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An ERG (*ets*-related gene)-associated histone methyltransferase interacts with histone deacetylases 1/2 and transcription co-repressors mSin3A/B

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Covalent modifications of histone tails play important roles in gene transcription and silencing. We recently identified an ERG (*ets*-related gene)-associated protein with a SET (suppressor of variegation, enhancer of zest and trithorax) domain (ESET) that was found to have the activity of a histone H3-specific methyltransferase. In the present study, we investigated the interaction of ESET with other chromatin remodelling factors. We show that ESET histone methyltransferase associates with histone deacetylase 1 (HDAC1) and HDAC2, and that ESET also interacts with the transcription co-repressors mSin3A and mSin3B. Deletion analysis of ESET reveals that an N-terminal region containing a tudor domain is responsible for interaction with mSin3A/B and association with HDAC1/2, and that truncation of ESET enhances its binding to mSin3. When bound to a promoter, ESET represses the transcription of a downstream luciferase reporter gene. This repression by ESET is independent of its histone methyltransferase activity, but correlates with its binding to the mSin3 co-repressors. In addition, the repression can be partially reversed by treatment with the HDAC inhibitor trichostatin A. Taken together, these data suggest that ESET histone methyltransferase can form a large, multi-protein complex(es) with mSin3A/B co-repressors and HDAC1/2 that participates in multiple pathways of transcriptional repression.

Key words: chromatin, ERG-associated protein with a SET domain (ESET), SETDB1, trichostatin A, tudor domain.

INTRODUCTION

Eukaryotic gene transcription is controlled by a cascade of signals and a network of proteins that interact with both the nucleosome and the transcriptosome [1]. The effectiveness of sequence-specific transcription factors is determined in large part by the biological activity and the physical conformation of chromatin. Proteins that remodel and modify chromatin include ATP-dependent remodelling engines and enzymes that covalently modify histone tails [2]. Recent studies have shown that acetylation, methylation, phosphorylation and ubiquitination of histone tails all have an impact on further modification of the same or nearby residues [3,4]. In addition, these covalent modifications result in the recruitment and/or dissociation of proper chromatin-associated factors that regulate gene expression both at the level of the nucleosome and at the level of transcription [5]. The combinatorial possibilities of various modifications have been hypothesized to function as a 'histone code' [6] that can be recognized by regulatory proteins to influence downstream events, such as immediate-early gene activation [7].

The evolutionarily conserved SET (suppressor of variegation, enhancer of zest and trithorax) domain is commonly found in proteins involved in chromatin modification [8]. The discovery two years ago that Suv39H1, a prototypical SET domain protein, can function as a histone H3-specific methyltransferase [9] has now been extended to include several other histone methyltransferases with similar SET domains [10–13]. Among these mammalian histone methyltransferases, the ESET [ERG (*ets*-related gene)-associated protein with a SET domain; also called SETDB1] protein was identified through its interaction with the *ets* transcription factor ERG [14]. ESET protein was initially reported to be a histone H3-specific methyltransferase [14], and it was later found to specifically methylate lysine-9 of histone H3 [15]. The ESET protein immunostains predominantly in euchromatic regions of interphase nuclei, suggesting its involvement in global euchromatic histone H3 Lys-9 methylation [15]. In addition to the catalytic SET domain, ESET also contains a tudor domain [16] and a methyl-CpG binding domain [17]. The tudor domain within the ESET protein is of particular interest, since a similar domain in the SMN (survival of motor neurons) protein reportedly plays a critical role in bringing various transcription and RNA processing factors together in Cajal bodies [18].

The Suv39H1 histone methyltransferase was shown recently to interact with the so-called 'histone deacetylase (HDAC) core complex', which contains one molecule each of HDAC1, HDAC2, retinoblastoma protein (Rb)-associated protein 46 (RbAp46) and RbAp48 [19,20]. This HDAC1/2 core complex itself can be a component of even larger complexes, such as the mSin3-containing nuclear receptor co-repressor (NcoR) complex [21–25] and the methyl-CpG binding protein 2 (MeCP2) complex [26,27]. HDAC1 and HDAC2 are also found in mSin3-independent complexes, such as the nucleosome remodelling and deacetylating (NuRD) complex [28], the HDAC–Rb–hSWI/SNF complex [29], and the co-repressor to the REST transcription factor (CoREST) complex [30]. In addition, association of HDAC1/2 with other cofactors is known to be affected by post-

Abbreviations used: ERG, *ets*-related gene; ESET, ERG-associated protein with a SET domain (also called SETDB1); HDAC, histone deacetylase; PAH2, paired amphipathic helix 2; Rb, retinoblastoma protein; RbAp, retinoblastoma protein-associated protein; SET, suppressor of variegation, enhancer of zest and trithorax; SMN, survival of motor neurons; TSA, trichostatin A.

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translational events, such as protein phosphorylation [31]. It has been proposed that a HDAC–histone-methyltransferase complex(es) containing multiple essential cofactors may work co-operatively in gene silencing [19,20,32].

