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EVI1 Recruits the Histone Methyltransferase SUV39H1 for Transcription Repression

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ABSTRACT

EVI1 is an oncoprotein inappropriately expressed in acute myeloid leukemia and myelodysplastic syndrome cells. In vitro studies indicate that diverse biological properties can be attributed to this protein. Its role in leukemogenesis is still unclear but it is thought that overall EVI1 can act mostly as a transcription repressor through its interaction with a subset of histone deacetylases. Studies with histone deacetylase inhibitors have however indicated that EVI1-mediated repression can be only partially rescued by deacetylase inhibitor drugs, suggesting that additional chromosomal modifications might occur to induce gene repression by EVI1. To investigate whether histone methylation contributes to the repressive potential of EVI1, we examined a potential association between EVI1, the histone methyltransferase (HMT) SUV39H1, and methyltransferase activity in vitro. We find that EVI1 directly interacts with SUV39H1 and that the proteins form an active complex with methyltransferase activity in vitro. Our data indicate that SUV39H1 enhances the transcription repressive potential of EVI1 in vivo. We suggest that EVI1 affects promoters' activity in two different pathways, by association with histone deacetylases and by recruiting chromatin-modifying enzymes to impose a heterochromatin-like structure establishing a lasting transcription repression. J. Cell. Biochem. 105: 344–352, 2008.

KEY WORDS: EVI1; ZINC FINGERS; SUV39H1; HISTONE METHYLATION; GENE SILENCING

The *EVI1* (*Ecotropic Viral Integration site 1*) gene, located on chromosome 3 band q26, was originally identified as a common retroviral integration site in murine myeloid leukemia [Morishita et al., 1988]. The protein has multiple zinc finger motifs organized into two domains of seven and three zinc finger motifs localized at the N- and C-terminus, respectively. Typically EVI1 is not detected in normal hematopoietic tissue and its inappropriate expression, which occurs mostly through chromosomal rearrangements, is closely associated with development and progression of human myeloid malignancies [Morishita et al., 1992; Wieser et al., 2003; Laricchia-Robbio et al., 2006; Nucifora et al., 2006; Poppe et al., 2006]. Although numerous data have been collected about the molecular events that lead to EVI1 activation, the role of the protein in the development and progression of hematological malignancies is still unclear. Studies in mice have shown that a spatial and temporally restricted pattern of expression of *Evi1* exists, suggesting that this gene plays a critical role in development, organogenesis, cell migration, and growth [Hoyt et al., 1997]. The undifferentiated phenotype of *EVI1*-positive leukemia supports the hypothesis that the inappropriate activation of this gene may interfere with cell differentiation. In vitro and in vivo studies indicate that the forced expression of EVI1 reduces the cellular response to interferon alpha [Buonamici et al., 2005] and the transforming growth factor-β (TGF-β), and that impairs terminal granulopoiesis and erythropoiesis [Kurokawa et al., 1998; Sitailo et al., 1999; Buonamici et al., 2004]. These effects could be explained in part by biochemical and in vitro studies showing that EVI1 acts as a transcription repressor that recruits several co-repressor proteins [Kurokawa et al., 1998; Chakraborty et al., 2001; Izutsu et al., 2001]. Recently, we showed that EVI1 directly interacts with RUNX1 and GATA1 transcription factors [Laricchia-Robbio et al., 2006; Senyuk et al., 2007]. These interactions alter the ability of the transcription factors to bind their recognition sites on the DNA, leading to the deregulation of their target genes and to the impairment of normal hematopoietic programs.

The transcription activity and the replication timing of a DNA sequence depend on the chromatin state of that region. The chromatin is normally regulated by potentially reversible covalent modifications of the DNA itself and/or the histones [Bannister et al., 2002]. In particular, the histone tails can undergo a variety of post-translational modifications such as acetylation, phosphorylation, methylation, and ubiquitination. All these changes, catalyzed

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by specific enzymes, can reduce or increase the affinity of the histones for the DNA and/or can serve as markers for the recruitment of proteins or protein complexes that control gene expression, DNA replication, and chromosome segregation [Zaidi et al., 2007]. Among all the possible modifications, lysine methylation has emerged as a central epigenetic change in the organization of eukaryotic chromatin with far-reaching implications for the regulation of cell proliferation, cell-type differentiation, gene expression, genome stability, overall development, and genesis of cancer [Czvitkovich et al., 2001]. SUV39H1 was the first histone lysine methyltransferase (HMTase) identified [Ait-Si-Ali et al., 2004]. SUV39H1 specifically catalyzes the addition of trimethyl groups to lysine 9 of histone 3 (H3-K9me3). A direct consequence of this modification is the creation of a high-affinity binding site for heterochromatin protein 1 (HP1), inducing chromatin packaging and gene silencing [Bannister et al., 2001; Lachner et al., 2001]. Several authors have shown that SUV39H1 methylates H3-K9 residues located at the pericentric heterochromatin [Aagaard et al., 1999, 2000; Melcher et al., 2000] and within euchromatic promoters [Mal, 2006; Jang et al., 2007]. Our group and others have found that SUV39H1 interacts with DNA-binding proteins involved in leukemogenesis such as AML1 and PML-RARα promoting the silencing of their target genes [Chakraborty et al., 2003; Carbone et al., 2006]. In this study we have examined whether EVI1 associates with the histone methyltransferase (HMT) SUV39H1 to promote gene repression. We have determined that the two proteins interact through the proximal zinc finger domain of EVI1 and the C-terminus domain of SUV39H1 and that the EVI1/SUV39H1 complex is enzymatically active and methylates a recombinant histone H3 in vitro. We propose that the transcription repression and the oncogenic effects of EVI1 are mediated by a pathway that requires both the deacetylation and the methylation of histone proteins.

