numbers of primary spheres were counted at day 7. Experiments were repeated three times and the mean and s.e.m. of sphere numbers were calculated. For the neurosphere differentiation assay, primary spheres were cultured for 1 day and plated onto PLL- and laminin-coated Lab-Tek chamber slides (Nalg Nunc) in differentiation medium (DMEM/F-12 supplemented with B-27 and 2% fetal bovine serum). Cells were examined immunocytochemically 1, 3 and 5 days after plating.

In utero electroporation
In utero electroporation was performed with E17.5 ICR pregnant mice as described previously (Ohtsuka et al., 2011). Neonates at postnatal day (P) 2 were harvested and analyzed, as described above.

Quantification and statistics
The total numbers of marker-positive cells were counted in more than five sections from at least three different animals per group. The ventricular zone, subventricular zone and cortical plate were defined according to Tbr2 staining. The percentage of cells from each compartment was calculated. Statistical differences were examined with Student’s t-test and P<0.05 was considered significant.

Chromatin immunoprecipitation (ChIP)
ChIP assays were performed as described previously (Matsui et al., 2010) using the primers and antibodies listed in supplementary material Table S1.

Bisulfite sequencing
Bisulfite sequencing was conducted using the EpiTect Plus DNA Bisulfite Kit (Qiagen). PCR products were cloned and individual inserts were sequenced using BigDye v3.1 chemistry. Sequencing data were analyzed using QUMA (RIKEN). Primer sequences are listed in supplementary material Table S1.

RESULTS

ESET is expressed by NPCs in the developing brain
It has been shown that ESET is expressed in ES cells and the postnatal brain (Ryu et al., 2006; Matsui et al., 2010; Jiang et al., 2010), but expression in the developing brain has not been examined extensively. We therefore performed in situ hybridization and immunohistochemical analyses with mouse embryos at different developmental stages. ESET was expressed in the telencephalon, olfactory vesicles, forelimbs and hindlimbs at E9.5 (Fig. 1A). At this stage, ESET was highly expressed in the ventricular zone where NPCs reside (Fig. 1B). ESET mRNA expression occurred in cells expressing Ki67, a marker for cell proliferation, but was excluded from Tuj1+ neuronal layers (Fig. 1B). At E11.5, ESET expression was found in similar regions (Fig. 1A,B). All radial glia/apical progenitors (Pax6+) and some basal progenitors (Tbr2+) co-expressed ESET (Fig. 1D, Ea,b), but neurons (Tuj1+) did not (Fig. 1Ec). ESET mRNA expression in the ventricular zone was considered significant.
further decreased at E15.5 (Fig. 1B) and was hardly detectable at E17.5 (Fig. 1B). qRT-PCR also showed that ESET mRNA expression in purified NPCs decreased as development proceeded (Fig. 1C). Similarly, ESET protein expression also decreased as development proceeded (supplementary material Fig. S1). From this we infer that ESET is expressed by NPCs (both apical and basal progenitors), but that the expression decreases over time and is very low by E17.5, when the transition from neurogenesis to astro genesis in the dorsal telencephalon occurs.

**ESET is required for proper regulation of neural and non-neuronal gene expression**

To determine whether ESET plays a role in cortical development, we crossed ESET flox mice with Emx2-Cre driver mice to generate forebrain-specific ESET cKO mice. By E11.5, ESET expression was mostly abolished in the dorsal telencephalon, but was not affected in the midbrain of the cKO mice, where the Emx2 promoter was inactive (supplementary material Fig. S2A). These mutant mice were born but did not survive beyond P10. We also generated ESET cKO mice using Nes-Cre mice, in which Cre is active in NPCs, and obtained virtually the same results (see below).

Because ESET catalyzes the trimethylation of H3K9 (H3K9me3), a modification that potentially alters gene expression, we examined which genes were misregulated in the absence of ESET by microarray expression profiling of the E14.5 dorsal telencephalon. In the ESET cKO brain, 185 genes were upregulated more than 2-fold relative to the WT brain (supplementary material Table S2), whereas 112 genes were downregulated to less than half the level of the WT (supplementary material Table S3). The misregulated genes were categorized into three groups by GeneSprint software clustering analysis (Fig. 2A). Cluster 1 included downregulated genes, whereas clusters 2 and 3 included upregulated genes; many of them, particularly the downregulated genes, might be indirectly regulated by ESET, which functions as a transcriptional repressor.

