Monoallele deletion of CBP leads to pericentromeric heterochromatin condensation through ESET expression and histone H3 (K9) methylation

Junghee Lee¹,⁴, Sean Hagerty¹,⁴, Kerry A. Cormier¹,⁴, Jinho Kim¹,⁴, Andrew L. Kung⁵, Robert J. Ferrante¹,²,³,⁴ and Hoon Ryu¹,⁴,*

¹Department of Neurology, ²Department of Pathology and ³Department of Psychiatry, Boston University School of Medicine, Boston, MA 02118, USA, ⁴Geriatric Research Education and Clinical Center, Bedford Veteran’s Affairs Medical Center, Bedford, MA 01730, USA and ⁵Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA

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Chromatin remodeling is tightly controlled under physiological conditions. Alterations in chromatin structure are involved in the pathogenesis of neuronal systems. We found that the monoallelic deletion of CREB binding protein (CBP) results in the induction of ERG-associated protein with SET domain (ESET) and increases trimethylation of histone H3 (K9) and condensation of pericentromeric heterochromatin structure in neurons. Nested deletion and mutational analysis of the ESET promoter further demonstrated that the Ets-2 transcription factor regulates transcriptional activity of the ESET gene. In CBP¹⁻/² mice, Ets-2 occupancy in the ESET promoter DNA was markedly elevated. Our results suggest that CBP is a transcriptional repressor of ESET gene expression by limiting Ets-2 transcriptional activity, while CBP siRNA enhances basal and Ets-2-dependent ESET transcriptional activity. Altered expression of the ESET gene and hypertrimethylation of H3 (K9) correlate with striatal neuron atrophy and dysfunction in CBP¹⁻/² mice. These results establish an alternative pathway that loss of CBP leads to the pericentric heterochromatin condensation through ESET expression and trimethylation of H3 (K9).

INTRODUCTION

The balance of chromatin remodeling through histone acetylation and methylation in the N-terminal lysine residues modulates the transcription of nuclear genes (1–3). As such, altered nucleosome dynamics via histone modification may result in transcriptional dysfunction (4–6). Histone H3 (K9) methylation is associated with decreased transcriptional activity, whereas H3 (K9) acetylation improves transcription (2,7,8). To date, however, the mechanisms of H3 (K9) methylation and H3 (K9) histone methyltransferase (HMT) gene expression, which is a marker of gene silencing, have not been fully investigated in the central nervous system (CNS) (9–11). We have recently found abnormal alterations of methylated H3 (K9) and HMT expression are correlated with transcriptional dysfunction and the subsequent neurodegeneration in animal models of Huntington’s disease (HD) (4–6,12). It has been reported that the sequestration of CBP by polyglutamine aggregates leads to transcriptional dysfunction (13–16). CREB binding protein (CBP) functions as a transcriptional cofactor and a histone acetyltransferase (HAT). CBP interacts with diverse transcription factors and with components of the RNA polymerase II (Pol II) complex, thereby acting as a co-activator or repressor of transcription. CBP also plays a role as a HAT in acetylating histones that contribute to transcription by remodeling the chromatin structure (17,18). It has been shown that a loss of CBP function interferes with transcription by inhibiting recruitment to the promoter of the basal transcription machinery and by altering the acetylation level of histones in neurons (17,18). Given the large number of transcriptional events in which CBP participates, the exact mechanism whereby loss of CBP contributes

*To whom correspondence should be addressed at: Department of Neurology, Boston University School of Medicine, 200 Springs Road, Bedford, MA 01730, USA. Tel: +1 7816872922; Fax: +1 7816873515; Email: hoonryu@bu.edu

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to the chromatin remodeling remains unclear (17,18). In light of counter-regulatory effects of acetylation and methylation on H3 (K9), we assessed the level of H3 (K9) methylation in CBP⁺/² mice. Since CBP dysfunction is concurrent with an abnormal increase of histone methylation, we hypothesized that CBP may directly affect H3 (K9) methylation through regulation of HMT.

This study investigates the association of CBP deficiency with HMT gene expression and histone methylation in the CNS. We show that the expression of ESET, a H3 (K9) trimethyltransferase (9–11) is markedly increased through Ets-2 transcriptional activation in CBP⁺/² mice, as well as H3 (K9) trimethylation. Our results suggest that the abnormal induction of ESET gene expression and hypertrimethylation of H3 (K9) by CBP deficiency may be linked to the neuropathogenesis of striatal neurons.