In the present study, we investigated whether the ESET histone methyltransferase also interacts with HDACs and other cofactors found in larger complexes. Here we demonstrate that ESET not only associates with HDAC1 and HDAC2, but also interacts with the mSin3A and mSin3B transcription co-repressors. Interestingly, the HDAC- and mSin3-interacting region of ESET overlaps with the tudor domain. In transient transfection assays, repression of a heterologous promoter by ESET does not require its SET histone methyltransferase catalytic domain, but instead requires its HDAC- and mSin3-interacting region. This repression can be partially reversed by an inhibitor of HDAC.

EXPERIMENTAL

Plasmids

The Flag-epitope-tagged expression plasmid pSG5-FL-ESET was generated by cloning full-length ESET cDNA into the *Eco*RI/*Sma*I sites of the pSG5-FL vector [33]. Myc-epitope-tagged expression plasmid pCS2-MT-ESET was generated by cloning full-length ESET into the *Eco*RI/*Xho*I sites of the pCS2-MT vector [33]. The cDNA encoding an ESET splicing variant (GenBank accession no. AF546078) was isolated from a mouse EML cell cDNA library [34] and cloned into the same *Eco*RI/*Xho*I sites of the pCS2-MT vector. Deletion mutant PCS2-MT-ESETΔ680–1307 was generated by *Nde*I/*Xba*I digestion, pCS2-MT-ESETΔ366–1307 was generated by *Xho*I digestion, pCS2-MT-ESETΔ168–1307 was prepared through PCR, and pCS2-MT-ESET(C1242T) was generated by site-directed mutagenesis. For generation of pCS2-Gal4-MT-ESET, the Gal4 DNA binding domain was cloned in-frame into the unique *Cla*I site within the pCS2-MT vector. The pG5-SP1-Luc reporter was generated by inserting five Gal4 binding sites and one SP1 binding site upstream of the luciferase gene.

Antibodies

All anti-HDAC and anti-mSin3 antibodies used in this study were available commercially (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The C-19 goat polyclonal anti-HDAC1 antibody was raised against the C-terminus of human HDAC1, and the H-11 mouse monoclonal anti-HDAC1 antibody was against amino acids 432–482 at the C-terminus of human HDAC1. The C-19 goat polyclonal anti-HDAC2 antibody was raised against the C-terminus of human HDAC2, and the C-8 mouse monoclonal anti-HDAC2 antibody was against amino acids 435–488 of human HDAC2. All polyclonal anti-mSin3 antibodies were raised in rabbits: AK-11 against the PAH2 (paired amphipathic helix 2) region of mouse mSin3A, K-20 against the N-terminus of mouse mSin3A, A-20 against the N-terminus of mSinB, and AK-12 against amino acids 172–228 within the PAH2 region of mSin3B. The M2 mouse monoclonal anti-Flag and the 9E10 mouse monoclonal anti-Myc antibodies were purchased commercially (Sigma, St. Louis, MO, U.S.A.). A rabbit polyclonal anti-ESET antibody was generated against the N-terminal 167 amino acids of ESET protein [14].

Immunoprecipitation

HEK-293 cells or mouse testis TM3 cells in a 10 cm-diam. dish were lysed with 1.2 ml of buffer A (10 mM Tris, pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100, 10 mM dithiothreitol) supplemented with a protease inhibitor cocktail and a phosphatase inhibitor cocktail (Sigma). A 20 μl portion of antibody was incubated with 40 μl of Protein A/G–agarose for 50 min at 4 °C in 0.3 ml of buffer A, and the antibody–Protein A/G–agarose complex was then incubated with 0.2 ml of fresh cell lysate overnight at 4 °C on a rotating wheel. After extensive washing with buffer A or the more stringent RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS), 40 μl of 1 × SDS sample buffer was added to the agarose beads. The protein samples were denatured at 95 °C for 5 min, separated by SDS/PAGE, and subjected to Western blot analysis. Protein bands were visualized using the ECL® Western Blotting Analysis System (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

HDAC assay

^{3}H-labelling of biotinylated histone H4 peptide substrate was carried out with a kit from Upstate (Lake Placid, NY, U.S.A.). After ^{3}H incorporation, the histone H4 peptide substrate was captured with streptavidin–agarose. For measurement of HDAC activity, 4 μl of ^{3}H-acetylated histone H4 peptide–streptavidin–agarose beads (4000 c.p.m./μl) was incubated with 15 μl of immunoprecipitate at 37 °C for 2 h in 200 μl of assay buffer (10 mM Tris, pH 8.0, 250 mM NaCl, 10% glycerol, 0.5 mM PMSF). The released [^{3}H]acetate was collected for measurement of radioactivity by scintillation counting.