Each cluster was subjected to Gene Ontology (GO) analysis with a P<0.05 cut-off. Cluster 1 GO analysis revealed enrichment for neurogenesis, neuronal differentiation and signal transmission (P-values of 0.014, 0.014 and 0.031, respectively; Fig. 2B). Genes in these categories included Reln, Etv1, Nr4a2, Emx2, Satb2, Rorb, Neurog1, Ntrk2 and other important genes required for the proper regulation of neuronal differentiation, maturation, as well as the maintenance of neuronal functions. For some of these genes, the changes in expression were validated by qRT-PCR of E14.5 and E16.5 cortical samples, and similar results were obtained at both stages (supplementary material Fig. S2B,C).

Interestingly, the GO terms of cluster 1 overlapped substantially with those of downregulated genes in ESET-null ES cells (Karimi et al., 2011), suggesting that ESET could be commonly involved in neuronal differentiation in these cells. Cluster 2 included genes involved in ossification, such as Sox9 (P=0.015; Fig. 2C). Sox9 is known to regulate gliogenesis in the brain (Stolt et al., 2003), and other glia-related genes such as Tspo and Ppp1r3c were also included in cluster 2 (supplementary material Table S2). These results suggest that glial differentiation was activated whereas neuronal differentiation was impaired in the absence of ESET. Cluster 3 contained strongly upregulated genes and revealed enrichment for spermatogenesis (P=0.0044), M phase of the meiotic cell cycle (P=0.00041), and chromosome organization involved in meiosis (P=0.00039) (Fig. 2D). This cluster included IAP, Mnd1, Tcfl5, Rec8, Rnf17, Sycp3 and Mael. These genes are strictly expressed by germ cells in a narrow time frame when meiosis or homologous recombination takes place. In the WT brain, many of these genes must be silenced so as to prevent genomic instability. For instance, IAP belongs to the endogenous retroviruses (ERVs), which could potentially bring about genetic disorders by retrotransposition (Mietz et al., 1987; Qin et al., 2010), whereas Mnd1, Rec8 and Sycp3 are the major players in facilitating proper crossing over during homologous recombination in meiosis.
Upregulated expression of cluster 2 and cluster 3 genes in the cKO brain was also validated by qRT-PCR (Fig. 3A; supplementary material Fig. S2D) and western blot analyses (Fig. 3B). Upregulation of these genes, such as IAP, was also observed in Nes-Cre-driven ESET cKO mice (supplementary material Fig. S3A). The GO terms of clusters 2 and 3 mostly differed from those of the upregulated genes in ESET-null ES cells, which included plasma membrane, vitamin binding and regulation of cell death (Karimi et al., 2011). In the ESET cKO brain, some genes, such as IAP and Mnd1, were ectopically expressed by neurons, whereas others, such as Sox9, were expressed by NPCs and some migrating cells (Fig. 3C, arrow), suggesting that the onset of ectopic expression differs from gene to gene. Together, these results indicated that ESET is required for the proper expression of neuronal genes and for the suppression of non-neuronal genes (Fig. 2E).

Derepression of ERVs is responsible for the upregulation of many genes in the ESET cKO brain