MATERIAL AND METHODS

DNA CLONING

The expression vectors pCMV-$(Myc)_3$SUV39H1 and the mutated pCMV-$(Myc)_3$SUV39H1(H324L) were a gift of Dr. T. Jenuwein (Research Institute of Molecular Pathology, Austria). The expression vector pEGFP-Flag-G9a was a gift of Dr. M. J. Walsh (Mount Sinai School of Medicine, NY). The $pCS2^+$MT-SET7/9 and $pCS2^+$MT-SETDB1 vectors were a gift of Shelby Blythe and Peter Klein (University of Pennsylvania). The entire open reading frame of EVI1 (EVI1-1051) and the deletion mutants EVI1-283 and EVI1-Δ283 were previously described [Laricchia-Robbio et al., 2006]. EVI1-1051 was also cloned into the pBK-CMV vector under the regulation of the T3 promoter to allow in vitro translation. The mutants EVI1(Δ5–7) lacking zinc fingers 5–7 and the EVI1(Δ1–4) lacking zinc fingers 1–4 were subcloned from the wild type EVI1. The single point mutants in zinc fingers 1, 2, 3, and 4 and the double mutant HA-EVI1(1 + 2)M containing the H39A/C44A and the H93A/H97A mutations were generated by PCR. The N-terminus domain (1–81 aa) amino acids, the central region (82–248 aa) and C-terminus domain (249–412 aa) of SUV39H1 were generated from the original plasmid used as a template for PCR. Appropriate primers were designed to obtain the three mutants in frame with the epitope tag and the nuclear localization signal (NLS) of the pCMV-Flag plasmid previously described [Chakraborty et al., 2001]. Glutathione *S*-transferase (GST) fusion proteins were generated by subcloning the appropriate restriction or PCR fragments into pGEX-2T vector (Amersham).

CELL CULTURE

The adherent cell lines 293T, HeLa, and NIH-3T3 were maintained as previously described [Chakraborty et al., 2001; Laricchia-Robbio et al., 2006].

TRANSFECTION

DNA transfection was performed with the calcium phosphate precipitation method or using the EscortV reagent (Sigma–Aldrich) according to the manufacturer's instructions.

CO-IMMUNOPRECIPITATION AND WESTERN BLOT ANALYSIS

The 293T cells were harvested 48 h after transfection with ice-cold PBS and lysed in JLB (50 mM Tris–HCl, pH 8, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100) containing a complete protease inhibitor cocktail (Roche). Lysis proceeded for 30 min at 4°C, after which the cellular debris was pelleted by centrifugation at 14,000 rpm for 30 min. The proteins were immunoprecipitated by incubation with commercially available antibodies overnight at 4°C under constant rotation. The following day, 30 μl of protein A-coupled sepharose beads slurry, pre-equilibrate in JLB, were added to the lysate for 2 h at 4°C under constant rotation. For Western blot analysis (WB) the beads were washed three times for 5 min at room temperature with MSWB (50 mM Tris–HCl, pH8, 150 mM NaCl, 0.1% NP-40) containing a complete protease inhibitor cocktail (Roche), and the proteins, after separation by SDS–PAGE, were transferred to a polyvinylidene difluoride (PVDF) membrane. Protein bands were identified by WB with commercially available antibodies.

IN VITRO BINDING ASSAY

In vitro translation of SUV39H1 and EVI1 was carried out as previously described [Laricchia-Robbio et al., 2006]. ^{35}S-EVI1 was mixed with unlabeled SUV39H1 and incubated at 37°C for 30 min. The reaction mixture of EVI1 and SUV39H1 was diluted to 250 μl with HND buffer (20 mM HEPES, pH 7.5, 200 mM NaCl, 2 mM DTT) and immunoprecipitated with anti-Myc antibody as described previously [Chakraborty et al., 2003]. The products were separated in a 10% SDS–PAGE and analyzed by WB using anti-EVI1 and anti-Myc antibodies (Cell Signaling Tech).

GST FUSION PROTEINS PULL-DOWN

The expression and purification of GST fusion proteins EVI1, SUV39H1(1–81), SUV39H1(82–248) and SUV39H1(249–412) and their interaction with ^{35}S-SUV39H1 or ^{35}S-EVI1 were carried out as previously described [Laricchia-Robbio et al., 2006].

HISTONE METHYLATION ASSAY

HMT assay was performed as follows. HA-EVI1, and wild type or H324L-mutant SUV39H1 was immunoprecipitated with the appropriate antibody. After 3 h of incubation at 4°C, 30 μl of 50%

protein G-sepharose beads were added to each tube and the mixture was further incubated for 2 h. The beads were collected by centrifugation and washed several times with the immunoprecipitation buffer plus a final wash with MAB buffer (50 mM Tris, pH 8.5, 20 mM KCl, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 250 mM sucrose). The beads were divided into two parts; one part was used for the HMT assay and the other one was used for input detection by WB analysis. For HMT assay the beads were incubated for 1 h at 30°C in 50 μl of MAB buffer containing 5 μg of recombinant histone 3 (Roche), as substrate, and 5 mM unlabelled S-adenosyl-L-methionine. The HMT reaction was stopped by boiling the samples in 5× SDS loading buffer and the proteins were separated by SDS–PAGE and analyzed using the Pan-Methyl Histone H3 (Lys9) antibody (Cell Signaling Tech).

REPORTER GENE ASSAY

FLAG-EVI1 was cloned in frame to the DNA binding domain of GAL4. The plasmid pG5Luc (Promega) with five GAL4 binding sites upstream of the luciferase transcription start site was used as reporter plasmid. For normalization of transfection efficiency, we used pRL-TK plasmid (Promega) that expresses Renilla luciferase. The assays were carried out by transient transfection in NIH-3T3 cells and the luciferase readings were recorded with a Promega Dual-Luciferase Assay System by luminometer (Turner Design TD20/20). The experiment was repeated three times.

IMMUNOFLUORESCENCE (IF) ANALYSIS

HeLa cells were plated at a concentration of 10^5 cells/ml on glass slides and transfected using EscortV Transfection Reagent (Sigma–Aldrich). After 48 h the transfected cells were fixed in 100% methanol at −20 °C and processed as previously described [Chakraborty et al., 2001].