Transfection and luciferase assay

The pG5-SP1-Luc reporter and the pCS2-Gal4-MT-ESET expression construct were introduced into mouse NIH 3T3 cells by electroporation. In duplicate 60 mm dishes, 4×10^6 cells were mixed with pG5-SP1-Luc (1.5 μg) and pCS2-Gal4-MT-ESET (3 μg) plus pCMV-β-galactosidase control DNA (0.5 μg) in 350 μl of PBS, then electroporated on a BTX 600 Electro Cell Manipulator at a setting of 250 V/48 Ω/200 μF in a 2 mm cuvette. After incubation for 24 h in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, trichostatin A (TSA) was added to a final concentration of 250 ng/ml. The cells were washed once with PBS 24 h later, and then lysed with 0.2 ml of Nonidet P40 lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P40). A 40 μl aliquot of the lysate was added to 50 μl of luciferin substrate (Promega Life Science, Madison, WI, U.S.A.), and luciferase activity was measured using a TD-201 luminometer. For β-galactosidase assay, 30 μl of the cell lysate was added to 3 μl of 100 mM Mg^{2+} solution, 66 μl of 1 × *o*-nitrophenyl β-D-galactopyranoside solution and 201 μl of 10 mM Tris, pH 8.0. After incubation at 37 °C, the reaction was stopped with 500 μl of 1 M Na_2CO_3, and β-galactosidase activity was measured at 420 nm using a Beckman DU 640 spectrophotometer.

RESULTS

ESET associates with HDAC1 and HDAC2

Proteins that enzymically remodel chromatin structure and covalently modify core histones are known to exist in large, multi-subunit complexes [2]. As a prototypical histone methyltransferase, the *Drosophila* Su(var) 3-9 and its mammalian homologue Suv39H1 have been reported to functionally and physically associate with HDACs [19,20]. The HDAC and histone methyltransferase activities co-operate to methylate pre-acetylated histones, and therefore their association has been implicated in heterochromatin silencing and transcriptional repression of particular regions within the genome.

The evolutionarily conserved SET domain is a signature motif for lysine-specific histone methyltransferases [10–14]. Other than

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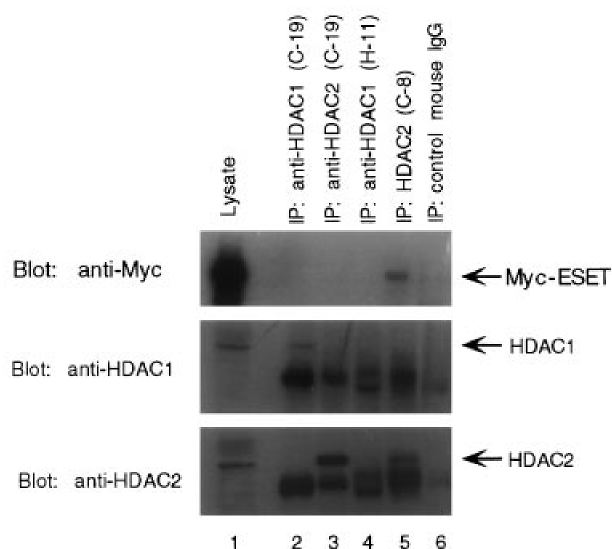

Figure 1 Association of ESET with HDAC2

HEK-293 cells were transfected with the pCS2-MT-ESET expression plasmid. The cell lysate (lane 1) was immunoprecipitated (IP) with C-19 goat polyclonal anti-HDAC1 antibody (lane 2), C-19 goat polyclonal anti-HDAC2 antibody (lane 3), H-11 mouse monoclonal anti-HDAC1 antibody (lane 4), C-8 mouse monoclonal anti-HDAC2 antibody (lane 5), and an unrelated control mouse IgG (lane 6). The immunoprecipitates were blotted with the anti-Myc antibody (top panel), the C-19 anti-HDAC1 antibody (middle panel), or the C-19 anti-HDAC2 antibody (bottom panel).

this SET domain, there is little sequence identity among these methylating enzymes. Of the histone methyltransferases specific for Lys-9 of histone H3 identified to date, Suv39H1 requires HDAC activity for transcriptional repression, whereas G9a does not [35]. To investigate whether association with HDACs is a unique property of Suv39H1 or is shared by other histone methyltransferases, we transfected a Myc-tagged ESET expression plasmid into HEK-293 cells, and the resultant lysate (Figure 1, lane 1) was used in immunoprecipitation experiments with various anti-HDAC antibodies. Two different goat polyclonal antibodies specific to the C-terminus of HDAC1 and HDAC2 worked well in bringing down these deacetylases, yet neither co-immunoprecipitated Myc–ESET (Figure 1, lanes 2 and 3). Although the mouse H-11 monoclonal anti-HDAC1 antibody did not immunoprecipitate any other protein under our experimental conditions (Figure 1, lane 4), the mouse C-8 monoclonal anti-HDAC2 antibody was able to co-immunoprecipitate Myc–ESET (Figure 1, lane 5). This association between HDAC2 and Myc–ESET appeared to be specific, as both proteins were absent from the sample obtained with a negative control mouse IgG (Figure 1, lane 6). Using an antibody against Flag-tagged ESET, we were also able to co-immunoprecipitate HDAC1 and ESET, as described below.

