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A novel interaction between the proto-oncogene Evi1 and histone methyltransferases, SUV39H1 and G9a

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Abstract **The transcription factor ecotropic viral integration site 1 (Evi1) is associated with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) in patients due to chromosomal aberration of chromosome 3. Here we show that Evi1 interacts with the histone methyltransferase SUV39H1. The interaction requires the N-terminal part of Evi1 and the H3-specific histone methyltransferase domain, SET, of SUV39H1 without Evi1 having an inhibitory effect on SUV39H1 methyltransferase activity. Presence of SUV39H1 enhances Evi1 transcriptional repression in a dose dependent manner. In addition, Evi1 also interacts with another histone methyltransferase, G9a, but not with SET9. Our data establish an epigenetic role of Evi1 in cell transformation by recruiting higher order chromatin remodeling complexes.**

Keywords: Evi1; SUV39H1; G9a; Histone methyltransferase; Transcription; Repression; AML

1. Introduction

Ecotropic viral integration site 1 (Evi1) encodes a zinc finger transcriptional regulator which has been identified as a common virus integration site in murine leukemia [1]. In humans, aberrant expression of Evi1 has been observed in myeloproliferative disorders including myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML), particularly in patients with translocations or inversions involving chromosome 3q26 [2]. In vitro and in vivo studies support the notion that inappropriate expression of Evi1 plays a critical role in the development of MDS and AML. Overexpression of Evi1 blocks the differentiation of myeloid progenitor cell line 32Dcl3 towards mature neutrophils [3], whereas in early erythroid lineage cell line model 32DEpo1 overexpression of Evi1 diminishes erythropoietin sensitivity [4].

Evi1 contains two DNA binding motifs. The N-terminal part of the protein possesses a domain containing seven zinc fingers, which recognizes GACAAGATAA(GATAA) nucleotide sequences, whereas a region of three zinc fingers at the C-terminus binds GAAGATGAG sequences [5–7]. One of the possible mechanisms that may explain the oncogenic effect of Evi1 is the direct binding and interaction with putative target genes [8,9]. Another possible mechanism of transformation may be through complex formation with other proteins that are critical for transcriptional regulation. In fact, Evi1 has been shown to interact with several transcriptional regulators like Smad3, HDAC's, and CtBP, to mediate transcriptional repression [10–14].

In recent years, the interest in post-translational modifications of histone tails has unveiled the important role of chromatin remodeling in transcriptional regulation. Modifications at specific histone residues have important biological effects on transcription [15]. Methylation has been one of the first modifications shown to play an important role in chromatin regulation. Histone H3 methylation at different lysine residues by specific methyltransferases has been linked to activation and silencing of transcription [16–18]. The first protein demonstrated to possess methyltransferase specificity towards histone H3 lysine 9 (H3-K9) was the mammalian SUV39H1 enzyme, a homologue of *Schizosaccharomyces pombe* Clr4 and *Drosophila* Su(Var)3.9 [19]. This preferential methylation of H3 depends on the SET domain (Suppressor of Variegation, Enhancer of Zeste and Trithorax [20]) of SUV39H1, a 130 amino acid domain that is conserved among different species. In addition to SUV39H1, several other methyltransferases have been identified, e.g. SET7, SET9 or G9a, which display methylation activities specific to histone H3-lysine 4, lysine 9 and lysine 27 [16–18,21].

Here we investigate whether Evi1 can form a complex with histone methyltransferases and show that it physically interacts with SUV39H1. This interaction involves the SET domain of SUV39H1 and the N-terminus of Evi1. These results establish that Evi1 can act as a transcriptional regulator that is able to form higher order complexes with histone methyltransferases and thereby influence the transcriptional repression of putative target genes.

2. Materials and methods

2.1. Expression constructs

A FLAG or an HA epitope tag was fused in frame with the first ATG of Evi1 cDNA and cloned into the pCMV mammalian expression vector (Clontech, Palo Alto, CA). All deletion mutants were generated from the original cDNA of FLAG-*Evi1* using available restriction sites (available upon request). Full length *SUV39H1* and its mutants were a kind gift from Dr. A.H. Peters (IMP, Vienna, Austria). All SUV39H1 constructs were tagged with triple-Myc at the amino terminus. The pCMV-FLAG-*G9a* expression vector was a kind gift from Dr. I. Talianidis (Institute of Molecular Biology & Biotechnology, Crete, Greece) and has been described previously [22]. The

