Myeloid Transforming Protein Evi1 Interacts with Methyl-CpG Binding Domain Protein 3 and Inhibits in Vitro Histone Deacetylation by Mbd3/Mi-2/NuRD[†]

Dominik Spensberger,[‡] Michiel Vermeulen,[§] Xavier Le Guezennec,[§] Renee Beekman,[‡] Antoinette van Hoven,[‡] Eric Bindels,[‡] Henk Stunnenberg,[§] and Ruud Delwel*[,‡]

Department of Hematology, Erasmus University Medical Center, Rotterdam 3015 GE, The Netherlands, and Department of Molecular Biology, University of Nijmegen, Nijmegen 6525 GA, The Netherlands

Received February 15, 2008; Revised Manuscript Received April 18, 2008

ABSTRACT: The ecotropic viral integration site 1 (*Evi1*) gene encodes a putative transcription regulator, which is aberrantly expressed in acute myeloid leukemias (AML) with chromosomal abnormalities involving the 3q26 locus. Repression and activation of transcriptional control have been reported, but it is currently unclear how Evi1 may evoke these opposing effects. Using a yeast two-hybrid screen, we identified a novel binding partner of Evi1, i.e., methyl binding domain 3b (Mbd3b) protein, a member of the Mi-2/NuRD histone deacetylase complex. Applying in vitro and in vivo assays, we found that Evi1 interacts with Mbd3b but not with other MBD family members Mbd1, -2, and -4 or MeCP2. We show that interaction of Evi1 with Mbd3 requires 40 amino acids that are adjacent and downstream of the methyl binding domain (MBD). We further demonstrate that the first three zinc fingers of Evi1 are needed for Mbd3 interaction. Evi1 acts as a transcriptional repressor when recruited to an active promoter, yet when present in the Mi-2/NuRD complex through Mbd3 interaction, it inhibits the histone deacetylation function of this multiprotein structure. Our data may in part explain how Evi1 could act as a repressor as well as an activator of transcription.

The ecotropic viral integration site 1 (*EVI1*) oncogene is associated with human and murine myeloid leukemias. In humans, *EVI1*[1] is found to be overexpressed in AML patients with translocations or inversions involving chromosome 3 at band q26, e.g., t(3;3)(q21;26) or inv(3)(q21;q26), the locus where the gene resides (*1, 2*). Recently, it has been shown that aberrant *EVI1* mRNA expression also occurs in patients without chromosome 3 abnormalities (*3, 4*).

Evi1 was initially identified in the mouse as a common proviral integration site in virus-induced leukemias (*5*), and overexpression of *Evi1* in mouse myeloid models revealed interference of myeloid development (*6*). Enforced expression of *Evi1* in the red blood cell lineage impairs erythroid differentiation (*7*), whereas in megakaryocytic cells, the gene appears to enhance differentiation (*8*). An in vivo model of *Evi1* expressed from the murine *Sca-1* promoter showed a reduced number of colony-forming units in the erythroid lineage (CFU-E) in the bone marrow (*9*). More recently, bone marrow cells transduced with *EVI1* and transplanted into recipient mice showed development of fatal myelodysplastic syndromes after a latency of 10 months (*10*). Although these in vitro and in vivo findings are in agreement with the clinical manifestations of malignant disease with a high level of *EVI1* expression, the mechanisms by which EVI1 evokes these distinct effects are largely unknown and appear complex.