RESULTS AND DISCUSSION

SUV39H1 ASSOCIATES WITH EVI1 IN MAMMALIAN CELLS

The methylation of H3-K9, catalyzed by SUV39H1, has a repressive effect on gene transcription. Because EVI1 acts as a transcription repressor, we investigated whether EVI1 can associate with SUV39H1 and whether this association alters SUV39H1 activity. Full length proteins or point- and deletion- mutants of the proteins were used in co-immunoprecipitation (co-IP) assays. A diagram of the clones used in this study is shown in Figure 1A. HA-EVI1 was transfected in 293T cells in absence or in presence of $(Myc)_3$-SUV39H1. As shown in Figure 1B, upper right panel, EVI1 can be immunoprecipitated by an anti-Myc antibody when co-expressed with SUV39H1 (lane 8). The antibody alone is unable to interact with EVI1 (lane 7). To detect the structural domains responsible for EVI1/SUV39H1 association, deletion mutants of EVI1 were cloned and used in co-IP experiments in 293T cells. A mutant lacking the first 283 aa containing the proximal seven zinc finger domain (HA-EVI1-Δ283) and a mutant consisting of only the proximal zinc finger domain (HA-EVI1-283) were used (Fig. 1A). As shown in Figure 1B, top right panel, EVI1-283 can be pulled-down by SUV39H1 (lane 12) whereas the mutant EVI1Δ283 cannot (lane 10), suggesting that the N-terminus of EVI1 contains the only/major interaction surface for SUV39H1. Lanes 9 and 11, upper right panel, represent the results when only the antibody is used as negative control. The bottom panel shows SUV39H1 detection after co-IP. Lanes 1–6 show the expression of the HA-EVI1 and the mutants used (upper left panel) and of $(Myc)_3$-SUV39H1 (lower left panel).

THE FIRST AND SECOND ZINC FINGERS OF EVI1 INTERACT WITH SUV39H1

To identify the motif(s) that mediate this interaction, we used deletion mutants of EVI1 that lack either the first four zinc fingers of the proximal domain, EVI1(Δ1–4), or the last three zinc fingers of the proximal domain, EVI1(Δ5–7; Fig. 1A). Co-IP experiments showed that whereas EVI1(Δ5–7) is still able of interacting with SUV39H1 (Fig. 1C, lane 10, upper right panel), the mutant EVI1(Δ1–4) does not significantly interact with the HMT (lane 12), suggesting that the region responsible for SUV39H1 binding is likely located between the first and the fourth zinc finger motifs. The full-length EVI1 is used as positive control (lane 8). Lanes 7, 9 and 11, top right panel, represent the reaction with only the antibody used as negative control. Lanes 7–12 (Fig. 1C, lower right panel) show SUV39H1 detection after immunoprecipitation. Lanes 1–6 (Fig. 1C) show the expression of HA-EVI1 and the deletion mutants used (upper left panel), and of $(Myc)_3$-SUV39H1 (lower left panel). Based on these results, we introduced different Cys-to-Ala and His-to-Ala mutations in each of the first four zinc fingers of EVI1 by themselves or in different combinations and we screened the mutants in co-IP experiments, as shown in Figure 1D,E. In Figure 1D, the full-length EVI1 is used as positive control (lane 10). Lanes 9, 11, 13, and 15 top right panel represent the reaction with only the antibody used as negative control. Lanes 9–16 (Fig. 1D, lower right panel) show SUV39H1 detection after immunoprecipitation. Lanes 1–8 (Fig. 1D) show the expression of HA-EVI1 and the point mutants used (upper left panel), and of $(Myc)_3$-SUV39H1 (lower left panel). Using double point mutants, we found that when both the first and the second zinc fingers were mutated the binding between EVI1 and SUV39H1 was compromised, suggesting that these two zinc fingers are responsible for the interaction (Fig. 1E, lane 8, upper right panel). The full-length EVI1 was used as positive control (lane 6). Lanes 5 and 7, top right panel, represent the reaction with only the antibody used as negative control. Lanes 5–8 (lower right panel) show SUV39H1 detection after immunoprecipitation. Lanes 1–4 (upper left panel) show the expression of wild type and EVI1 double point-mutant used. The lower left panel confirms $(Myc)_3$-SUV39H1 expression.

GST PULL-DOWN AND IN VITRO BINDING ASSAYS CONFIRM THE INTERACTION BETWEEN EVI1 AND SUV39H1

We used GST pull-down assays to confirm the interaction between the two proteins and to determine whether the binding is direct. As shown in Figure 2A, ^{35}S-labeled SUV39H1 does not interact with GST alone (lane 1) but interacts with purified GST-EVI1 (lane 2), confirming that the two proteins interact and suggesting that the interaction is direct. To further validate the results and to exclude that bacterial proteins might mediate the contact, we performed a binding assay using both proteins translated in vitro. A mixture of ^{35}S-labeled EVI1 and unlabeled $(Myc)_3$-SUV39H1, or ^{35}S-labeled EVI1 and the in vitro translated unlabeled $(Myc)_3$-HP1α, used as