Genome database analysis indicated that many of the upregulated genes are located near ERVs such as IAP sequences, suggesting that activation of the long terminal repeat (LTR) of ERVs leads to ectopic upregulation of neighboring genes in the absence of ESET. Indeed, one of the most highly upregulated genes in cluster 3, Mnd1 (Fig. 3A,C), has an IAP sequence in the first intron. PCR and sequence analysis showed that the major Mnd1 transcript started from the intronic IAP in the absence of ESET (Fig. 3D,E; supplementary material Fig. S3D,E). RNA deep sequencing analyses revealed that, among the upregulated genes, six were chimeric transcripts with IAPs (supplementary material Table S4) and three (Mnd1, Slec20a2 and Sec14l4) were verified as fusions with IAP by PCR and sequencing analyses (Fig. 3D,E; supplementary material Fig. S3D,E). Among these six chimeric transcripts with IAPs, three (Adk, Gramd1c and Slec20a2) were shown to be astrocyte-enriched genes (Cahoy et al., 2008). RNA deep sequencing additionally revealed the IAP-Capn11 chimeric transcript, which was also verified by PCR and sequencing analyses (supplementary material Fig. S3F). Other derepressed ERVs, such as MMERVK, also formed five upregulated chimeric transcripts (supplementary material Table S4). Therefore, among the 185 upregulated genes, a total of 11 were expressed as chimeric transcripts with ERVs in the ESET cKO brain (supplementary material Table S4). In addition, 60 other upregulated genes were found to have ERVs within 10 kb of the transcription initiation site (supplementary material Table S4). Because deep sequencing analyses detected enhanced transcription over 10 kb regions from some activated ERVs in the absence of ESET (data not shown), these results raised the possibility that as many as 71 of the upregulated genes could be influenced by nearby ERVs, representing ~38% of the upregulated genes in the ESET cKO brain. This possibility remains to be analyzed, and the actual number of genes activated by derepressed ERVs could be smaller.

ESET maintains global and localized H3K9me3

Because ESET is an H3K9 methyltransferase, we next examined the H3K9me3 level in the ESET cKO brain. In the absence of ESET (Fig. 4A), the global H3K9me3 mark in the chromatin was slightly reduced at E14.5 and E18.5 (Fig. 4B; supplementary
material Fig. S4A), whereas the H3K9me2 mark was unaffected (Fig. 4C; supplementary material Fig. S4A). Because the microarray data suggested that there was no overwhelming global upregulation of gene expression, it is likely that the role of ESET in H3K9me3 regulation could be, to a certain extent, specific or localized. Hence, we investigated whether ESET binds directly to, or if so whether it modifies the H3K9me3 status of, the chromatin localized. Therefore, we examined whether ESET binds directly to, and if so whether it modifies the H3K9me3 status of, the chromatin localized. Hence, we investigated whether ESET binds directly to, and if so whether it modifies the H3K9me3 status of, the chromatin localized. Moreover, we examined whether ESET binds directly to, and if so whether it modifies the H3K9me3 status of, the chromatin localized.

Fig. 3. Misregulated gene expression in the ESET cKO brain. (A) qRT-PCR analysis of misregulated gene expression in the WT and ESET cKO mouse brain at E14.5 (n=6). Values were normalized to that of Gapdh, and a ratio relative to the WT level ± s.e.m. was calculated. ***P<0.001, t-test. (B) Expression of intracisternal A-particle (IAP), Sox9 and Tbr2 protein was examined in WT, heterozygote (Het) and ESET cKO by western blot with E14.5 whole-cell lysate from the dorsal telencephalon. In each case, tubulin provides a loading control. (C) Expression of IAP gag protein, Mnd1 mRNA and Sox9 mRNA was examined in coronal sections from both WT and cKO brains. IAP and Mnd1 were expressed by neurons of the ESET cKO brain, whereas Sox9 mRNA was expressed by both NPCs and migrating neurons (arrow). (D) RNA deep sequencing revealed emergence of IAP- Mnd1 chimeric transcripts in the ESET-ablated cortex. Red bar indicates the position of IAP. (E) Conventional PCR was performed with IAP- and Mnd1-specific primers using E14.5 cortical cDNA. PCR products were detectable in the ESET cKO samples but not in the WT. Scale bars: 100 μm.

Ablation of ESET impairs neurogenesis
Because many neuronal and non-neuronal genes were misregulated, we examined the effects of ESET loss on neural development. At E11.5, the number of radial glia/apical progenitors (Pax6+) was not significantly affected, whereas basal progenitors (Tbr2+*) were significantly reduced (supplementary material Fig. S5A). At E12.5, Ctip2+ early-born layer V and VI neurons and Tbr2+ basal progenitors were markedly reduced in the ESET cKO cortex compared with the WT (87.4% in the cKO versus 99% in the WT; Fig. 4G; supplementary material Fig. S4D,E). Thus, in addition to ESET loss, demethylation of CpG might be required for activation of IAP, suggesting that this additional process might contribute to delayed onset of IAP expression in neurons.