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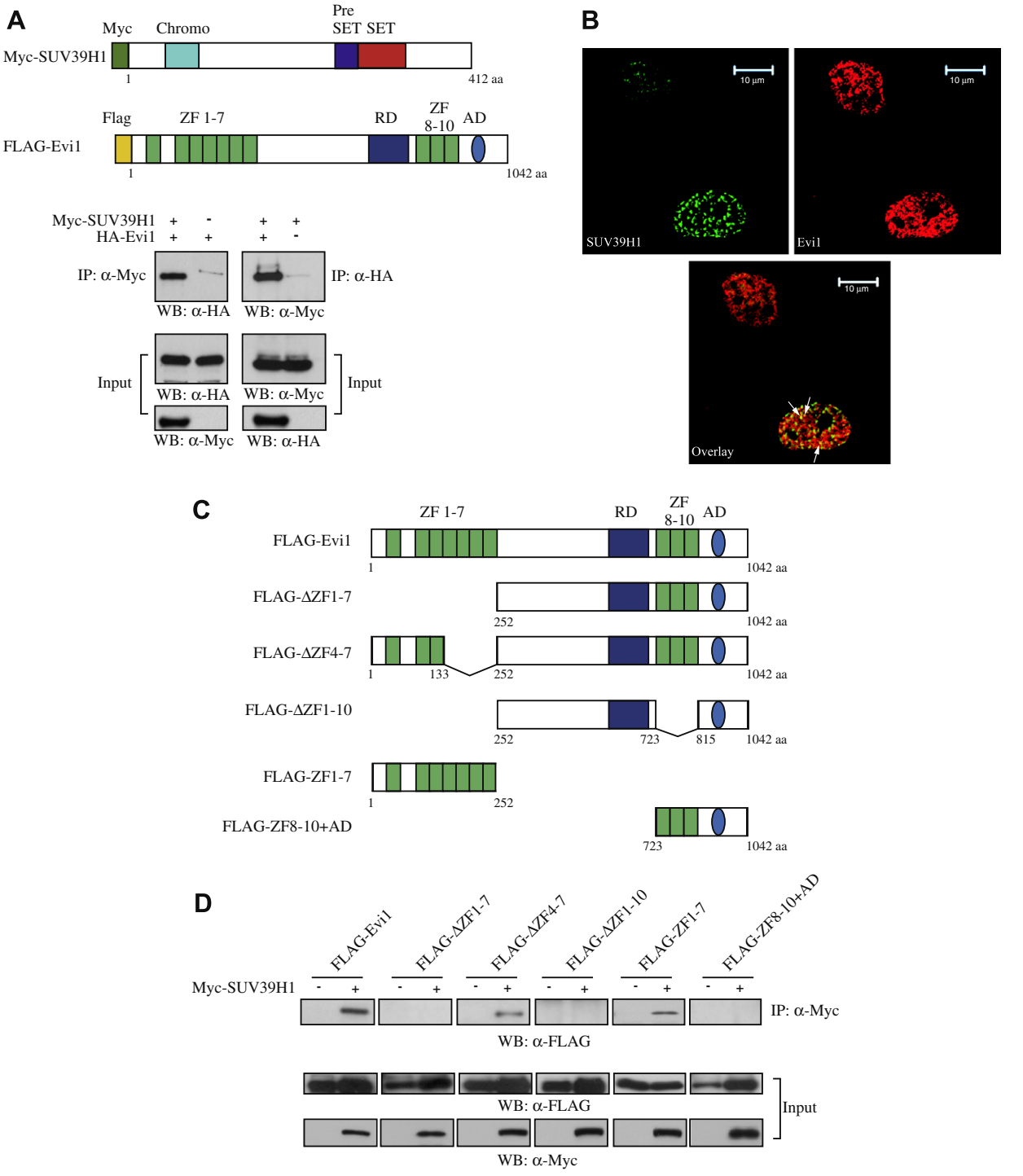

Fig. 1. Evi1 interacts with SUV39H1 via its N-terminal zinc finger domain. (A) Schematic representation of SUV39H1 and Evi1. Protein extracts from cells transiently transfected with Myc-SUV39H1 and HA-Evi1 constructs were immunoprecipitated with anti-Myc (left panel) or anti-HA (right panel) antibodies followed by Western blot (WB) analysis. In control experiments, immunoprecipitations were performed with lysates that were transfected with HA-Evi1 alone (left panel) or Myc-SUV39H1 alone (right panel). Ten percent of total protein lysates were used as input for WB. (B) Cells were co-transfected with Myc-SUV39H1 and HA-Evi1 and stained with anti-Myc and anti-HA antibodies followed by secondary TRITC (Red) and FITC (Green) staining, respectively. Merged images revealed the association of both proteins in speckled structures of the nucleus. (C) Diagram representing full length Evi1 and various mutants used in the mapping analysis with full length SUV39H1. Evi1 domains: zinc fingers (ZF) 1–7, RD (repression domain), ZF 8–10, AD (acidic domain). In all constructs the Tag was cloned such that it was expressed at the N-terminus of the proteins. (D) Full length Myc-SUV39H1 was transiently transfected with full length FLAG-Evi1 or with one of its mutants. Immunoprecipitation was performed using an anti-Myc antibody. Proteins were detected by WB analysis using an anti-FLAG antibody. Ten percent of total protein lysates were used as input for the WB analysis and are depicted in the lower panel.