RESULTS

Abnormal increase in the level of TMH-H3 (K9) in CBP⁺/− mice

In the first series of experiments, since CBP⁺/− mice died prenatally, we characterized the effects of heterozygous CBP⁺/− on the level of H3 (K9) methylation in neurons in comparison to littermate control mice (19). There was robust TMH-H3 (K9) immunoreactivity in striatal tissue sections from CBP⁺/− mice, as compared to wild-type mice. Interestingly, intensely immunostained TMH-H3 (K9)-positive puncta were co-localized with condensed heterochromatin within the nucleus in CBP⁺/− mice (Fig. 1A). Further characterization of neuronal filament structures, using combined immunofluorescence for neurofilament-200 and MAP2, showed a marked reduction in the dendritic aborization in CBP⁺/− mice (Fig. 1 and Supplementary Material, Fig. S1). In addition, the level of TMH-H3 (K9) was increased in the nucleus of neuronally differentiated CBP null embryonic stem (ES) cells (Supplementary Material, Fig. S2A–H). Condensed and punctate structures of heterochromatin were co-localized with TMH-H3 (K9) in differentiated CBP null ES cells. Western blot analysis supported the tissue section findings, showing that TMH-H3 (K9) levels were increased by 1.7-fold in CBP⁺/² mouse brain (Fig. 1B).

Expansion of pericentromeric heterochromatin condensation and TMH-H3 (K9) aggregation by CBP deficiency

We next determined the spatial distribution of pericentromeric heterochromatin and TMH-H3 (K9) in striatal neurons using confocal microscopy and image analysis program (Ip Lab, and AQI-X-COMBO-CWF, Media cybernetics Inc., Bethesda, MD). It has been well known that mouse cells have revealed that the majority of TMH-H3 (K9) is localized to the prominent clusters of pericentromeric heterochromatin (chromocenters) that are observed in this species, whereas the mono- and dimethylation states could not be resolved (7). The immunoreactivity of TMH-H3 (K9) (red color) was distributed in small foci that is spatially different and distinguished from chromocenters (blue color stained with DAPI) in neurons of the wild-type (CBP⁺/⁺) mouse brain (Fig. 2A). In contrast, the majority of TMH-H3 (K9) immunoreactivity was co-localized with prominent clusters of pericentromeric heterochromatin in neurons of CBP-deficient (CBP⁺/−) mouse brain (Fig. 2B). Deconvolved images (Fig. 2A and B) and image analysis data (Fig. 2G) showed a 10-fold increase in the condensed structure of TMH-H3 (K9)-positive heterochromatin along with the volume expansion of chromocenters in the neurons.
CBP-deficient (CBP−/−) mice (Fig. 3A). In contrast, ESET protein levels were increased by 2-fold in CBP+/− mouse brain (Fig. 3A). When normalized to 18S, mRNA levels of ESET were significantly increased (P < 0.05) in striatal tissue samples by 1.7-fold, in comparison to control brain samples (Fig. 3B). Consistent with the western and RT–PCR results, ESET immunoreactivity was predominantly found in the nucleus of striatal neurons (Fig. 3C). Confocal microscopy showed neuronal and nuclear localization of ESET protein (Fig. 3D). In addition, confocal microscopic analysis confirmed increased co-localization of ESET and TMH-H3 (K9) in striatal neurons of CBP+/− mice (Supplementary Material, Fig. S3). Otherwise, ESET knock down by ESET shRNA reduces the endogenous level of ESET (Supplementary Material, Fig. S2M–P) and TMH-H3 (K9) (Supplementary Material, Fig. S2U–X and Y) compared with control shRNA in neurally differentiated CBP null (−/−) ES cells.