Sox9 regulates the differentiation of many cell types, including osteoblasts and glial cells (Stoll et al., 2003; Liu et al., 2007), and it is normally expressed in the developing brain, but was significantly upregulated in the absence of ESET (Fig. 3A-C). As described above, IAP element expression was mostly negative in the WT brain but was derepressed in the absence of ESET (Fig. 3A-C). ChIP assay of E14.5 cortical samples showed that ESET bound to the promoters of Sox9, IAP and other genes (Fig. 4D; supplementary material Fig. S4B). In addition, upon ESET depletion, the H3K9me3 level at these loci was significantly decreased (Fig. 4E; supplementary material Fig. S4C), whereas the H3K9ac level was enhanced (supplementary material Fig. S4C). These results indicate that ESET directly binds to these genes and represses their expression via elevated H3K9me3 modification.

Although there are ~1000 copies of IAP sequences in the mouse genome, only six genes were activated to form chimeric transcripts with IAPs (supplementary material Table S4), suggesting that derepression of IAPs is not global but localized. It was previously shown that CpG sequences in the IAP LTR regions are highly methylated, and that demethylation of CpG sequences by inactivation of DNA methyltransferase 1 (Dnmt1) leads to robust IAP expression (Walsh et al., 1998). Therefore, we examined whether the DNA methylation status of the IAP LTR was affected in the ESET cKO brain. Bisulfite sequencing analysis revealed that although global DNA demethylation was not observed in the global IAP LTR sequences (94% in the cKO versus 92% in the WT; Fig. 4F), CpG sequences of the IAP in Mnd1 and other derepressed genes were partially demethylated in the ESET cKO brain compared with the WT (87.4% in the cKO versus 99% in the WT; Fig. 4G). Conventional PCR was performed with IAP- and Mnd1-specific primers using E14.5 cortical cDNA. PCR products were detectable in the ESET cKO samples but not in the WT. Scale bars: 100 μm.
that the population of upper layer neurons might expand at the expense of deep layer neurons in the ESET cKO brain.

In order to determine whether late-born upper layer neurons differentiate prematurely, we performed a BrdU birthdating assay at E13.5 and E16.5 and analyzed BrdU co-labeling with various layer-specific markers at E18.5. The percentage of Cux1+ upper layer neurons born at E13.5 was significantly increased whereas that of Tbr1+ and FoxP2 + deep layer neurons was significantly decreased in the cKO (Fig. 5J), revealing that the production of late-born upper layer neurons was enhanced whereas that of early-born deep layer neurons was prematurely decelerated (Fig. 5J). Approximately 20-30% of Cux1+ and Brn2+ upper layer neurons were still generated at E16.5 in the WT, whereas only 12-15% of such neurons were born at the same stage in the cKO (Fig. 5K). These results suggest that the production of upper layer neurons was accelerated at E13.5 at the expense of deep layer neurons but prematurely decelerated at E16.5.

At E18.5, GABAergic neurons, which are derived from the medial ganglionic eminence, tangentially migrated to the dorsal telencephalon. However, this migration was somewhat reduced in the ESET cKO brain compared with the WT (supplementary material Fig. S6A), a defect that could be due to a lack of proper signals from excitatory neurons (Lodato et al., 2011).

The ESET cKO cortex was smaller than that of the WT at E18.5 (Fig. 5C) and P7 (supplementary material Fig. S6B). At P7, by which time neurogenesis and migration were completed, the number of Ctip2+ layer V and VI neurons was severely reduced in the ESET cKO cortex compared with the control, whereas Brn1+ layer II-III neurons (supplementary material Fig. S6Ca-d) and Cux2+ layer II-IV neurons were not significantly affected, and Id2 expression was slightly enhanced in the ESET cKO (supplementary material Fig. S6Ce-h). These results showed that production of deep layer neurons was most severely impaired, whereas upper layer neurons were only modestly affected in the absence of ESET.