cDNA of *SET9* was amplified from the human cell line HL-60 using *Pfu* polymerase and cloned into a pCMV-FLAG expression vector to generate a pCMV-FLAG-*SET9* construct.

2.2. Immunoprecipitation, Western Blot, protein purification, and in vitro histone methyltransferase (HMTase) assay

2×10^6 phoenix E (φE) cells were seeded in 100 mm dishes (Becton Dickinson, Franklin Lakes, NJ) and cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Paisley, UK) supplemented with 10% FCS and transfected with 20 μg DNA using calcium phosphate co-precipitation [23]. Cells were harvested after 48 h, washed with ice-cold PBS and lysed in ice-cold lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% NP-40, 10% glycerol) containing protease inhibitor mix and Pefablock (Roche, Zwijndrecht, NL). Protein complexes were incubated overnight at 4 °C. Proteins bound to sepharose G-beads were collected, washed extensively with ice-cold lysis buffer and resuspended in 1× SDS Laemmli buffer [24]. Complexes were denatured, separated on an 8–10% SDS–PAGE gel and transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Primary antibodies were detected with a secondary horseradish peroxidase-conjugated antibody (Dako Diagnostic BV, Denmark) and visualized by enhanced chemiluminescence (ECL, Boston, MA). HMTase activity was assayed as described previously [21]. Proteins were visualized by staining SDS–PAGE gels with Coomassie Brilliant Blue R-250 (N.V. Life Technologies S.A., Merelbeke, NL).

2.3. Immunofluorescence microscopy

COS-7 cells were grown on cover slips and transfected with equal amount (4 μg) of Myc-SUV39H1 and HA-Evi1 constructs using the FuGENE transfection reagent (Roche, Zwijndrecht, NL). Forty-eight hours post-transfection, cells were washed with cold PBS and processed as described previously [25]. Cells were stained with anti-HA and anti-Myc primary antibodies (Santa Cruz Biotechnology) and tetramethylrhodamine isothiocyanate (TRITC) and fluorescein isothiocyanate (FITC) conjugated secondary antibodies (Dako Diagnostics BV, Denmark). Cells were analyzed on a Zeiss confocal laser scanning microscope (LSM510).

2.4. Histone H3 pull down assay

Mouse histone H3 cDNA cloned into the pGEX-4T-3 (Amersham Pharmacia Biotech) bacterial expression vector was a kind gift from Dr. Y. Shinkai (Department of Cell Biology, Kyoto University, Japan). The isolation and purification of the fusion protein was performed as described previously [21]. Recombinant proteins were expressed in *Escherichia coli* and purified using glutathione beads. The concentration was quantified by Coomassie Brilliant Blue R-250 staining. Equal concentrations of the GST-H3 recombinant protein were incubated with cell extracts from φE cells transfected with Myc-*SUV39H1* with or without HA-*Evi1*. The complexes were incubated overnight at 4 °C, washed with ice-cold lysis buffer and separated on an 8% SDS–PAGE gel. Detection with antibodies was performed as described above.

2.5. Luciferase reporter assays

The luciferase reporter assays were performed as described previously [26]. In short, φE cells were grown in 24-well plates at a density of 1×10^5 cells per well and transfected with 300 ng 4xGAL-TK-Luc, 100 ng pcDNA3-DBD-*Evi1*, 300 ng pRSVLacZ and various concentrations of pCMV-*SUV39H1*. The total amount of plasmid DNA was normalized to 1 μg using empty pCMV expression vector.