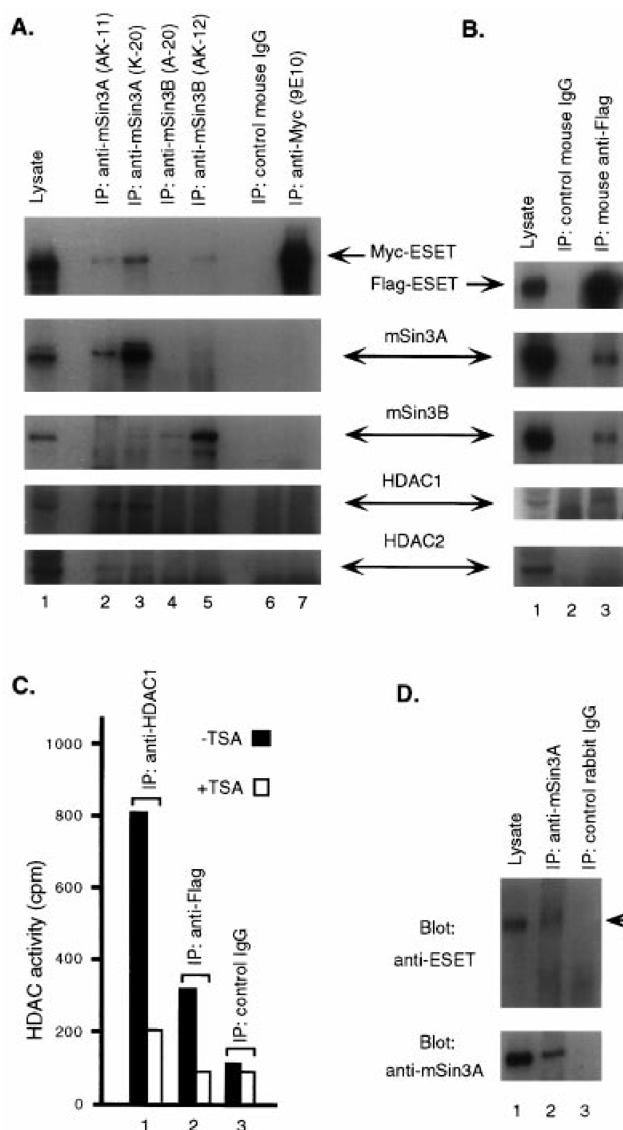

Figure 2 Association of ESET with mSin3 transcription co-repressors

(**A**) HEK-293 cells were transfected with the pCS2-MT-ESET expression plasmid. The cell lysate (lane 1) was immunoprecipitated (IP) with AK-11 and K-20 rabbit polyclonal anti-mSin3A antibodies (lanes 2 and 3), A-20 and AK-12 rabbit polyclonal anti-mSin3B antibodies (lanes 4 and 5), a mouse control IgG (lane 6), and the 9E10 mouse monoclonal anti-Myc antibody (lane 7). The immunoprecipitates were blotted with the anti-Myc antibody (top panel), K-20 anti-mSin3A antibody (second panel), AK-12 anti-mSin3B antibody (third panel), C-19 anti-HDAC1 antibody (fourth panel) or C-19 anti-HDAC2 antibody (bottom panel). (**B**) A HEK-293 cell lysate expressing Flag–ESET (lane 1) was immunoprecipitated with a control mouse IgG (lane 2) or the M2 mouse monoclonal anti-Flag antibody (lane 3). (**C**) A HEK-293 cell lysate expressing Flag–ESET was immunoprecipitated with the indicated antibodies. The immunoprecipitates were assayed for HDAC activity, and results from one representative experiment are shown. (**D**) Mouse TM3 cell lysate (lane 1) was immunoprecipitated with the AK-11 anti-mSin3A antibody (lane 2) or a control rabbit IgG (lane 3), then blotted with the anti-ESET antibody (top panel) and K-20 anti-mSin3A antibody (bottom panel).

ESET associates with the transcription co-repressors mSin3A and mSin3B

Our finding that epitope-tagged ESET co-immunoprecipitates with HDAC1 and HDAC2 is in agreement with the recently reported association between Suv39H1 and HDACs [19,20], and suggests that it may be a common phenomenon for a histone methyltransferase to complex with HDACs. One molecule each of HDAC1, HDAC2, RbAp48 and RbAp46 together form a so-called 'core HDAC complex' [28], and this core complex has been shown to interact with Suv39H1 [20]. Since the enzymic activity of a HDAC requires additional cofactors, we investigated the association between ESET histone methyltransferase, HDAC1 and HDAC2, and the transcription co-repressors mSin3A and mSin3B.