ESET promotes neuronal survival and proliferation
To further investigate the reason for the smaller cortex of ESET cKO mice (supplementary material Fig. S6B) and the severe loss of deep layer neurons in the ESET cKO cortex (supplementary material Fig. S6C), we examined whether increased apoptosis, reduced proliferation, or a combination of both could be involved. Apoptotic cells, as indicated by TUNEL and cleaved caspase 3 staining, significantly increased in number from E13.5 in the cKO brain (Fig. 6A,B). Furthermore, these apoptotic cells were mostly located in layers V-VI (Fig. 6B, graph), and many of them co-expressed the deep layer neuronal marker Ctip2 (Fig. 6B, enlargements), suggesting that apoptosis preferentially occurred in deep layer neurons.

Expression of the basal progenitor marker Tbr2, which is required for the production of a sufficient number of neurons (Sessa et al., 2008), was reduced in the ESET cKO brain (Fig. 5A,D,F; supplementary material Fig. S5A) and, in agreement with this observation, the number of dividing cells [phosphohistone H3 (PH3)+] was reduced in the subventricular zone of the mutant brain, where basal progenitors reside (Fig. 6C,D). By contrast, expression
of the radial glial marker Pax6 was not noticeably changed (supplementary material Fig. S5A) and the number of dividing cells in the ventricular zone was not significantly affected in the ESET cKO brain (Fig. 6C,D). Primary neurosphere culture assays with NPCs extracted from E14.5 WT and ESET cKO dorsal telencephalon showed that mutant NPCs were less likely to develop into neurospheres of greater than 50 μm diameter than WT NPCs within 7 days of culture (Fig. 6E,F), indicating that the proliferation potential of NPCs was compromised in the absence of ESET.

Together, these results suggest that the decrease in basal progenitors from early stages and the increase in apoptosis contribute to the size reduction of the ESET cKO cortex, with particular effect on the deep layer populations.

**Astrocyte formation is accelerated in the absence of ESET**

Our BrdU birthdating and Tbr2 immunostaining analyses showed that the neurogenic phase seemed to have ended prematurely in the ESET cKO brain, and we therefore examined whether the timing of astrocyte formation was affected. We performed immunostaining of the astrocyte marker Gfap at E18.5 (Fig. 7A). The enhanced expression of Gfap continued at later stages (Fig. 7A). Upregulation of Gfap also occurred in the ESET cKO driven by Nes-Cre (supplementary material Fig. S7A). Furthermore, expression of the astrocyte markers Gfap, Aldh1l1, Aqp4 and Slc14a1 (Cahoy et al., 2008) was

**Fig. 5. ESET deficiency impairs neural development.**

Immunohistochemistry (Ctip2, Tbr1, Tbr2, Cux1, Satb2 and FoxP2) or in situ hybridization (Fezf2 and Nurr1) on sections of the telencephalon of WT and ESET cKO mice. (A, C) At E12.5 (A) and E18.5 (C), the numbers of Ctip2+ and Tbr2+ cells were decreased in the ESET cKO brain compared with the WT. (D, F) At E14.5 (D) and E16.5 (F), the numbers of Ctip2+, Tbr1+ and Tbr2+ cells were decreased in the ESET cKO brain compared with the WT. (H) At E18.5, the numbers of Cux1+ and Satb2+ upper layer neurons were increased in the ESET cKO brain, whereas Ctip2+, Fezf2+, Tbr1+, FoxP2+ and Nurr1+ deep layer neurons were decreased in the ESET cKO brain compared with the WT. (B, E, G, I) Quantification of neuronal number with s.e.m. at E12.5 (B), E14.5 (E), E16.5 (G) and E18.5 (I). n=3 per group. **P<0.01, ***P<0.001, t-test. (J, K) Pulse-chase experiments with BrdU administered at E13.5 (J) or E16.5 (K) (n=3 for both) followed by analysis at E18.5. Quantification shows the percentage with s.e.m. of cells that were double labeled with BrdU and layer-specific markers. At E13.5, a higher proportion of Cux1+ neurons incorporated BrdU, whereas lower proportions of Tbr1+ and FoxP2+ neurons incorporated BrdU. At E16.5, lower proportions of Cux1+ and Brn2+ neurons incorporated BrdU. *P<0.05, **P<0.001, t-test. Scale bars: 100 μm in A, D, F; 1 mm in C; 50 μm in H.
increased at P7 in the ESET cKO cortex (supplementary material Fig. S7B). These results indicated that astrocyte differentiation was accelerated and enhanced in the absence of ESET.