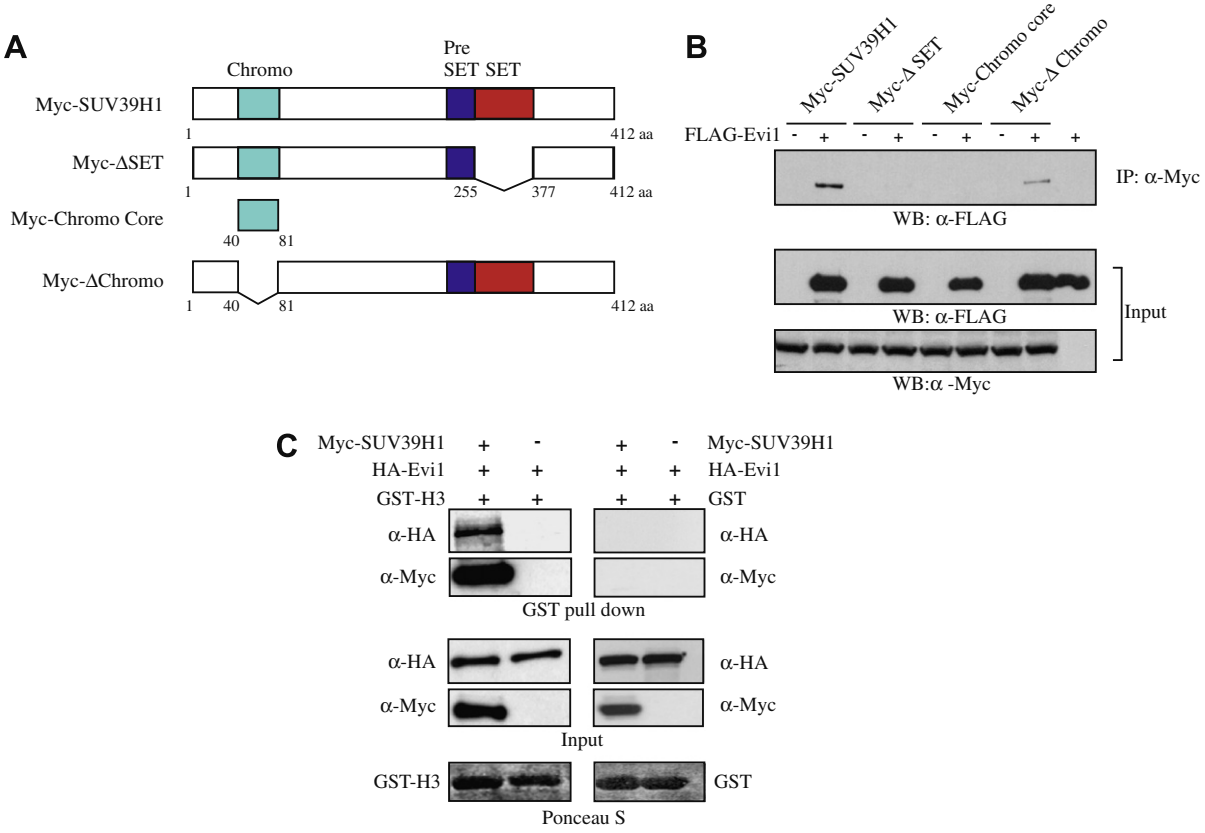

Fig. 2. The SET domain of SUV39H1 is required for its interaction with Evi1 and this complex binds the H3 N-terminus. (A) Schematic representations of SUV39H1 and its mutants. (B) Full length Myc-SUV39H1 and its various mutants were transfected with full length FLAG-Evi1. Immunoprecipitation was performed using an anti-FLAG antibody. Proteins were detected by WB analysis using an anti-Myc antibody. Ten percent of total protein lysates were used as input for WB. (C) Pull-down assays using GST-H3 (left panel) or GST alone (right panel) from cell extracts transfected with HA-Evi1 and Myc-SUV39H1 or HA-Evi1 alone. Proteins were detected by WB. Purified GST-H3 or GST concentrations were determined by Ponceau S staining (bottom panel).

3. Results and discussion

3.1. Evi1 interacts with SUV39H1 via its N-terminal zinc finger domain

The Evi1 protein has been shown to physically and functionally interact with different classes of nuclear proteins involved in transcriptional control [11–13]. To establish whether Evi1 is able to interact with SUV39H1, φE cells were transiently transfected with Myc-*SUV39H1* and HA-*Evi1*. SUV39H1 and Evi1 complexes were immunoprecipitated using anti-Myc or anti-HA antibodies. Analysis of these complexes on Western blots revealed the presence of Myc-SUV39H1 in HA-Evi1 immunoprecipitates. Likewise, HA-Evi1 could be detected in Myc-SUV39H1 immunoprecipitates (Fig. 1A). The interaction is specific, as only very low background levels of HA-Evi1 or Myc-SUV39H1 were observed in single transfected cells using anti-Myc or anti-HA antibodies, respectively. Furthermore, immunofluorescence analysis and confocal microscopy showed partial co-localization between Evi1 and SUV39H1 in cells transiently transfected with both constructs (Fig. 1B). The results of these experiments illustrate that the two proteins interact with each other in intact cells.