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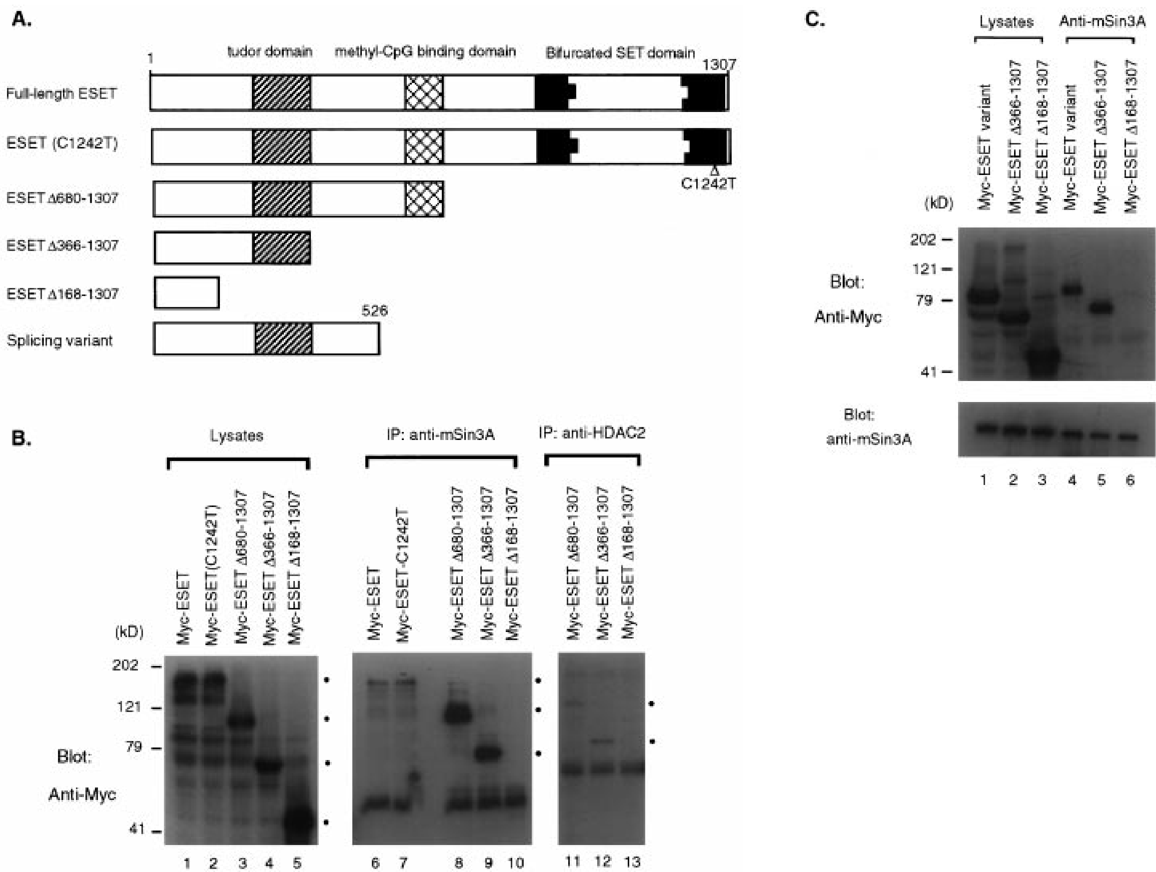

Figure 3 mSin3-interacting region within ESET protein

(**A**) Schematic representation of ESET. The tudor domain is indicated by the hatched box, the methyl-CpG binding domain by the crossed box, and the bifurcated SET domain by the black boxes. The position of the C1242T point mutation, deletion mutants and the splicing variant are indicated. (**B**) Plasmids encoding Myc–ESET and its mutants were transfected into HEK-293 cells. The resultant lysates (lanes 1–5) were immunoprecipitated (IP) with the K-20 anti-mSin3A antibody and washed with buffer A (lanes 6–10). Lysates for the deletion constructs were also immunoprecipitated with the C-8 anti-HDAC2 antibody and washed with the more stringent RIPA buffer (lanes 11–13). The immunoprecipitates were then blotted with the 9E10 anti-Myc antibody. Filled circles indicate the positions of the protein bands. (**C**) HEK-293 cell lysate expressing the Myc–ESET splicing variant was immunoprecipitated with the K-20 anti-mSin3A antibody and compared with two Myc–ESET deletion mutants (lanes 1–6).

In our experiment, a HEK-293 cell lysate expressing Myc-tagged ESET (Figure 2A, lane 1) was treated with two rabbit polyclonal anti-mSin3A antibodies (Figure 2A, lanes 2 and 3) or two rabbit polyclonal anti-mSin3B antibodies (Figure 2A, lanes 4 and 5). When the samples were blotted for Myc–ESET, three out of the four antibodies were shown to co-immunoprecipitate mSin3 and Myc–ESET (Figure 2A, top three panels). Both AK-11 and K-20 anti-mSin3A antibodies were also able to co-immunoprecipitate HDAC1 and HDAC2 (Figure 2A, bottom two panels). The A-20 anti-mSin3B antibody did not work in the immunoprecipitation experiment, as it was not effective in bringing down mSin3B itself, while the AK-12 anti-mSin3B antibody was unable to bring down either HDAC1 or HDAC2. Using a control mouse IgG and the 9E10 anti-Myc antibody, the reciprocal immunoprecipitation was attempted, but Western blotting failed to detect either mSin3A or mSin3B in the immunoprecipitates (Figure 2A, lanes 6 and 7).