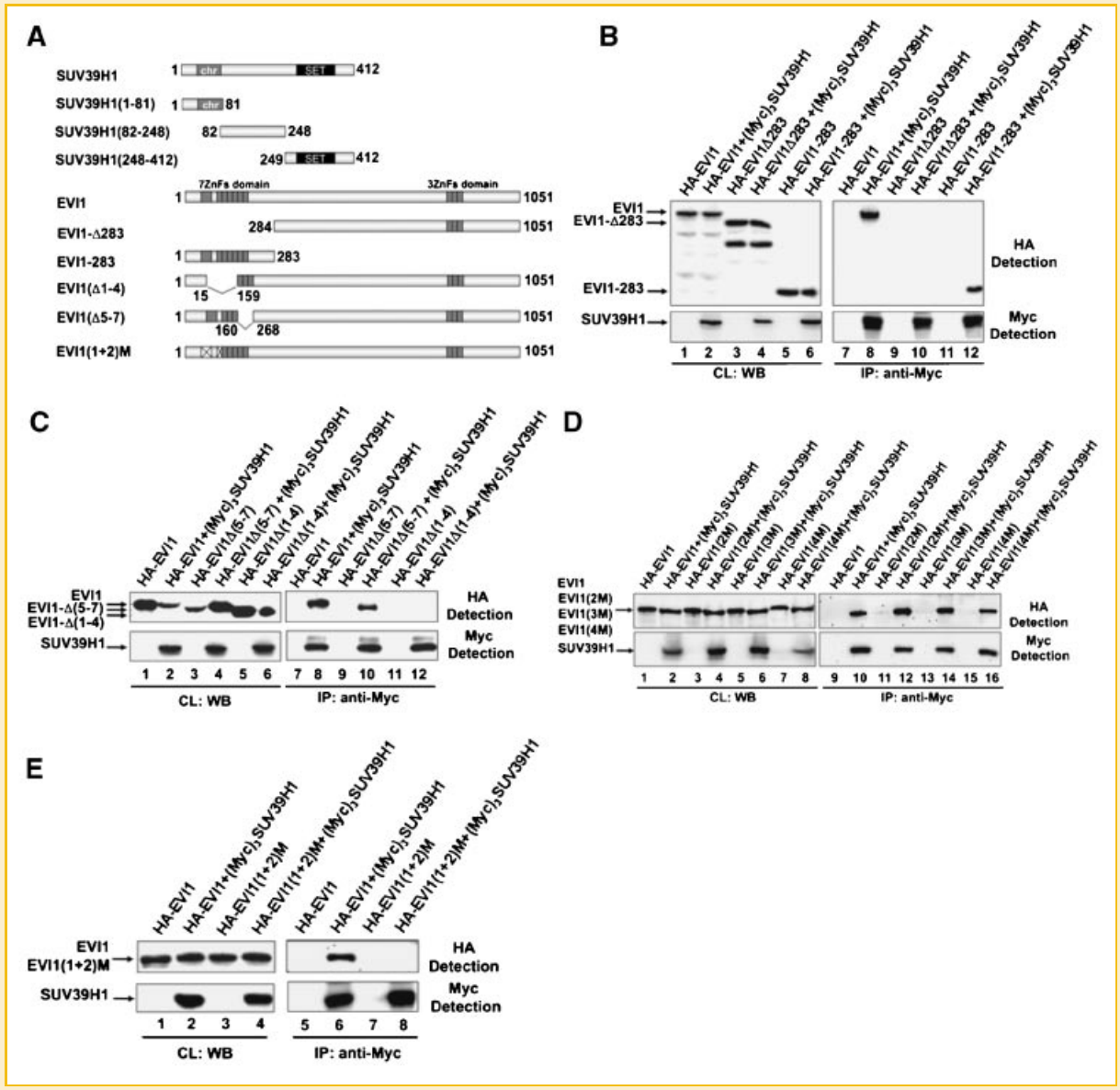

Fig. 1. EVI1 interacts with the histone methyltransferase SUV39H1 through the first and second zinc fingers of the proximal domain. A: Diagrams of SUV39H1, EVI1, and the deletion mutants used in this work are shown. The zinc finger domains of EVI1 (seven and three Znfs), and the chromo (chr) and catalytic (SET) domains of SUV39H1 are indicated. B: 293T cells were transfected with HA-EVI1, HA-EVI1Δ283 and HA-EVI1-283 alone (lanes 1, 3 and 5) or in presence of $(Myc)_3$-SUV39H1 (lanes 2, 4 and 6). Whole cell extracts were immunoprecipitated with anti-Myc antibody. Lanes 1–6 show the expression of full-length and mutant EVI1 (upper panel) and SUV39H1 (lower panel). Lanes 7–12 show the co-IP results. C: 293T cells were transfected with HA-EVI1, HA-EVI1(Δ5–7), and HA-EVI1(Δ1–4) alone (lanes 1, 3, and 5) or in presence of $(Myc)_3$-SUV39H1 (lanes 2, 4, and 6) and analyzed as described above. In lanes 1–6 the expression of full-length and mutant EVI1 (upper panel) and SUV39H1 (lower panel) are shown; in lanes 7–12 the immunoprecipitated proteins are shown. D: 293T cells were transfected with HA-EVI1, single point mutants in zinc fingers 2, 3 and 4 (lanes 1, 3, 5, and 7) or in presence of $(Myc)_3$-SUV39H1 (lanes 2, 4, 6, and 8) and analyzed as described above. In lanes 1–8 the expression of full-length and mutants EVI1 (upper panel) and SUV39H1 (lower panel) are shown; in lanes 9–16 the immunoprecipitated proteins are shown. E: 293T were transiently transfected with HA-EVI1 and HA-EVI1(1 + 2)M alone (lanes 1 and 3) or in presence of $(Myc)_3$-SUV39H1 (lanes 2–4) and analyzed as indicated. Lanes 1–4 show total cell proteins. Lanes 5–8 show the immunoprecipitated proteins.

negative control, were co-immunoprecipitated using an anti-Myc antibody (Fig. 2B, lanes 1–3). As shown in lane 2 (Fig. 2B, upper panel) a band corresponding to EVI1 was detected only in the presence of SUV39H1 confirming the specificity of this interaction. Lane 3 (Fig. 2B, upper panel) represents the reaction with an unlabeled $(Myc)_3$-HP1α protein used as negative control, which does not bind to EVI1 (data not shown). Lanes 1–3 (Fig. 2B, lower panel) show the detection of the proteins after immunoprecipitation. Lane 4 and 5 (Fig. 2B, upper and lower panels) shows the proteins input.