It is possible that increased neuronal death induced astrogliosis in the ESET cKO brain. To rule out this possibility, we performed Gfap and TUNEL double staining in P4 brains. Although the total number of TUNEL+ cells increased in the cKO, the majority of Gfap+ cells were present in regions where there were no apoptotic cells (Fig. 7B), suggesting that enhanced Gfap expression was not due to cell death-induced gliosis. Furthermore, higher magnification images revealed that these Gfap+ cells exhibited typical astrocyte morphology (Fig. 7B, lower panels). In addition, we performed an in vitro differentiation assay using NPCs prepared from E14.5 WT and cKO dorsal telencephalon to investigate the gliogenic potential of these progenitors. Indeed, NPCs from the ESET cKO cortex more readily differentiated into Gfap+ cells than the WT NPCs, although the frequency of cell death did not differ between ESET cKO and WT NPCs (Fig. 7C; supplementary material Fig. S7C). The percentage of Gfap+ colonies was also significantly increased in the absence of ESET on the third day upon differentiation (Fig. 7D). By contrast, overexpression of ESET by in utero electroporation decreased astrocyte differentiation (supplementary material Fig. S7D), although overexpression of ESET alone was not able to block astrocyte differentiation completely (supplementary material Fig. S7D). These results suggest that ESET represses the premature onset of astrocyte formation in the developing cortex.

To determine whether the Gfap gene is a direct target of ESET, we performed ChIP assays on the Gfap promoter. ChIP assay with E14.5 cortical samples showed that ESET directly binds to the Gfap promoter (Fig. 7E,F). In addition, upon ESET depletion, the H3K9me3 level at this promoter was significantly decreased (Fig. 7G), whereas the H3K9ac level was enhanced (Fig. 7H). These results indicated that ESET directly binds to the Gfap promoter and represses its expression via elevated H3K9me3 marks.

DISCUSSION

Epigenetic regulation is necessary for the establishment and maintenance of heritable gene expression during development, but very little is known about the significance of histone modifications such as H3K9me3 in neural development. Here, we showed that the H3K9 methyltransferase ESET controls the proper expression of neural genes and suppresses the expression of non-neural genes, thereby regulating brain development.

ESET is essential for temporal-specific gene expression

ESET is highly expressed by NPCs at an early stage, but is downregulated over time, and expression is very low during the neuron-to-astrocyte fate switch. In the ESET cKO brain, generation of early-born deep layer neurons was markedly impaired, whereas generation of late-born upper layer neurons was not significantly affected (Fig. 5; supplementary material Fig. S6C). By contrast, the generation of astrocytes was significantly enhanced in the absence of ESET...
Thus, in the ESET cKO brain, generation of astrocytes is accelerated at the expense of neurons, most notably early-born neurons. These results suggest that decreasing the expression of ESET during development might be one of the internal clock mechanisms that regulate the timing of cell fate switches of NPCs. The mechanism of gradual downregulation of ESET during development remains to be determined, but both extrinsic and intrinsic factors could be involved. It was reported that some extrinsic factors such as ciliary neurotrophic factors, leukemia inhibitory factor and cardiotrophin 1 promote astrocyte differentiation (Kahn et al., 1997; Nakashima et al., 1999; Miller and Gauthier, 2007), and these factors could lead to downregulation of ESET expression. It was previously reported that the bHLH genes Hes1 and Ngn2 are expressed in an oscillatory manner in NPCs (Shimojo et al., 2008), and it has been proposed that this oscillation is one of the internal clock mechanisms (Kageyama et al., 2007). Interestingly, there are multiple Hes1 binding sites in the ESET promoter (data not shown), suggesting that Hes1 oscillations could lead to gradual downregulation of ESET expression, although further analyses are required to clarify any such relationship.