We speculated that the interaction between Myc–ESET and mSin3A/B might be disrupted by the 9E10 anti-Myc antibody, and therefore another reciprocal immunoprecipitation experiment was carried out by transfection of HEK-293 cells with the Flag–ESET construct (Figure 2B, lane 1). The lysate was then immunoprecipitated with a control mouse IgG or the mouse M2 monoclonal anti-Flag antibody (Figure 2B, lanes 2–3, top panel). Both mSin3A and mSin3B were found in the anti-Flag immunoprecipitate (Figure 2B, second and third panels). In addition, HDAC1, but not HDAC2, was detected in the same anti-Flag immunoprecipitate (Figure 2B, bottom fourth and fifth panels). To investigate whether the anti-epitope antibodies can precipitate HDAC activity from these Flag–ESET-expressing cells, the immunoprecipitates were incubated with ^{3}H-labelled histone H4 peptide and the released [^{3}H]acetate was measured. HDAC activity in the anti-HDAC positive control or in the anti-Flag sample was significantly higher than that in the mouse IgG negative control, and the HDAC activity in the immunoprecipitates was inhibited by the HDAC inhibitor TSA (Figure 2C, lanes 1–3).

To rule out the possibility that the association between epitope-tagged ESET and mSin3A/B represented an adventitious weak interaction resulting from overexpression of exogenous ESET, similar immunoprecipitation experiments were carried out with lysate from mouse testis TM3 cells, which express a high level of endogenous ESET (Figure 2D, lane 1). Endogenous ESET was present in the anti-mSin3A immunoprecipitate, but absent from the negative control sample (Figure 2D, lanes 2 and 3). While we

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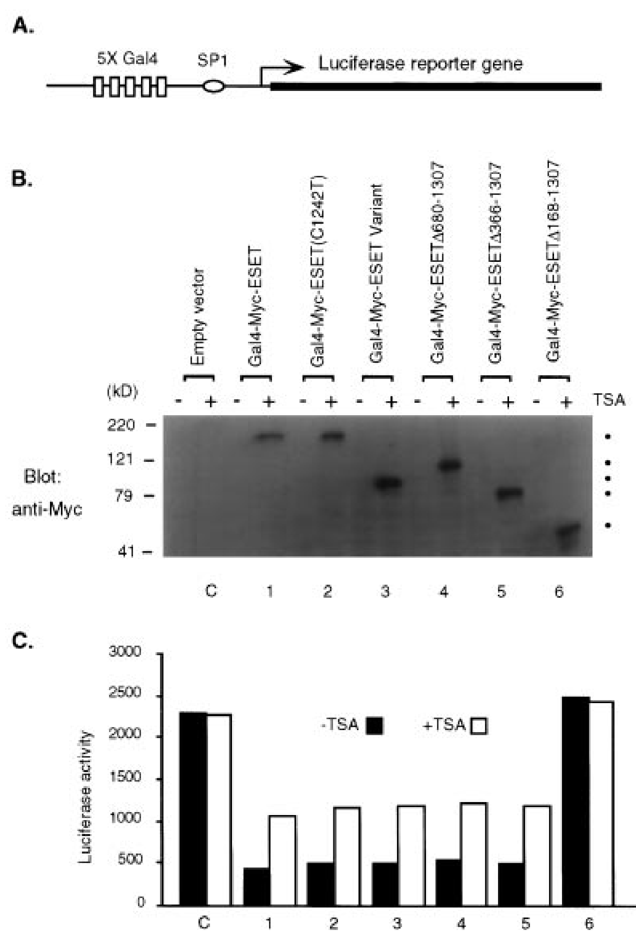

Figure 4 Transcriptional repression by ESET protein

(**A**) Schematic representation of the pG5-SP1-Luc reporter. Five Gal4 binding sites upstream of the SP1 site and the luciferase reporter gene are indicated. (**B**) NIH 3T3 cells were transfected with the pG5-SP1-Luc reporter and the pCS2-Gal4-MT-ESET expression plasmid plus a pCMV-β-Gal control. At 48 h after electroporation, untreated ($-$) and TSA-treated ($+$) cells were lysed for Western blotting using the 9E10 anti-Myc antibody. Note that the CMV (cytomegalovirus) promoter of the pCS2-Gal4-MT-ESET constructs was activated by TSA, resulting in increased expression of ESET and its mutants. (**C**) Luciferase activities were measured with the same lysates. In the absence of pCS2-Gal4-MT-ESET constructs, TSA had no effect on the promoter of pG5-SP1-Luc (see control lane C). TSA reduced the suppression of pG5-SP1-Luc in the presence of all ESET constructs containing the mSin3-interacting region (lanes 1–5). When the mSin3-interacting region was removed from the ESET construct, TSA was no longer able to reduce suppression (lane 6). Since TSA did activate the promoter of pCMV-β-Gal, the luciferase activity was not divided by the β-galactosidase activity in TSA-treated samples. Results from one representative experiment are shown.

did not attempt to identify all components present in the immunoprecipitates, these results lend support to the notion that ESET histone methyltransferase interacts with a large protein complex(es) containing both HDAC1/2 and mSin3A/B transcription co-repressors.