Evi1 encodes a nuclear protein of 145 kDa with domains characteristic of transcription regulators. It has 10 Cys_2His_2 type zinc fingers, seven of which are at the N-terminus and three of which are in the C-terminal part of the protein. A proline rich region separates the two zinc finger domains, and a small acidic domain is located C-terminal of the second zinc finger domain (*5*). Both zinc finger domains are able to bind specific DNA consensus sequences in vitro: the N-terminal zinc finger domain recognizes the nucleotide sequence (GACAA) GACAAGATAA (*11–13*), and the C-terminal domain is capable of binding to GAAGATGAG (*14*). Although the recognition sequence of the N-terminal zinc finger domain is similar to GATA1 binding sequences and it has been proposed that Evi1may interfere with GATA1 binding sites to deregulate its targets (*7*), binding of Evi1 to GATA1 specific promoter sequences has not been demonstrated. Intriguingly, in a recent study, Laricchia et al. (*15*) reported that EVI1 interacts with GATA1 and interferes with the activity of this erythroid specific transcription factor. These results suggest that Evi1 can bind to transcription regulators and thereby execute its capacity to transform erythroid precursor cells. It has also been demonstrated that

[†] This work was supported by grants from the Dutch Cancer Society Koningin Wilhelmina Fonds (KWF), The Netherlands Organisation for Scientific Research (NWO), and the Association for International Cancer Research.

* To whom correspondence should be addressed: Department of Hematology, Erasmus University Medical Center, Dr. Molenwaterplein 50, Rotterdam 3015 GE, The Netherlands. Telephone: +31 10 40887843. Fax: +31 10 4089470. E-mail: h.delwel@erasmusmc.nl.

[‡] Erasmus University Medical Center.

[§] University of Nijmegen.

[1] Abbreviations: Evi1, ecotropic viral integration site 1; Mbd3b, methyl binding domain 3b; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; GST, glutathione *S*-transferase; CMV, cytomegalovirus; Gal4AD, Gal4 activating domain; Gal4DBD, Gal4 DNA binding domain; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TAP, tandem affinity purification.

10.1021/bi800267f CCC: $40.75
Published on Web 05/24/2008

Smad3 interacts with the first zinc finger domain of Evi1. Consequently, Evi1 disturbs the TGFβ pathway, which is known to be a negative regulator for cellular growth and differentiation (*16*). The repression activity of Evi1 may be due to interaction with the corepressor carboxyl-terminal binding protein (CtBP) (*17, 18*), and with histone deacetylases HDAC1 and HDAC2 (*19*). Although Evi1 may repress transcription of genes critical for development, it may also act as a transcriptional activator (*20*). Thus, the effects of Evi1 in myeloid precursors and its role in transformation appear complex.

Here we demonstrate the identification of a novel Evi1 interacting protein, methyl-CpG binding domain protein Mbd3b, a member of the Mi-2/NuRD transcriptional repressor complex. Mbd3 belongs to the family of five MBD proteins, i.e., MeCP2 and MBD1−4. Each of these protein shares the methyl-CpG binding domain (MBD), although Mbd3, in contrast to the other family members, does not harbor specific methyl CpG binding activity (*21, 22*). In fact, two distinct Mi-2/NuRD-like complexes have been reported, i.e., one that contains Mbd2 and another that harbors Mbd3, called Mbd2/NuRD and Mbd3/NuRD, respectively (*23, 24*). Mi-2/NuRD complexes contain several components, of which MTA2, Mi-2, RbAp46/p48, HDAC1, HDAC2, and either Mbd2 or Mbd3 form the core (*24*). Although the exact difference between the two complexes needs further elucidation, the two differ in a number of proteins present in Mbd2/NuRD that are absent in Mbd3/NuRD. We investigated the interaction of Evi1 and Mbd3b and studied the role Evi1 may have within this complex. Evi1 interaction appears highly specific for Mbd3, as other members of the MBD motif-containing family do not interact with Evi1. We show that the interaction with Mbd3 recruits Evi1 into the Mi-2/NuRD complex. We demonstrate that Evi1, when recruited into the Mi-2/NuRD complex, interferes with the deacetylase activity function evoked by this large molecular structure. These different effects are in line with the observations that Evi1 may repress as well as activate expression of genes critical in hematopoietic development.