CBP modulates Ets-2-dependent ESET transcriptional activity

The murine ESET promoter has been found to have a DNA binding site for the Ets (E26: avian erythroblastosis virus oncogene-E twenty-six) protein family, which are transcriptionally active guanosine–adenine-specific DNA binding proteins (20,21). To examine the basal activity and to identify the specific purine-rich binding site in the ESET/SETDB1 promoter, murine primary neurons were transiently transfected with nested-deletion reporter plasmids, including −450/+80, −300/+80, −150/+80, −90/+80 and −10/+80, and a pGL3 basic vector (6,22). Transcriptional activities of the −300/+80 and the −150/+80 ESET reporters were increased, as compared to the pGL3 control vector (Fig. 4A). The shorter element binding region and the comparable transcriptional activity of −150/+80 to −300/+80 makes the former a highly suitable reporter for examining ESET promoter activity. In order to investigate the relative influence of Ets isoforms on ESET promoter activity, the ESET (−150/+80) reporter was induced by Ets-1 and Ets-2 in SH-SY5Y cells. Both Ets-1 and Ets-2 up-regulated ESET promoter activity more than 4-fold in a concentration-dependent manner (Fig. 4B). While Ets-1 has not been shown to be present in the CNS, its regulation of ESET confirms Ets-2’s own regulation of the promoter through their shared homology. The data suggest that Ets-2 acts as a transcriptional activator of the ESET promoter in neurons. Moreover, mutation of the ESET promoter at the Ets-2 binding site reduces promoter activity below basal levels, presumably preventing Ets-2 binding (Fig. 4C). Consistent with the mutation data, siRNA of Ets-2 diminished both basal activity and overexpression of the ESET promoter at the Ets-2 binding site at the −150/+80 ESET promoter activity below basal levels (Fig. 4D). Our data provide evidence that ESET promoter activity is modulated by purine-rich elements, especially through an Ets-2-dependent mechanism.

To determine whether CBP influences ESET gene expression, incremental dosages of CBP were transiently transfected, in conjunction with the control vector (pCMV), Ets-1 and Ets-2. Ets-dependent transcriptional activity of the −150/+80 ESET promoter was dose dependently repressed by CBP (Fig. 4E). We further tested whether CBP’s HAT activity
activity resulted in the modulation of ESET gene expression. We transiently transfected the control vector (pCMV), Ets-1, Ets-2, wild-type CBP, and CBP ΔHAT (a deletion mutant of the catalytic subunit of HAT) plasmids with the ESET promoter into SH-SY5Y cells. As expected, ESET promoter activity enhanced by the Ets transcription factors was diminished to basal levels by both wild-type CBP and CBP ΔHAT (Fig. 4D). This data confirms that CBP represses Ets-2-induced ESET promoter activity independent of its HAT function.

Interestingly, the total level of Ets-2 protein was not changed in CBP+/− mice as compared to CBP+/+ control mice (Fig. 5A). However, the trans-localization of Ets-2 from the cytosol to the nucleus was increased in striatal tissue sections in CBP+/− mice (Fig. 5B and Supplementary Material, Fig. S4). Chromatin immunoprecipitation (ChIP) assays were used to detect if Ets-2 DNA occupancy was altered within the ESET gene promoter in vivo. The Figure 5C displays the PCR product of pulled-down DNA with primers specific to the ESET gene. A ~246-bp DNA fragment was increased in CBP+/− mice neostriatal tissues. In contrast, little or no PCR product was generated in CBP+/+ control mice tissues or with normal rabbit IgG as a negative control (Fig. 5C). These results validate that CBP deficiency enhances the DNA occupancy of Ets-2 with ESET gene loci in vivo. In addition, we found that CBP binds to the ESET promoter through the Ets-2 binding element by ChIP (Fig. 5D). There was no significant change of CBP occupancy in Ets-2 binding element between CBP+/+ control and CBP+/− mice.

CBP suppresses Ets-2-enhanced ESET promoter activity via direct physical interaction

siRNA of CBP elevates ESET promoter activity both in the presence and absence of Ets-2 (Fig. 6A and B). These data show that wild-type CBP modulates Ets-2-induced ESET promoter activity and ESET gene expression. siRNA of CBP elevated the protein level of ESET, whereas siRNA of Ets-2 reduced the level of ESET (Fig. 6C). This data suggests that ESET gene expression is negatively modulated by CBP and limits the Ets-2-dependent ESET transcriptional activity. Otherwise, co-immunoprecipitation analysis of CBP and Ets-2 from striatal neuronal lysates of wild-type (CBP+/+) and CBP+/− mice confirmed the physical interaction between CBP and Ets-2 in neurons (Supplementary Material, Fig. S5).