The tudor region of ESET interacts with mSin3

As a protein of 180 kDa, ESET contains several sequence motifs found in other nuclear proteins. In addition to the bifurcated SET domain that plays a critical role in its histone H3-specific methyltransferase activity [14], ESET protein also possesses a potential methyl-CpG binding domain and a tudor domain (Figure 3A). While the methyl-CpG binding domain is a well known feature of proteins involved in methylated DNA silencing [17], the functions of the tudor domain are less well understood, but it may mediate protein–protein interactions [16].

To investigate whether the bifurcated SET domain, as well as the histone methyltransferase activity itself, are required for the interaction of ESET with the mSin3A/B transcription co-repressors, the highly conserved cysteine residue at position 1242 within the SET domain of ESET was mutated to threonine. This point mutation has been shown to abolish the histone methyltransferase activity of ESET [14], and the corresponding mutation in Suv39H1 [9] is also known to render the enzyme inactive. In addition to this C1242T point mutation, three Myc-tagged mutants in which the entire SET domain (Δ680–1307), the methyl-CpG binding domain (Δ366–1307) or the tudor domain (Δ168–1307) was deleted were also generated (Figure 3A) and transfected into HEK-293 cells.

The levels of expression of these ESET mutants appeared to be comparable, as determined by Western blotting (Figure 3B, lanes 1–5). When these lysates were immunoprecipitated using the anti-mSin3A antibody, the enzymically inactive Myc–ESET(C1242T) was brought down in an amount similar to wild-type Myc–ESET (Figure 3B, lanes 6–7). Interestingly, strong Myc–ESETΔ680–1307 and Myc–ESETΔ366–1307 bands were detected from the anti-mSin3A immunoprecipitates (Figure 3B, lanes 8 and 9), and a rough calculation revealed that approx. 25 % of the mutant proteins were in complexes with mSin3A. The mSin3A-interacting region of ESET apparently resides between amino acids 168 and 366, a region that overlaps with the tudor domain, as Myc–ESETΔ168–1307 was barely detected from the anti-mSin3A immunoprecipitate (Figure 3B, lane 10).

When the same lysates were immunoprecipitated with the anti-HDAC2 antibody and washed under mild conditions, low levels of all three ESET deletion mutants were present in the immunoprecipitates (results not shown). Under stringent washing conditions, however, Myc–ESETΔ680–1307 and Myc–ESETΔ-366–1307 were detected in the anti-HDAC2 immunoprecipitates, whereas Myc–ESETΔ168–1307 was barely visible (Figure 3B, lanes 11–13). Even though these results suggest that HDAC2 associates with the tudor region of ESET via mSin3, we cannot rule out the possibility that a weak interaction between HDAC2 and the N-terminal 167 amino acids of ESET is stabilized by the adjacent tudor region.

These deletion studies also suggested an enhanced affinity of truncated ESET for mSin3. To test this notion further, we sequenced an ESET splicing variant (GenBank accession no. AF546078) from a cDNA library constructed from the EML mouse cell line, which is capable of erythroid/myeloid/lymphoid differentiation [34]. This ESET splicing variant encodes the N-terminal 526 amino acids, containing the tudor domain but lacking the SET and the methyl-CpG binding domains (Figure 3A). When expressed in HEK-293 cells, binding of this ESET variant to mSin3A was comparable with that of the ESETΔ366–1307 deletion mutant (Figure 3C, lanes 1–6).

Transcriptional repression by ESET correlates with its interaction with mSin3

Since ESET is a histone H3-specific methyltransferase and associates with HDAC1/2 and mSin3A/B, ESET protein is likely to have a repressive effect on gene transcription. Our initial efforts were focused on a promoter construct containing an ERG binding site, but co-expression of ESET had a minimal effect on ERG-mediated transactivation. We speculated that this lack of repression by ESET might be due to inefficient targeting of ESET

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to the promoter because of a weak interaction between ERG and ESET.

To assay for the effect of ESET on gene transcription, we fused the Gal4 DNA binding domain to Myc–ESET and various ESET mutants. This approach is based on previous reports that similar Gal4 fusion proteins allow efficient targeting to artificial promoters containing Gal4 binding sites [36,37]. When co-transfected into NIH 3T3 cells with a luciferase reporter (Figure 4A) and treated with TSA, different Gal4–Myc–ESET fusion proteins were expressed at similar levels, as shown by Western blotting (Figure 4B). Measurement of luciferase activity in the same lysate revealed that the full-length Gal4–Myc–ESET fusion protein was a potent repressor of transcription from the reporter gene (Figure 4C, compare control with lane 1). The C1242T point mutation that abolishes histone methyltransferase activity did not result in a loss of transcriptional repression, nor did the ESET splicing variant or mutants in which the entire SET domain and the methyl-CpG binding domain were deleted. In addition, treatment of the cells with TSA was able to partially reduce suppression by these Gal4–Myc–ESET constructs containing the mSin3-interacting region (Figure 4C, lanes 1–5). When the mSin3-interacting tudor domain was deleted from the ESET protein, the resultant Gal4–Myc–ESETΔ168–1307 protein was no longer able to repress transcription or respond to TSA (Figure 4C, lane 6), suggesting that repression by ESET is due, at least in part, to recruitment of HDACs. Thus it appears that, in this assay system, repression by ESET of transcription from the artificial promoter correlates with the mSin3-interacting tudor region, and that the SET catalytic domain is dispensable for such repression.