To identify the region of Evi1 required for the interaction with SUV39H1 we constructed a panel of FLAG-*Evi1* mutants (Fig. 1C). Full length Myc-SUV39H1 was only able to interact with FLAG-Evi1 mutants that contained an intact N-terminal zinc finger domain (FLAG-ZF 1–7) or that only missed zinc fingers 4–7 (FLAG-*Evi1*Δ4–7). This indicates that the region required for interaction is located within the first zinc finger domain (Fig. 1D).

Our data thus demonstrate an association between Evi1 and SUV39H1, a member of the histone methyltransferase family of genes.

4. The SET domain of SUV39H1 binds Evi1 and the complex specifically interacts with the histone H3 N-terminus

SUV39H1 is a transcriptional repressor that methylates histone residues, but requires specific DNA binding proteins for its recruitment to particular promoter regions [27]. To identify which domain of SUV39H1 is responsible for the interaction with Evi1, we carried out co-immunoprecipitation experiments with full length HA-Evi1 and various Myc-SUV39H1 mutants

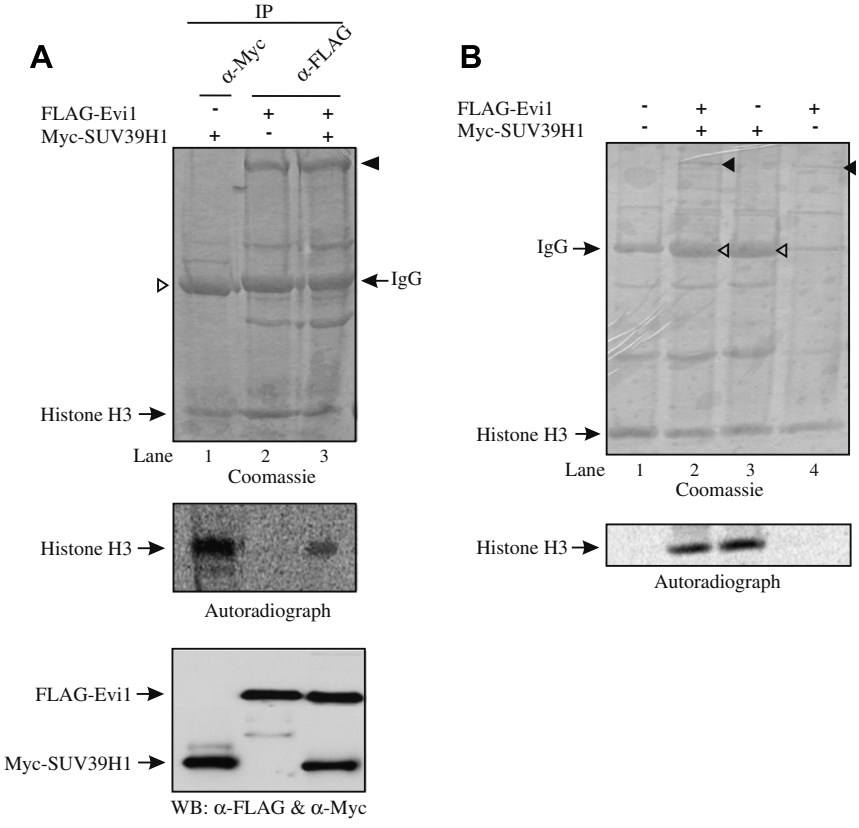

Fig. 3. Histone H3 methylation occurs in an Evi1 immunoprecipitate only when SUV39H1 is present. Evi1 does not interfere with SUV39H1 histone methyltransferase activity. (A) Immunoprecipitates obtained using anti-FLAG or anti-Myc antibodies from cells transfected with FLAG-Evi1/Myc-SUV39H1, or FLAG-Evi1 or Myc-SUV39H1 alone were analyzed in HMTase assay. Closed arrow heads indicate purified FLAG-Evi1; open arrow heads indicate Myc-SUV39H1. The presence of histone H3 peptide is highlighted in each lane by an arrow. Myc-SUV39H1 was immunoprecipitated with anti-Myc antibody and used as a positive control (left lane). Histone H3 methylation is demonstrated by autoradiography in the middle panel. Lower panel: FLAG-Evi1 and Myc-SUV39H1 concentrations as determined by WB. (B) FLAG-Evi1 and Myc-SUV39H1 were transfected separately into φE cells. Immunoprecipitated complexes either alone or in combination were analyzed in an HMTase assay. The upper figure represents a Coomassie Blue staining and demonstrates the presence of FLAG-Evi1 in lanes 2 and 3 (closed arrow heads). Myc-SUV39H1 are in lanes 2 and 3 (open arrow heads), but are not visible as they migrate together with the IgG heavy chain. Lane 1 is a control precipitation using an irrelevant antibody. Precipitates were incubated with histone H3 peptide and an HMTase assay was performed followed by SDS–PAGE electrophoresis. Equal amounts of histone H3 peptide were present in each lane (upper figure). Specific H3 methylations in the lysates were demonstrated by autoradiography (lower panel).