In order to further characterize how CBP regulates ESET promoter activity, a series of Gal-4 fused CBP deletion
CBP

mice present atropic striatal neurodegeneration

Along with a marked reduction in the dendritic arborization (Fig. 1A and F, and Supplementary Material, Fig. S1) and chromatin condensation (Fig. 1A and G) in CBP

mice, we observed additional neuropathological alterations. CBP

mice present with both gross and microscopic abnormalities, particularly associated within the neostriatum (Fig. 7A). Gross brain atrophy, mild ventricular hypertrophy and striatal neuron atrophy was observed in the CBP

mice. The level of tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of serotonin, was markedly reduced in neostriatal neurons from CBP

mice, as compared to CBP

mice (Fig. 7B). These data suggest that altered CBP may lead to striatal neuron dysfunction as a result of structural change of heterochromatin and overall transcription (Fig. 7C).

DISCUSSION

CBP binds to a multitude of transcription factors and facilitates transcription through recruitment of the basal transcriptional machinery as well through chromatin remodeling through intrinsic and associated HAT activities (13,14,16). In this study, we show that monoallelic loss of CBP results in hypermethylation of H3 (K9) directly through induction of ESET gene expression. Histone methylation, in particular, lysine (K) 9 trimethylation has been correlated with both local and global repression of transcription and the formation of large constitutive heterochromatin domains (7). The complexity that can be achieved with this single modification is remarkable. Our current findings strongly suggest that the relationship between H3 (K9) trimethylation and heterochromatin condensation may be closely linked to CBP deficiency-induced transcriptional repression in neurons (7,8).

ESET, a novel H3 (K9) methyltransferase, has been shown to mediate histone methylation (9–11). ESET contains both tudor and methyl-CpG binding domains that converge transcription and RNA processing factors, as well as acting as a signature motif for proteins regulating methylated DNA

Figure 5. CBP deficiency leads to the activation of Ets-2 to target the promoter of ESET gene. (A) The nuclear level of Ets-2 protein is increased in CBP

mice. Nu, nucleus; Cy, cytoplasm. (B) Ets-2 translocates to the nucleus of striatal neurons in CBP

mice. Scale bar: 10 μm. (C) The DNA occupancy of Ets-2 to ESET gene promoter is elevated in the neurons of CBP

mice. (D) CBP binds to the promoter of ESET gene through the Ets-2 binding element. There is no significant change in the CBP ChIP of CBP

mice compared to CBP

mice. Blk, blank; IgG, rabbit immunoglobulin G immunoprecipitation; CBP, anti-rabbit CBP immunoprecipitation.

Figure 4. ESET promoter activity is up-regulated by transcription factor Ets-2 and suppressed by CBP. (A) Mouse ESET promoter activity was determined using a series of deletion reporter constructs (ESET1, −450/+80; ESET2, −300/+80; ESET3, −150/+80; ESET4, −90/+80; ESET5, −10/+80).

Primary cortical neurons were transfected with reporter constructs for 48 h. (B) ESET promoter (−150/+80) activity is up-regulated by Ets-2 in a dose-dependent manner. (C) Mutation of the ESET promoter (−150/+80) at Ets-2 binding element (mtEBE) down-regulates ESET promoter activity below basal levels. (D) Ets-2 siRNA diminishes the basal activity of ESET promoter (−150/+80). ESET promoter activity was normalized to protein levels and the error bars indicate the SEM of three combined experiments. (E) CBP down-regulates ESET promoter (−150/+80) activity enhanced by Ets-2. (F) CBP represses Ets-2-induced ESET promoter (−150/+80) activity independent of HAT activity. CBPΔHAT (1431–1596) (pCMV2N3T-CBP-ΔHAT) was constructed by partial digestion of pCMV2N3T-CBP (WT). SH-SY5Y cells were used for the luciferase assay (B–F).
silencing. Recently, we have found that ESET is involved in neuronal dysfunction through its histone methyltransferase activity and the epigenetic silencing of neuronal genes (6). ESET mRNA and protein levels are elevated in human HD patients and transgenic HD mice (6). We report, herein, that ESET mRNA and protein levels are elevated in human HD patients and transgenic HD mice (6). We report, herein, that ESET mRNA and protein levels are elevated in human HD patients and transgenic HD mice (6). We report, herein, that ESET mRNA and protein levels are elevated in human HD patients and transgenic HD mice (6). We report, herein, that ESET mRNA and protein levels are elevated in human HD patients and transgenic HD mice (6). ESET mRNA and protein levels are elevated in human HD patients and transgenic HD mice (6). ESET mRNA and protein levels are elevated in human HD patients and transgenic HD mice (6). ESET mRNA and protein levels are elevated in human HD patients and transgenic HD mice (6). ESET mRNA and protein levels are elevated in human HD patients and transgenic HD mice (6). ESET mRNA and protein levels are elevated in human HD patients and transgenic HD mice (6). ESET mRNA and protein levels are elevated in human HD patients and transgenic HD mice (6). ESET mRNA and protein levels are elevated in human HD patients and transgenic HD mice (6).
It has been proposed that CBP plays a role in the maintenance of genomic integrity and that a loss of CBP function may contribute to genomic instability (19,27–30). However, the mechanism of epigenetic modification by CBP is not known. Our current study confirms that CBP deficiency results in hypermethylation of histone molecules and contributes to neuronal atrophy. The epigenetic regulation and alteration by CBP deficiency can possibly occur in the pathogenesis of the CNS. For example, the sequestration of CBP by mutant Huntingtin (mtHtt) expression causes the hypermethylation and hypoacetylation of histone proteins and the subsequent transcriptional dysfunction of neurons in HD (4–6). The polyQ stretches in mtHtt interact physically with CBP and block a transcriptional co-activator function, as well as intrinsic CBP transcriptional HAT activity (13,14,16). These specific interactions and transcriptional dysregulation is attributable to pathological epigenetic modifications in HD (4,6). In addition, CBP\textsuperscript{+/−} is haploinsufficient for a variety of developmental tasks in mice and humans, such as Rubinstein–Taybi Syndrome (RTS). Therefore, the increased H3 (K9) methylation by CBP deficiency may also be linked to mental retardation of RTS (17).