MATERIALS AND METHODS

Expression Constructs. Mouse *Evi1* cDNA was fused in frame with FLAG (FLAG-*Evi1*) or HA (HA-*Evi1*) nucleotide sequences and inserted into the pCMV mammalian expression vector (Clontech, Palo Alto, CA). All deletion mutants were generated from the original cDNA of FLAG-*Evi1* using the following restriction sites: *Msc*I for ΔZ1−7, *Eco*47III/*Drd*I for ΔZF8−10, *Msc*I/*Eco*47III/*Drd*I for ΔZF1−10, *Pst*I for Evi1 439, *Eco*47III for Evi723, *Xba*I for Δacidic domain, and *Eco*57I/*Msc*I for ΔZF4−7. Full-length mouse *Mbd3b* was subcloned from the pACT2 vector using *Bgl*II restriction sites and ligated into the *Bam*HI site of the pCMV vector. *Mbd2* mouse cDNA was amplified from a day 17 total embryonic cDNA library using *Pfu* polymerase (Promega, Madison, WI) and primers containing specific restriction sites. All inserts were cloned in-frame with the HA epitope using pBluescript (Stratagene, Amsterdam, The Netherlands) followed by subcloning into the pCMV vector. All constructs were sequenced for correct in-frame fusion, and the expression of all proteins was confirmed by Western blot analysis.

For yeast two-hybrid assays, the Mbd3b mutant constructs were generated using available restriction sites within the pACT2 Mbd3 constructs. Deletion constructs of *Mbd3b* were generated using *Age*I and *Xho*I to generate a Mbd3b mutant lacking the MBD and 40 adjacent amino acids (ΔMBD−40), *Bgl*II and *Age*I to generate a construct expressing the *Mbd3b* MBD and 40 adjacent amino acids (MBD+40), *Nhe*I and *Xho*I to generate a construct expressing the *Mbd3b* glutamic acid repeat domain (EE), and *Bgl*II and *Nhe*I for a *Mbd3b* mutant lacking the EE domain (ΔEE). The *Mbd3b* MBD was amplified using *Pfu* polymerase (Promega) and cloned using *Eco*RI and *Xho*I sites. Amplified fragments were cloned in-frame with GAL4 AD (pACT2 vector) and fully sequenced.

For the preparation of an Mbd3b glutathione *S*-transferase (GST) fusion protein, *Mbd3b* was subcloned into the *Eco*RI site of pGEX-2Tk (Amersham Pharmacia Biotech).

For the luciferase assay experiment, full-length *Mbd3b* was cloned into the pcDNA3.1 GAL4 vector (kindly donated by R. Deplus, Free University of Brussels, Brussels, Belgium), which drives expression of the Gal4 DNA binding domain (DBD) by the CMV promoter. The reporter construct 4xGal4-TK-Luc (kindly donated by R. Deplus, Free University of Brussels) contains four GAL4 duplicate binding sites upstream of the thymidine kinase promoter driving expression of the luciferase gene (*25, 26*).

Yeast Two-Hybrid Protein Interaction Screen. Mouse *Evi1* cDNA was subcloned in-frame into the pGBT9 (Clontech) expression vector with the Gal4 DNA binding domain (BD) to produce a fusion transcript. The construct was introduced by a standard polyethylene glycol/lithium acetate (PEG/LiAc) transformation method (*27*) into yeast strain PJ69-4A (kind gift from P. James, University of Wisconsin, Madison, WI). Subsequently, an expression library from mouse 17 day total embryo cDNA (Clontech) was then introduced into these yeast cells and screened by selection on plates lacking tryptophan (Trp), leucine (Leu), histidine (His), and adenine (Ade), but containing 4 mM 3-aminotriazole (*28*). Selected clones were further analyzed in a liquid culture assay for β-galactosidase expression according to the manufacturer's protocol (Clontech).