(Fig. 2A). *SUV39H1* contains several functional domains that have all been shown to play an important role in the function of the protein. Our results illustrate that absence of the SET domain, which is responsible for the methylation of H3-K9, completely abolishes the interaction with Evi1 (Fig. 2B). In contrast, co-expression with Myc-SUV39H1-Δ chromo or Myc-SUV39H1 constructs, which retain the SET domain, preserved the interaction with Evi1.

Since H3-K9 is known to be a specific target of the SUV39H1 enzyme, we investigated if SUV39H1 and Evi1 can form a complex at the H3 tail using GST-pull down experiments with GST recombinant protein fused to the histone H3 N-terminal tail (amino acids 1–57). φE cells transfected with HA-Evi1 alone or in combination with Myc-SUV39H1 were lysed and incubated with equal concentrations of GST-H3 (Fig. 2C). Staining with an anti-HA antibody revealed the presence of Evi1 in the samples that also contained SUV39H1, while no HA-Evi1 signal was observed in the control samples with HA-Evi1 and GST-H3 alone. Collectively, these data demonstrate that the Evi1–SUV39H1 complex is able to bind the histone H3 tail in vitro, which may affect the expression of putative Evi1 target genes.

5. The Evi1–SUV39H1 complex displays methyltransferase activity, however Evi1 does not interfere with SUV39H1 activity

In accordance with the "histone code", modifications of the histone H3 N-terminus influence the chromatin state, resulting in either silenced heterochromatin, due to specific lysine methylation, or active euchromatin, due to acetylation. SUV39H1 possesses histone methyltransferase activity specific for H3-K9 [19]. To investigate whether the Evi1–SUV39H1 complex retains this activity, we transfected φE cells with FLAG-Evi1, Myc-SUV39H1 or with both constructs. Immunoprecipitation of SUV39H1 from single transfected cells clearly showed methylation of histone H3 as expected (Fig. 3A, lane 1), while immunoprecipitated Evi1 alone did not show any activity (Fig. 3A, lane 2). Notably, Evi1 immunoprecipitated from cells transfected with both constructs showed a clear methylation signal, indicating that the Evi1–SUV39H1 complex possesses methyltransferase activity (Fig. 3A, lane 3).

The in vitro binding analyses (Fig. 2) suggest that the interaction between Evi1 and SUV39H1 requires a specific region within the SET domain of SUV39H1. As this domain is also required for the enzymatic activity of SUV39H1 [27], we asked the question if the interaction between Evi1 and SUV39H1 might influence its histone methyltransferase activity. φE cells were transfected with Myc-SUV39H1 or FLAG-Evi1 and the proteins were subsequently immunoprecipitated with anti-Myc or anti-FLAG antibodies (Fig. 3B, upper panel). Purified immunoprecipitates were combined and the methyltransferase activity of the complexes was analyzed in HMTase assays (Fig. 3B; lower panel). The results demonstrate that the activity of Myc-SUV39H1 is unchanged in the presence of the FLAG-Evi1 protein (Fig. 3B, lower panel lane 2) as compared to a sample without FLAG-Evi1 (Fig. 3B, lower panel lane 3). Therefore, the interaction between Evi1 and SUV39H1, although it occurs through the SET domain, appears not to influence the histone methyltransferase activity of SUV39H1.