ChIP assays showed that ESET directly binds the promoters of the astrocyte-related genes Gfap and Sox9. Furthermore, in the absence of ESET, the H3K9me3 mark on these astrocyte-related gene loci was reduced, leading to upregulation of astrocyte gene expression. These results suggest that ESET prevents premature astrocyte formation by repressing Gfap and other astrocyte-related gene expression via H3K9me3. However, overexpression of ESET at E17.5 reduced astrocyte formation but failed to inhibit this process completely. This suggests that additional regulation, such as DNA methylation (Takizawa et al., 2001), might be required to inhibit astrocyte formation.

The expression of neuronal genes was downregulated in the ESET cKO brain, but the precise mechanism of this downregulation remains to be determined, especially as ESET is known to function as a transcriptional repressor. It was previously reported that Sox9 represses the expression of neuronal genes (Stolt et al., 2003; Scott et al., 2010). Furthermore, it was shown that the neurogenic and gliogenic programs inhibit each other by sequestering the CBP-Smad1 complex (Sun et al., 2001). Thus, one of the likely mechanisms for downregulation of neuronal genes is the upregulation of astrocytic genes.
ESET in neural development

Epigenetic regulation in the nervous system
Recent studies revealed that epigenetic regulation is essential for the maintenance of neuronal identity. Ablation of the H3K9 methyltransferase G9a (Ehmt2 − Mouse Genome Informatics), which catalyzes dimethylation of H3K9 (Tachibana et al., 2001), leads to derepression of non-neuronal genes and NPC-specific genes in neurons and to abnormal behaviors in mice (Schaefer et al., 2009; Maze et al., 2010). The precise role of ESET in neurons is not known, but it was shown that overexpression of ESET in neurons leads to the repression of some neuronal genes and to abnormal behaviors in mice, and that ESET expression is highly upregulated in patients with Huntington’s disease (Ryu et al., 2006; Jiang et al., 2010). Thus, the proper level of ESET is important for normal neuronal functions, although a loss-of-function analysis for ESET in neurons remains to be performed.

ESET is essential for repression of non-neuronal genes
In addition to the regulation of neural genes, we found that ESET is important for repressing the endogenous retroelement IAP in somatic tissues. Among the upregulated genes in the ESET cKO brain, many were associated with derepressed IAPs and other ERVs. In particular, genes near derepressed IAPs were strongly upregulated in the ESET cKO brain. One such gene is Mnd1, which regulates meiotic recombination between homologous chromosomes (Petukhova et al., 2005). Normally, Mnd1 is not expressed in the developing nervous system, but in the ESET cKO cortex chimeric transcripts consisting of the IAP and Mnd1 sequences were robustly expressed. Although it remains to be determined whether any defects occur upon the ectopic expression of such chimeric transcripts, these transcripts might lead to abnormal chromosomal recombination, which is possibly prevented by ESET in normal tissues. Similarly, loss of ESET results in derepression of ERVs in ES cells, causing these sequences to be expressed ectopically, resulting in defects in the maintenance of ES cells (Bilodeau et al., 2009; Yuan et al., 2009; Matsui et al., 2010; Karimi et al., 2011). These results indicate that one of the major roles of ESET is the permanent suppression of ERV expression in many cell types.

It was previously shown that methylation of CpG sequences in the LTR region is required for repression of IAP expression (Walsh et al., 1998). Interestingly, the CpG sequences of the IAP LTR region located in the Mnd1 gene are highly methylated in the WT brain but hypomethylated in the ESET cKO brain, suggesting that hypomethylation of CpG sequences is involved in the increased expression of IAP in the ESET cKO brain. Because ESET is known to interact with DNA methyltransferases (Li et al., 2006), ESET might regulate DNA methylation by recruiting DNA methyltransferases.

IAP sequences can be copied and retrotransposed to other regions (Heidmann and Heidmann, 1991), which might induce insertional mutagenesis. However, in our preliminary analysis, a clear increase in the number of IAP copies in the genome of mutant mice was not detectable. Nevertheless, the retrotransposition could occur at a low frequency in the absence of ESET, as transcription of full-length IAP sequences increased robustly. This retrotransposition might cause carcinogenesis and other diseases induced by insertional mutagenesis.

Thus, in summary, ESET appears to be very important for the maintenance of neural identity by suppressing non-neuronal gene expression and retrotransposition.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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