THE C-TERMINUS OF SUV39H1 IS RESPONSIBLE FOR THE INTERACTION WITH EVI1

Three distinct structural domains can be identified in SUV39H1. The chromatin organization modifier, or Chromo domain (1–81 aa) is

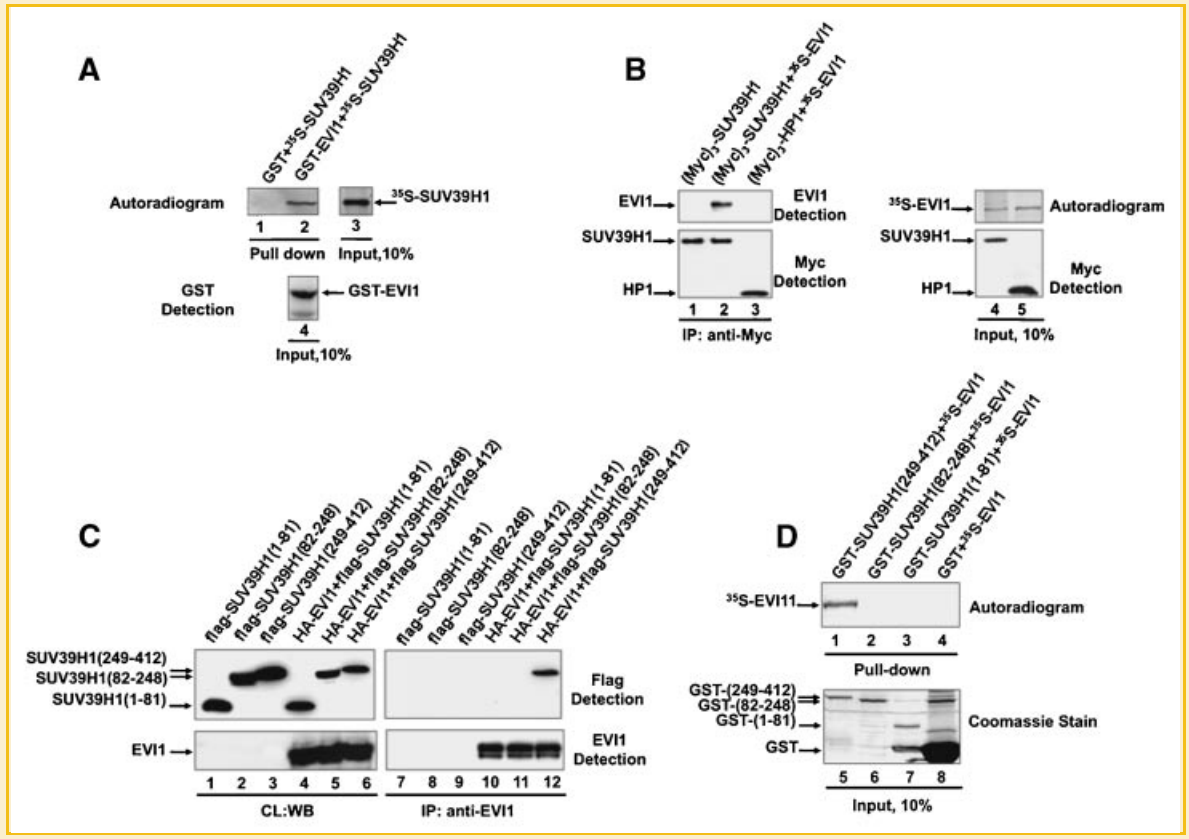

Fig. 2. A: The interaction between EVI1/SUV39H1 is direct. Purified GST and GST-EVI1 conjugated to glutathione-sepharose beads were incubated with in vitro translated ^{35}S-SUV39H1. After extensive washing, the beads were subjected to SDS–PAGE and the separated proteins were analyzed by autoradiography. ^{35}S-labeled SUV39H1 interacts with purified GST-EVI1 (lane 2) but not with GST (lane 1). Lane 3 shows in vitro translated ^{35}S-labeled SUV39H1 input and lane 4 shows GST-EVI1 input. B: In vitro binding assay confirms that the interaction is direct. In vitro translated unlabeled $(Myc)_3$-SUV39H1 (lane 1), a mixture of the two proteins (lane 2), or in vitro translated ^{35}S-labeled EVI1 and the negative control protein $(Myc)_3$-HP1α were co-immunoprecipitated with anti-Myc antibody, separated by electrophoresis, and analyzed by WB. A band corresponding to EVI1 was detected only when the protein was mixed in vitro with $(Myc)_3$-SUV39H1 (lane 2, upper panel). Lanes 1–3, lower panel, show the immunoprecipitated Myc–tagged proteins. In lane 4 and 5 (upper and lower panels) the input proteins are shown. C: The catalytic domain of SUV39H1 physically interacts with EVI1. 293T cells were transfected with Flag-tagged SUV39H1 deletion mutants alone (lanes 1–3) or in presence of HA-EVI1 (lanes 4–6). Total cells extracts were co-IP using an anti-EVI1 antibody and tested in Western blot with anti-Flag antibody. Lanes 1–6 show the total cell proteins while lanes 7–12 indicate the immunoprecipitated proteins. D: Purified GST and GST-SUV39H1(1–81), –(82–248) and –(249–412) conjugated to glutathione-sepharose beads were incubated with in vitro translated ^{35}S-EVI1. The assay was performed as previously described. ^{35}S-Labeled EVI1 interacts with purified GST-SUV39H1(249–412; lane 1) and not with the other SUV39H1 deletion mutants or GST alone (lane 2–4). Lanes 5–8 show the input proteins stained with Coomassie blue.