6. SUV39H1 binding enhances transcriptional repression by Evi1

To determine if the interaction between SUV39H1 and Evi1 may directly influence the transcriptional repression by Evi1, we performed a luciferase reporter assay. We used the Gal4-DBD and Gal4-AD-Luc system, which is commonly used to measure transcriptional activity of interacting proteins [28,29]. A GAL4-DBD-*Evi1* construct in combination with a Myc-*SUV39H1* plasmid and the luciferase reporter gene (GAL4-AD-Luc) were transiently transfected into φE cells (Fig. 4A). Expression of low concentrations of Evi1 did not significantly repress luciferase expression (Fig. 4B). However, cells transfected with GAL4-DBD-Evi1 plus increasing concentrations of Myc-SUV39H1 showed significant repression

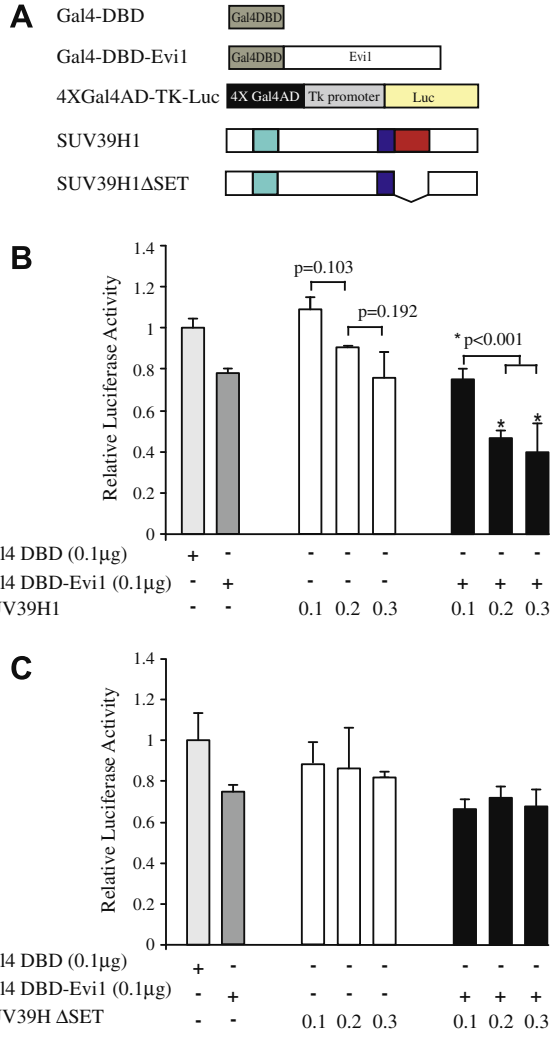

Fig. 4. SUV39H1 enhances the transcriptional repression activity of Evi1. (A) Schematic representation of the constructs used for the luciferase assay: Gal4 DNA binding domain (Gal4-DBD), Evi1 fused to the Gal4 DNA binding domain (Gal4-DBD-Evi1), Luciferase reporter plasmid with four Gal4 binding sites upstream of the thymidine kinase promoter (4xGal4AD-TK-Luc). (B) Cells were co-transfected with 4xGal4AD-TK-Luc, Gal4-DBD-Evi1 and different concentrations of the SUV39H1 expression plasmid; concentrations shown are in micrograms of DNA. All luciferase assays were performed in triplicates in two independent experiments. (C) Luciferase experiment using SUV39H1ΔSET instead of SUV39H1. The experimental set-up was as described above.