Isolation of the plasmid DNA from strain PJ69-4A was performed using QIAprep Spin Miniprep columns (Qiagen, Westburg BV, Leusden, The Netherlands). Positive clones were analyzed by restriction enzyme analysis and nucleotide sequencing (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) to confirm the correct open reading frame with regard to the Gal4-activating domain (AD). The identity of the candidate Evi1 binding partners was determined by using public databases (NCBI).

Immunoprecipitation and Western Blot Analysis. Phoenix E (φE) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Paisley, U.K.) supplemented with 10% FCS. Cells (2×10^6) were seeded in 100 mm^2 dishes (Becton Dickinson, Franklin Lakes, NJ) and transfected with 20 μg of DNA using calcium phosphate transfection (*29*). Cells were harvested 48 h post-transfection, washed once with ice-cold PBS, and lysed on ice for 10 min in ice-cold lysis buffer [20 mM Tris (pH 8.0), 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% NP-40, and 10% glycerol] containing protease inhibitor mix and Pefablock (Roche, Zwijndrecht, The Netherlands). Cell debris was removed by centrifugation for 15 min at 13000 rpm at 4 °C. Supernatants were incubated at 4 °C with an appropriate antibody (1 μg)

for 2 h, followed by overnight incubation with Sepharose G-protein beads (Sigma-Aldrich, St. Louis, MO). Beads containing the bound proteins were collected, washed four times with ice-cold lysis buffer, and resuspended in 1× SDS Laemmli buffer (*30*). Purified complexes were denatured and separated on an 8 to 10% sodium dodecyl sulfate−polyacrylamide electrophoresis (SDS−PAGE) gel, transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), blocked in 4% blocking milk (Bio-Rad, Hercules, CA),and incubated with the appropriate antibodies. Detection was performed using a secondary horseradish peroxidase-conjugated antibody (Dako Diagnostic BV) and visualized by enhanced chemiluminescence according to the manufacturer's protocol (ECL, Boston, MA). The following antibodies were used in this study: anti-HA and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-FLAG (Sigma-Aldrich), anti-EVI1 (a kind gift of J. N. Ihle, Memphis, TN), and anti-MBD3 (IBL, Naka, Japan).

GST Pull Down. Mbd3b was fused in-frame with GST recombinant protein in the pGEX-2TK vector and expressed in *Escherichia coli* DH12. Cells were grown overnight at 37 °C, diluted to an OD_{600} of 0.2, and grown to an OD_{600} of 0.6 before being induced with 1 mM IPTG for 3 h at 37 °C and harvested at 4 °C. Bacteria were resuspended in TEND lysis buffer (1× TBS, 0.5% NP-40, 1 mM EDTA, and 1 mM DTT) containing complete protease inhibitor mix and Pefablock, sonicated, and centrifuged for 10 min at 13000 rpm. The supernatant was incubated with Glutathione beads (Amersham Pharmacia Biotech), for 2 h at 4 °C, and washed extensively with TEND buffer. Purified GST−Mbd3 recombinant protein was incubated overnight at 4 °C with total cell lysates from different FLAG-EVI1 mutants expressed in φE cells. Glutathione beads were pelleted, washed four times in ice-cold TEND lysis buffer, and resupended in 1× SDS Laemmli buffer. Bound proteins were analyzed by SDS−PAGE using Coomassie Brilliant Blue R-250 (N.V Life Technologies S.A., Merelbeke, Belgium).