located at its N-terminus. This domain is frequently found in proteins involved in the assembly of complexes on the chromatin. The SET domain (249–412 aa), which methylates lysine residues in histone and other proteins, is found at the C-terminus. The central region (82–248 aa) contains a regulatory domain enriched in Cys residues. Based on these structural motifs, three deletion mutants ofSUV39H1 were generated (Fig. 1A) to determine the domain responsible for the interaction SUV39H1/EVI1. Immunoprecipitation experiments using Flag-tagged mutants co-expressed with full-length HA-EVI1 were carried out. As shown in Figure 2C (upper right panel) the carboxyl terminus of SUV39H1, including the catalytic domain, appears to contain the major interaction site (lane 12) in that the mutants lacking this region were not able to associate with EVI1 (Fig. 2C, lanes 10 and 11). Lanes 7–9 (upper panel) represent the reaction with only the antibody used as negative control. Lanes 1–6 show the expression of Flag-SUV39H1 deletion mutants (upper panel) and of HA-EVI1 (lower panel). To validate these results, we performed GST pull-down assays. Purified GST-SUV39H1(1–81), GST-SUV39H1(82–248), and GST-SUV39H1(249–412) conjugated to glutathione-sepharose beads were incubated with in vitro translated EVI1. As shown in Figure 2D, lane 1 (upper panel) we found that ^{35}S-labeled EVI1 interacts with purified GST-SUV39H1(249–412) but not with GST-SUV39H1(1–81), GST-SUV39H1(82–248) or GST alone (Fig. 2D, lanes 2–4), confirming the results previously described. Lanes 5–8 in the lower panel show the input proteins used for the pull down.

THE EVI1/SUV39H1 COMPLEX POSSESSES HISTONE METHYLTRANSFERASE ACTIVITY

Histone methylation assays confirmed that in the context of the EVI1/SUV39H1 complex SUV39H1 is fully capable of methylating

H3-K9 in vitro. No HMT activity was observed in cells transiently expressing only EVI1 (Fig. 3A, lane 3); in contrast, in presence of SUV39H1 the recombinant protein histone H3 could be methylated (Fig. 3A, lanes 2 and 4). As positive control cells expressing SUV39H1 alone were used (Fig. 3A, lane 1). A catalytically inactive enzyme harboring a His to Leu mutation at position 324 ($SUV39H1^{H324L}$) was used as negative control. As expected, in the pull-down assay in which EVI1 is transfected with the mutant H324L no histone methylation activity is observed (Fig. 3A, lane 5). Figure 3B, lanes 1–5, shows the input proteins for SUV39H1 (upper panel) and EVI (lower panel), while lane 6 shows the Coomassie stain of the recombinant histone 3 used as a substrate for the reaction. It should be noted that the $SUV39H1^{H324L}$ mutant is still able to interact with EVI1 as shown in Figure 3C (lane 4, upper right panel).

G9a, BUT NOT SET7/9 OR SETDB1, ASSOCIATES WITH EVI1 IN MAMMALIAN CELLS

Tachibana et al., [2001] described a novel lysine HMTase called G9a. In contrast to SUV39H1, G9a is able to methylate not only lysine 9 but also lysine 27 of histone H3. Because its nuclear localization differs significantly from that of SUV39H1, it was suggested that G9a could contribute to the regulation of chromatin structure in other loci rather than being involved solely in the organization of repressive chromatin domains. Another H3K9 methyltransferase studied is SETDB1 that mainly functions in euchromatic region, playing a central role in the silencing of euchromatic genes [Schultz et al., 2002]. SET7/9 is a H3K4 methyltransferase that is associated with transcription activation by competing with histone deacetylases and by precluding H3-K9 methylation by SUV39H1 [Nishioka et al., 2002]. Sequences alignment of the SET domains of these HMTs indicates that the enzymatic domains of SUV39H1 and G9a are highly homologous whereas only few residues are conserved with the domains of SETDB1 and SET7/9 (Fig. 3D). Therefore to determine to which extent the interaction EVI1-SET domain is determined by the domain sequence, we carried out co-IP experiments in 293T using G9a, SETDB1, and SET7/9. As shown in Figure 3D, G9a can be immunoprecipitated by an antibody that recognizes EVI1 when both proteins are co-expressed (upper right panel, lane 3). In contrast, SETDB1 and SET7/9 cannot (upper right panel, lanes 7 and 11). The antibody alone is unable to interact with the proteins alone (lanes 4, 8 and 12). The bottom right panel shows EVI1 detection after co-IP. Lanes 1,2, 5,6, and 9,10 show the expression of the G9a, SET7/9, and SETDB1 (upper left panel). The expression of EVI1 is shown in the lower left panel.

SUV39H1 ENHANCES THE REPRESSIVE POTENTIAL OF EVI1

It has been shown that in reporter gene assays SUV39H1 has repressive potential [Firestein et al., 2000; Nielsen et al., 2001]. Therefore, we investigated whether SUV39H1 could act as a co-repressor of EVI1. Figure 4A shows that SUV39H1 represses the activity of a promoter containing five GAL4 sites in a concentration-dependent manner when Gal4-EVI1 is expressed (bars 3 and 4). This concentration-dependent repression, not observed in absence of EVI1, depends specifically on the wild type SUV39H1, and it is not observed when the methyltransferase domain is inactivated by point mutation (bar 5). The effect of different DNA amounts of SUV39H1 alone on the GAL4 promoter is shown in bar 6 and 7. It should be noted again that both wild type and inactive SUV39H1 physically interact with EVI1 (Figs. 1B–D and 3C).

EVI1 CO-LOCALIZE WITH SUV39H1 IN THE NUCLEUS OF HeLa CELLS

We previously reported that in the majority of EVI1-positive cells EVI1 shows a nuclear diffused localization pattern and that EVI1 appears to be assembled in nuclear speckles only in a minority of nuclei [Chakraborty et al., 2001]. We also showed that the percentage of cells with EVI1 nuclear speckles significantly increases when acetyltransferases are co-expressed with EVI1 [Chakraborty et al., 2001]. It has been reported that SUV39H1 is prevalently organized in nuclear speckles. To determine whether there is any change in nuclear localization when EVI1 and SUV39H1 are co-expressed, we used IF in HeLa cells expressing EVI1, SUV39H1, or both proteins. We confirmed that EVI1 has either a diffused or a speckled appearance (Fig. 4B, red color) and that SUV39H1 (green color) is observed mostly in speckled bodies. When both proteins are expressed, EVI1 and SUV39H1 form speckles that partially overlap (yellow color), confirming the association between the two proteins in vivo. In addition we found that when SUV39H1 is co-expressed the number of nuclei in which EVI1 is assembled in nuclear speckles increases from 21.5% (43 out of 200 cells counted) to 36.5% (73 out of 200 cells counted; not shown), suggesting a influence of the HMTase in the nuclear localization of EVI1.