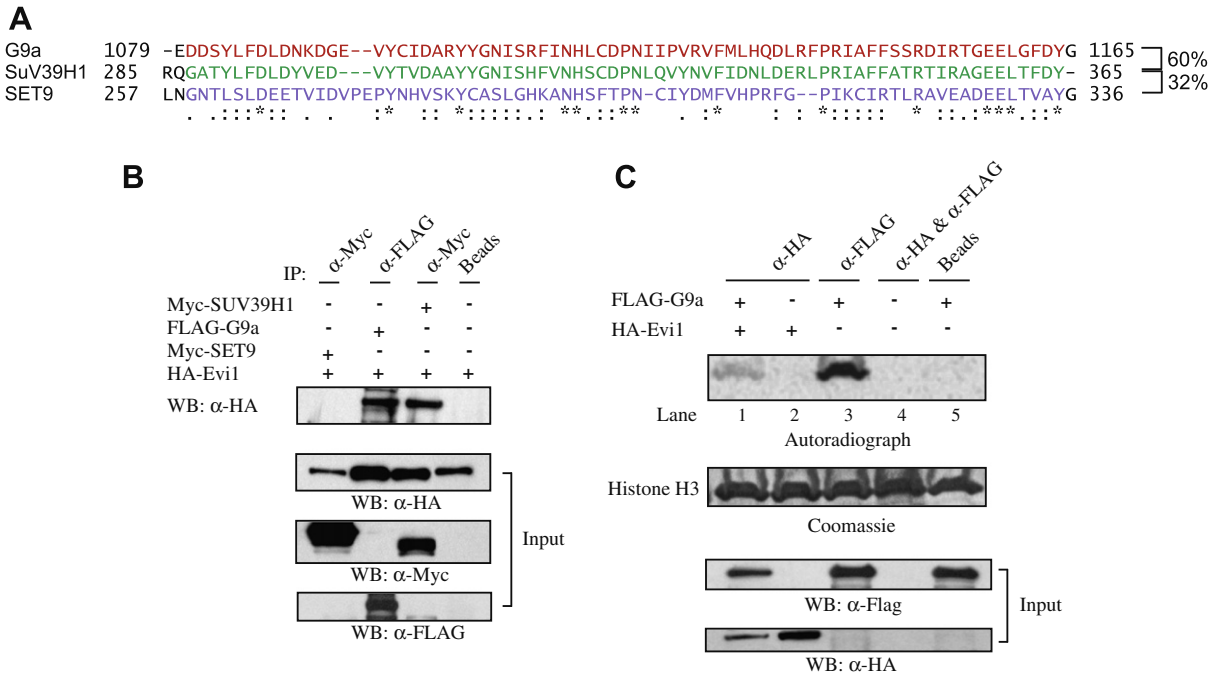

Fig. 5. Evi1 binds to the methyltransferase protein G9a but not to SET9. (A) Protein sequence alignment of the SET domains of SUV39H1, G9a and SET9 (B). φE cells were transiently transfected with Myc-SET9, FLAG-G9a, Myc-SUV39H1, HA-Evi1 or control (*ϕ*). The immunoprecipitates were analyzed with an anti-HA antibody for the presence of Evi1. Protein lysates were analyzed by WB for the presence of input proteins (lower three panels). (C) HMTase assay with FLAG-G9a and HA-Evi1. Lysates from cells transiently transfected with FLAG-G9a and HA-Evi1 were immunoprecipitated with an anti-HA antibody and analyzed for specific histone H3 methylation activity (top panel). FLAG-G9a was purified with an anti-FLAG antibody and used as a positive control. Presence of G9a and Evi1 proteins in samples was detected by WB.

of luciferase activity using *T*-test analysis (Fig. 4B, $P = 0.001$). In contrast, we did not observe significant changes in the repression of the luciferase activity in an experiment in which the SET domain of SUV39H1 was deleted (Fig. 4C). In summary, SUV39H1, through its SET domain, cooperates with Evi1 in vivo and enhances the transcriptional repression activity of Evi1 in a dose dependent manner.

7. Evi1 interacts with selected SET domain containing proteins

SET domains are found in many proteins and consist of a 130 amino acid motif first identified in members of the Polycomb group (PcG) and Trithorax group (TrG) of genes [20]. To date, several mammalian SET domain proteins have been identified and shown to associate directly or indirectly with chromatin [15,29]. We performed immunoprecipitation assays with two other known methyltransferase proteins, as the amino acid alignment of the SET domains showed high conservation between SUV39H1, G9a and SET9 (Fig. 5A).

HA-*Evi1* was co-transfected with Myc-SET9, FLAG-G9a or Myc-SUV39H1. Complexes were immunoprecipitated with anti-FLAG/anti-Myc antibodies and analyzed for the presence of Evi1. The experiment in Fig. 5B demonstrates that FLAG-G9a (lane 2) and SUV39H1 (lane 3) each form a complex with Evi1, whereas Myc-SET9 did not show any interaction with HA-Evi1 (lane 1). No HA-Evi1 was detected in immunoprecipitation experiments in the absence of anti-FLAG/anti-Myc antibody (beads only; lane 4). Similarly, when anti-HA was used for the immunoprecipitation experiments, only G9a and SUV39H1 could be detected by anti-FLAG Western blot analysis (data not shown).

To confirm that the enzymatic activity of G9a could be specifically precipitated in a complex with Evi1, we performed an HMTase assay on H3-histones using HA-Evi1/FLAG-G9a transfected cells. H3 peptide methylation was demonstrated with immunoprecipitated FLAG-G9a methyltransferase (Fig. 5C, lane 3), whereas this activity was not detectable when the same lysate was exposed to beads without anti-FLAG antibody (Fig. 5C, lane 5). Using an anti-HA antibody, we immunoprecipitated HA-Evi1 with G9a and observed a clear methylation of purified H3 (Fig. 5C, lane 1). Autoradiography revealed no signal in the precipitates obtained in the absence of either anti-HA or anti-FLAG antibodies (Fig. 5C, lanes 4 and 5) or when no G9a (lane 2) or Evi1 (lane 4) were introduced into cells.

Taken together, the results presented in this paper illustrate that Evi1 is able to form a complex with two methyltransferases, SUV39H1 and G9a, which may affect transcriptional repression of putative Evi1 target genes.

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