Immunofluorescence Microscopy. φE cells grown on glass coverslips were calcium phosphate transfected with FLAG-*Evi1* and HA-*Mbd3b* constructs and harvested 48 h post-transfection. Cells were washed once with ice-cold PBS and subjected to immunofluorescence staining as described previously (*31*). Briefly, cells were fixed for 15 min in a 4% paraformaldehyde/PBS mixture and permeabilized with a 0.2% Triton X-100/PBS mixture for 60 min at room temperature. Cells were washed once with 0.05% Tween 20, blocked overnight at 4 °C in 1% BSA with a 0.05% Tween 20/PBS mixture, stained with anti-FLAG and anti-HA MAbs for 2 h at room temperature, washed extensively with a 0.05% Tween 20/PBS mixture, and detected with tetramethylrhodamine isothiocyanate (TRITC) and fluorescein isothiocyanate (FITC) conjugated secondary antibodies (Dako Diagnostic BV) for 1 h at room temperature. After being washed with a 0.05% Tween 20/PBS mixture, cells were embedded in Vectashield (Vector Laboratories, Burlingame, CA). Stained cells were analyzed using a Zeiss confocal laser scanning microscope (LSM510).

Luciferase Reporter Assays. φE cells were plated on 24-well plates at a density of 0.1×10^6 cells/well in 1 mL of DMEM and 10% FCS and cultured overnight, followed by transfection with 100 ng of 4xGAL-TK-Luc, 20 ng of pcDNA3-DBD-*Mbd3b*, 300 ng of pRSVLacZ, and various concentrations of the indicated expression plasmid, and normalized to 1 μg of plasmid using an empty pCMV expression vector. Forty-eight hours post-transfection, cells were washed in PBS and lysed in 100 μL of lysis buffer [25 mM Tris phosphate (pH 7.8), 15% glycerol, 1% Triton X-100, 1 mM DTT, and 8 mM $MgCl_2$]. Cell lysates (25 μL) were transferred to 96-well flat bottom plates (Costar, Corning Inc., Corning, NY), and 25 μL of a 16 mg/mL luciferase substrate-containing buffer (Steady-Glo luciferase assay system, Promega) was added to each well. Light emission intensity was measured using a TopCount luminometer (Packard, Meriden, CT). To control for transfection, β-galactosidase activity was determined using *o*-nitrophenyl β-D-galactopyranoside (ONPG, Sigma). Absorption was measured at 450 nm in a Bio-Rad 450 microplate reader (Bio-Rad, Veenendaal, The Netherlands). All assays were carried out in triplicate.

Purification of the NuRD Complex using TAP-MBD3a. 293T cells, stably expressing TAP (tandem affinity purification)-tagged mouse MBD3a (*23*), were transiently transfected with pCMV-HA-*Evi1*. Cells were lysed in ice-cold lysis buffer [20 mM Tris (pH 8.0), 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% NP-40, and 10% glycerol] and incubated with Sepharose IgG beads for 2 h at 4 °C. Sepharose beads were collected by a brief centrifugation and washed extensively in lysis buffer with an increased salt concentration [20 mM Tris (pH 8.0), 400 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% NP-40, and 10% glycerol]. Members of the complex were visualized using HDAC2 (Santa Cruz Biotechnology), RbAp46, and MTA2 (Abcam, Cambridge, U.K.) antibodies.

Nucleosomal templates reconstituted with recombinant histones were acetylated by the *Saccharomyces cerevisiae* SAGA complex and subsequently incubated with the TAP−MBD3 complex in the absence or presence of Trichostatin-A (TSA) (*32*). H3 acetylation was assessed by Western blotting using antibodies against diacetylated histone H3 Lys-9,14 or tetra-acetylated H4. The binding of the MBD3 complex to the nucleosomal templates was assessed by Western blotting using a Myc antibody. The effects of Evi1 were studied by adding anti-HA immunoprecipitates (IP) of lysates obtained from φE cells transfected with HA-Evi1. As controls we used the same IP lysates from mock or HA-Evi1 mutant infected φE cells.