DISCUSSION

In this study, we report an association between the recently identified histone methyltransferase ESET and the well characterized HDAC1/2. Since similar interactions between the histone methyltransferase Suv39H1 and HDACs have also been reported recently [19,20], it appears that the physical association of different histone modification enzymes represents a common feature in the epigenetic regulation of gene expression.

In addition, we also found that ESET interacts with the transcription co-repressors mSin3A/B. To the best of our knowledge, this is the first demonstration that mSin3A/B are associated with a histone methyltransferase. mSin3A/B were originally identified as Mad binding proteins that mediate Mad:Max transcriptional repression [38], and this repression was later found to require mSin3-associated HDAC1/2 [23,39]. Since HDAC1/2 complexes do not always contain mSin3A/B, a model has been proposed [30] in which mSin3 is recruited to HDAC1/2 complexes for the simple deacetylation of dynamic genes (such as those within euchromatin), whereas mSin3 is excluded from HDAC1/2 complexes involved in stable repression (such as those within heterochromatin). If this is the case, then ESET protein may be able to regulate dynamic gene expression by functioning as a recruiter of HDAC1/2 and mSin3A/B. The recently reported localization of ESET in euchromatin appears to lend support to such a notion [15].

The *in vivo* association between HDAC1 and HDAC2 has been well documented [23,28,39], but HDAC1 alone can also be part of a repressor complex [40]. In the present study, epitope-tagged ESET was unable to co-immunoprecipitate HDAC1 and HDAC2 simultaneously (Figure 2B); however, both HDAC1 and HDAC2 did associate individually with tagged ESET. Not finding HDAC1 and HDAC2 together in the same complex probably reflects experimental limitations rather than functional exclusion of HDAC1 and HDAC2 in the same ESET multi-protein complex.

When the SET domain and the methyl-CpG binding domain are deleted from ESET protein, the deletion mutants surprisingly have much higher affinities towards mSin3. The enzymic activity of ESET apparently has been abolished in these mutants, yet it is interesting that the loss of the SET histone methyltransferase catalytic domain results in stronger association of mSin3 with these ESET mutants. This unexpected finding raises the intriguing possibility that the mSin3-interacting region within ESET might be able to undergo a conformational change after post-translational modification(s) and/or partial degradation of the full-length ESET protein. This change in protein conformation would be expected to facilitate the interaction of ESET with mSin3.

Upon further examination, we found that truncated versions of ESET exist naturally in mouse cells due to alternative splicing/polyadenylation. We have identified a mouse ESET splicing variant that only encodes the N-terminal 526 amino acids of the full-length ESET protein. This short protein product does not contain the catalytic SET domain or the methyl-CpG binding domain, but has enhanced mSin3A binding ability comparable with that of the ESETΔ366–1307 deletion mutant. For human ESET, a similar splicing/polyadenylation variant has also been identified (GenBank accession no. BC009362) that lacks the SET domain and the methyl-CpG binding domain. It is not clear at the present time how cells regulate the expression of different ESET splicing/polyadenylation isoforms. It is tempting to speculate that the histone methyltransferase activity of full-length ESET, along with its potential binding to methylated DNA, synergistically induces stable repression of genes, whereas the stronger mSin3-recruiting ability of shorter ESET products is utilized for transient regulation of gene expression.

The mSin3-interacting region of ESET overlaps with the tudor domain. This latter domain was named after the tudor protein that is important for abdominal segmentation and pole cell formation in *Drosophila* [41]. While there are 10 repeats of the tudor domain in the *Drosophila* tudor protein, the molecular functions of this tudor protein remain unknown. The SMN protein is mutated in spinal muscular atrophy [42], and the tudor domain of SMN protein has an essential role in spliceosomal uridine-rich small ribonucleoprotein assembly [43,44]. Even though further deletion and site-specific mutants are needed, our present study suggests that a putative function of the tudor domain may be the recruitment of mSin3A/B; therefore tudor-domain proteins may also be regulators of gene transcription.

The existence of multiple functional domains in full-length ESET suggests that this protein may function as a platform to aggregate various factors involved in nucleosome remodelling, histone modification and transcriptional regulation. Upon this platform, HDAC–histone methyltransferase multi-protein complexes could be assembled to recognize methylated DNA and facilitate the heritable transmission of the inactive state. Shorter splicing variants of ESET may function to dynamically regulate the transcription of active genes. Since the interaction between full-length ESET histone methyltransferase and mSin3/HDAC appeared to be substoichiometric, other ESET complexes comprising different cofactors may also exist inside the cells, and warrant further investigation.

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