The present study validates the role of CBP in epigenetic modulation through the expression of the ESET gene and via trimethylation of H3 (K9) in neurons. Our results present an alternative mechanism of pericentromeric heterochromatin condensation by CBP that is regulated independently from its HAT activity and is linked to the atrophy of striatal neurons. Thus, neuronal CBP levels may be considered a predictive indicator of the chromatin remodeling relevant in neuronal dysfunctions.

**MATERIALS AND METHODS**

**CBP \textsuperscript{+/−} knock-out mice**

A 129 mouse genomic phage library (Stratagene) was screened with a probe corresponding to the CH1 region of the murine CBP cDNA (351-bp MfeI fragment) (19). The three exons spanning the CH1 region were mapped by Southern blotting and the precise exon–intron boundaries were determined by sequencing. Two of the exons were deleted by MfeI and NcoI digestion and replaced with a PGK-neomycin cassette. PGK-TK was also inserted for negative selection. T/C ES cells were electroporated with the targeting plasmid, linearized with NorI. From 350 ES cell colonies surviving selection with G418 and Gancyclovir, two contained a correctly targeted allele. One of the ES cell clones gave rise to chimeric mice with a C57BL/6 background. These mice then transmitted the targeted allele via the germ line.

**Histopathology and confocal microscopy**

At 50–90 days of age groups CBP\textsuperscript{+/−} and littermate wild-type control mice were deeply anesthetized and transcardially perfused with 4% buffered paraformaldehyde. Brains were cryoprotected and serially sectioned (50 μm). Mouse tissue sections were stained for Nissl substance and immunostained for ESET/SETDB1 (ESET antibody; dilution, 1:200; Upstate Biotech), and trimethylated histone (TMH)-H3 (K9) (rabbit antibody; dilution, 1:200; Upstate Biotech) using a previously reported conjugated second antibody method (6). For the immunocytochemistry, antibody complexes were visualized using diaminobenzidine. For the confocal microscopy, the specimens were incubated for 1 h with fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (Vector, Burlingame, CA, USA) and Cy3-conjugated anti-rabbit IgG antibody (Jackson Lab) after the incubation of primary antibody. Images were analyzed using a Spinning Disk Confocal microscope (IX2-DSU, Olympus, Tokyo, Japan). Preabsorption with excess target protein or omission of primary antibody was used to demonstrate antibody specificity and delete background generated from the detection assay.