Even though the association between the inappropriate expression of EVI1 and the development of acute leukemia or myelodysplastic syndrome has been known for many years, only recently very large studies have shown how frequently this gene is activated in these diseases and how poor is the prognosis for EVI1-positive patients. Despite the combined results of several investigators, the normal or inappropriate role of this protein is still unclear, at least in part because EVI1 is a complex transcription factor with multiple structural domains potentially capable of forming high molecular size nuclear complexes that include co-activators (i.e., CBP and P/CAF), by which EVI1 itself is acetylated, and co-repressors (i.e., HDACs and CtBP1) [Chakraborty et al., 2001; Izutsu et al., 2001; Palmer et al., 2001; Nitta et al., 2005]. In this work we describe a new interaction partner of EVI1. We found that EVI1 contacts SUV39H1 through its N-terminus where the proximal zinc finger domain is located and that the HMTase function is preserved in a complex between the two proteins. Even though we here propose a possible mechanism of transcription repression by EVI1 that involved H3K9 methylation, we cannot exclude that in vivo the interaction between the two proteins could have also positive effect on gene transcription. Recent studies have shown, in fact, that this particular histone modification might be associated not only with gene silencing but also with gene activation [Komashko et al., 2008; Wiencke et al., 2008].

In recent years several reports have shown that many co-activators and co-repressors can interact with each other even though they have opposite functions. For example, we and others reported that CtBP interacts with P/CAF and that SUV39H1 interacts with HDACs [Vaute et al., 2002; Senyuk et al., 2005]. Despite the fact