**ChIP–PCR assay of Ets-2 DNA interaction**

ChIP for Ets-2 binding to DNA was performed using a CHIP assay kit (Upstate Biotech) as described previously (31). CBP\textsuperscript{+/−} and littermate control mice brain tissue fractions were cross-linked with 1% formaldehyde for 20 min at room temperature. The lysates were sonicated six times with each time for 20 s using Branson Sonifier 450 (Branson Ultrasonics Corp., Danbury, CT, USA). After centrifugation, the supernatant was diluted in CHIP dilution buffer and then incubated overnight at 4°C with, anti-Ets-2 antibody. Immune complexes were recovered by the addition of 60 μl of salmon sperm DNA/protein A agarose-50% slurry and incubation for 2 h at 4°C with rotation. The beads were pelleted and washed with low and high salt buffer, LiCl buffer and finally twice with TE buffer. The immune complexes were eluted by incubation for 15 min at 37°C with fresh elution buffer (1% SDS, 0.1 M NaHCO\textsubscript{3}). To reverse the cross-linking of DNA, 10 ml of 5M NaCl were added to the combined elute (150 ml) and incubated for overnight at 65°C. DNA was purified using QIAquick DNA purification spin column and eluted in 50 ml of nuclease-free water. PCR amplification was carried out for 35 cycles and PCR products were separated on 2% agarose gels. Forward primer was 5′-TGGAAGAGTAGAGCTAGAG-3′ and reverse primer was 5′-AAGGTACAATCAGGATGG-3′.

**RT–PCR analysis**

Fifty nanograms of RNA were used as a template for qRT–PCR amplification, using Superscript One-Step RT–PCR with platinum Taq (Invitrogen, Carlsbad, CA USA). Primers were standardized in the linear range of cycles prior to onset of the plateau. The sequence of the primers is as follows: ESET/SETDB1 forward, 5′-ACATCCTCAGCCTCT. GCAC-T3′; ESET/SETDB1 reverse, 5′-TTCCAGTACGGGTCAGATCC-3′; 18S RNA forward, 5′-CCGAGATTGGACAATAACAGG-3′; 18S RNA reverse, 5′-AGTTGCAAGCT. TCTTACAGG-3′. The conditions of one-step RT–PCR for ESET primers: 30 min at 50°C, 2 min at 94°C, 35 cycles of amplification for 15 s at 94°C for 15 s, 30 s at 68°C, 1 min at 70°C, 10 min at 72°C and 4°C. 18S RNA primers were complete at 30 cycles and 55°C for the annealing step. Differences were assessed using an unpaired, two-tailed Student’s t-test.
Acid extraction of histone protein and histone methylation assay

Tissue lysate was obtained by homogenizing minced mouse brain in 500 µl of phosphate-buffered saline containing 0.4 mM sodium butyrate, 5% Triton X-100, 3 mM DTT, 1 mM sodium orthovanadate, 5 mM sodium fluoride, 3 mM PMSF, 3 mM DTT, 0.5 µg/ml leupeptin and 10 µg/ml aprotinin as previously described (4–6). Two hundred microliter of the lysate was washed twice with the above-described 5% Triton buffer and histones were extracted by solubilization in 0.2 M HCl. After neutralizing the pH of the acid extracted solution containing the histone pool with ammonium acetate, protein was quantified and separated in a 15% SDS–PAGE for the western analysis of methylated H3 (K9) using anti-trimethyl-histone H3 (lysine 9) antibody (Upstate Biotech).

Western blot analysis

Thirty micrograms of tissue were subjected to SDS–PAGE (10%) and blotted with anti-ESET/SETDB1 (Upstate Biotech), anti-CBP (A-22 and C-20) (Santa Cruz Biotech) and anti-Ets-2 (C-20) (Santa Cruz Biotech) antibody. Protein loading was controlled by probing for α-tubulin (Sigma) on the same membrane.

ESET/SETDB1 promoter activity analysis

ESET promoter activity analysis was performed using a reporter-construct containing −435 to +87, including nested deletion constructs, of the 5′ regulatory region of the mouse ESET/SETDB1 gene (6). The site-directed mutagenesis was performed to generate Ets binding site mutant promoter (Stratagene). The sequence of the primers is as follows: forward, 5′-GTCGGAGAGCCCTTAAGGAGGCTCCC-3′; reverse, 5′-GGAGGCGCTCTTAAAGGCTCTCCGAC-3′. The mouse primary neurons and SH-SY5Y cells were used in the transient transfection assays of ESET promoters. The same amount of primary neurons and SH-SY5Y cells were used in the transient transfection assays of ESET promoters. The same amount of

RNA interference experiment

Mouse primary neurons (1 × 10⁶ cells/ml) or SH-SY5Y (2 × 10⁶ cells/ml) were transiently transfected with 100–400 nM of Stealth control RNAi, CBP RNAi and Ets-2 RNAi (Invitrogen Life Tech) using DMRIE-C transfection reagent (Invitrogen Life Tech) in the presence or absence of ESET promoter for 48 h.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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