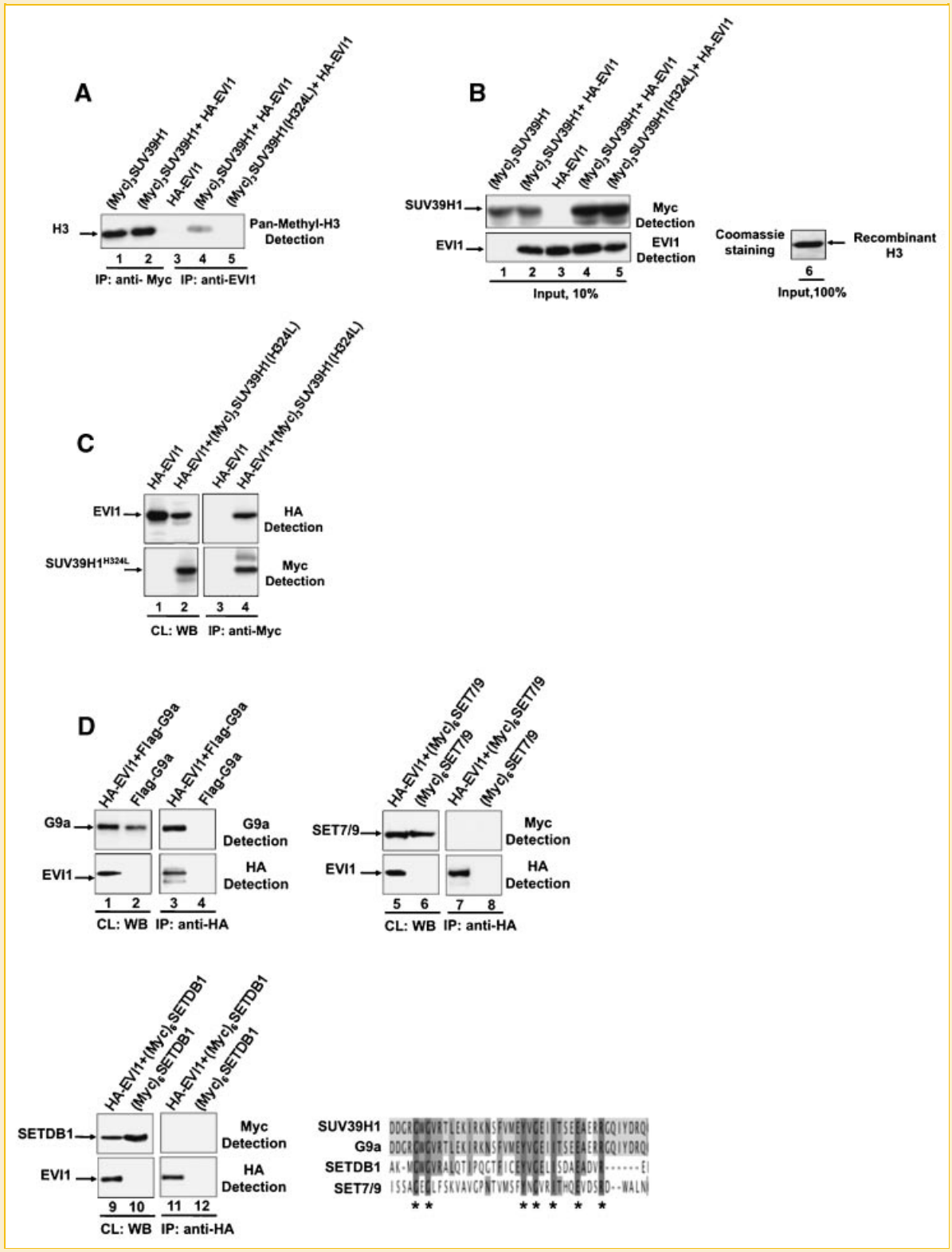

Fig. 3. A: EVI1 does not impair the histone methyltransferase activity associated with SUV39H1. Detection of H3-K9 methylation after HMT assay using a Pan-MethylH3Lys9 antibody. In lanes 1 and 2 anti-Myc was used to immunoprecipitate the complex, in lanes 3–5 the EVI1 antibody was used. Histone methylation can be detected only when EVI1 is co-expressed with SUV39H1 (lanes 2 and 4). B: Lanes 1–5 show the Western blot results using anti-Myc antibody (upper panel) or anti-EVI1 antibody (lower panel). Lane 6 shows the Coomassie blue staining of the recombinant histone H3 used as input. C: The inactive mutant SUV39H1 is still able to interact with EVI1. 293T cells were transiently transfected with expression vectors for HA-EVI1 and (Myc)3-SUV39H1^{H324L} as indicated. Whole cell extracts were immunoprecipitated with anti-Myc antibody and the presence of EVI1 was detected by Western blot using an anti-HA antibody. Lanes 1 and 2 show the expression level of HA-EVI1 (upper panel) and $(Myc)_3$-SUV39H1^{H324L} (lower panel). Lanes 3 and 4 show that the interaction between EVI1 and the SUV39H1 mutant is maintained (lane 4, upper panel). D: EVI1 can interact with the HMTase G9a but not with SET7/9 and SETDB1. 293T cells were transfected with Flag-G9a (lanes 2 and 4), $(Myc)_6$SET7/9 (lanes 6 and 8), $(Myc)_6$SETDB1 alone (lanes 11 and 12) or with HA-EVI1 (lanes 1 and 3, 5 and 7, 9 and 11). Whole cell extracts were immunoprecipitated with anti-HA antibody and the presence of G9a, SET7/9 or SETDB1 was detected by Western blot analysis using an anti-G9a or an anti-Myc antibody. Lanes 1 and 2 show the expression level of Flag-G9a (upper panel) and HA-EVI1 (lower panel). Lanes 3 and 4 show the immunoprecipitated proteins. Lanes 5 and 6 show the expression level of $(Myc)_6$SET7/9 (upper panel) and HA-EVI1 (lower panel). Lanes 7 and 8 show the immunoprecipitated proteins. Lanes 9 and 10 show the expression level of $(Myc)_6$SETDB1 (upper panel) and HA-EVI1 (lower panel). Lanes 11 and 12 show the immunoprecipitated proteins. Alignment of part of the SET domains of the four proteins is shown. The amino acids conserved between the proteins are indicated below the sequences with an asterisk (*).

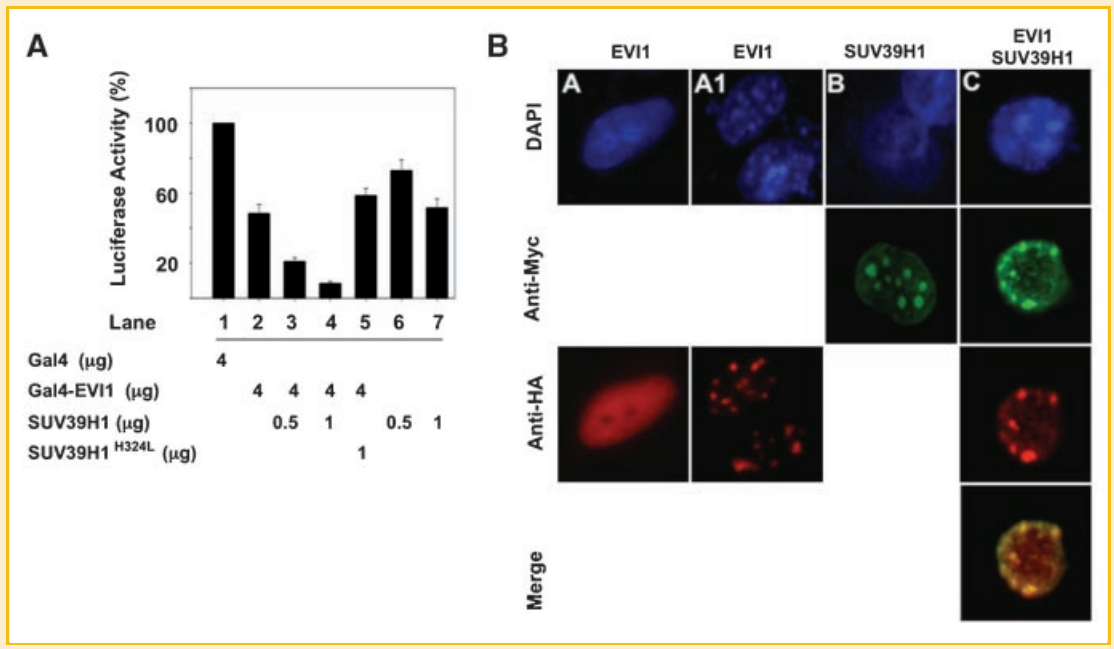

Fig. 4. A: Active SUV39H1 enhances the transcription repression potential of EVI1. Expression plasmids encoding the Gal4 DNA-binding domain (Gal4) or the Gal4-EVI1 fusion protein were used. EVI1 represses the activity of the promoter (bar 2). SUV39H1 by itself has a moderate repressive effect on the promoter (bars 6 and 7). However, when both EVI1 and SUV39H1 are co-expressed, there is a very significant repression that depends on SUV39H1 concentration (bars 3 and 4). This effect is not seen with the dominant negative SUV39H1 (bar 5). B: Immunofluorescence microscopy analysis of EVI1 and SUV39H1 in HeLa cells. EVI1 and SUV39H1 co-localize in the nucleus. Diffused and speckled appearance of EVI1 (panels A and A1), and nuclear speckles of SUV39H1 (panel B). Panel C shows colocalization of the two proteins in the nucleus.

that EVI1 has been known for well over 20 years, there is only one report of EVI1 binding to a specific promoter and most reporter gene studies suggest that while EVI1 can generally function as a non DNA-binding transcription repressor, in specific cases it could also activate a promoter. The absence of a clear DNA consensus target, the lack of evidence that EVI1 behaves as a classic transcription factor, and the diverse chromatin modifiers with which EVI1 interacts suggest that perhaps this oncoprotein could function also as a tissue- and development-specific central factor with scaffold properties around which co-activators and co-repressors assemble to generate higher order complexes associated with chromatin repression.

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