RESULTS

Evi1 Interacts with the 40-Amino Acid Region Adjacent to the Methyl-CpG Binding Domain of Mbd3 in a Yeast Two-Hybrid Assay. PJ69-4A yeast cells containing full-length *Evi1* fused to a Gal4 binding domain (Gal4-BD-*Evi1*) were transformed together with a mouse 17 day embryo cDNA library, in which the cDNA transcripts were cloned in-frame with the GAL-4 transactivation domain. Of the approximately 1×10^7 positively transformed cells, 124 viable clones survived selection on medium lacking Trp, Leu, His, and Ade. These clones were analyzed for β-galactosidase activity using an X-gal plate assay, revealing 25 positive clones, which were reconfirmed in a β-galactosidase liquid culture assay (data not shown). These positive clones were nucleotide sequenced, and six encoded full-length methyl-CpG binding domain protein 3 isoform b (Mbd3b), a member of the Mi-

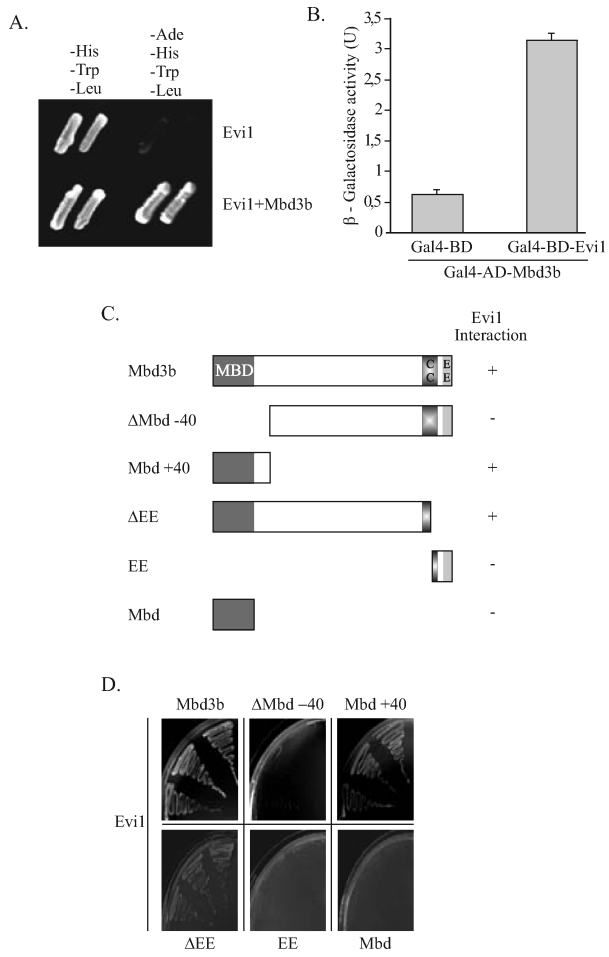

FIGURE 1: Identification and mapping of the regions of a novel Evi1 binding partner (Mbd3b) in a yeast two-hybrid screen. (A) Analysis of the interaction between Evi1 and Mbd3b in a yeast two-hybrid screen. Evi1 containing plasmid (pGBT9-Gal4-BD-Evi1) alone is not able to support growth on medium lacking the adenine (−Ade) amino acid, whereas in the absence of histidine (−His), no inhibition of growth was observed. Introduction of an Mbd3b-containing plasmid (pGBT9-Gal4-AD-Mbd3b) caused Ade transactivation due to the interaction between Evi1 and Mbd3b and allows yeast to grow. (B) pGBT9-Gal4-AD-Mbd3b was assayed in liquid culture to examine β-galactosidase activity in the presence of either pGBT9-Gal4-BD-Evi1 or an empty vector pGBT9-Gal4-BD. Increased β-galactosidase activity demonstrates the interaction between Mbd3b and Evi1. (C) Schematic representation of *Mbd3b* and *Mbd3b* mutants used in the yeast two-hybrid assay: (dark grey area) MBD, (EE) 12-glutamate repeat domain, and (CC) coiled-coil domain. (D) Full-length *Evi1* was transformed together with full-length *Mbd3b* or its mutants. Yeast was grown on selective plates lacking tryptophan (Trp), leucine (Leu), histidine (His), and adenine (Ade).

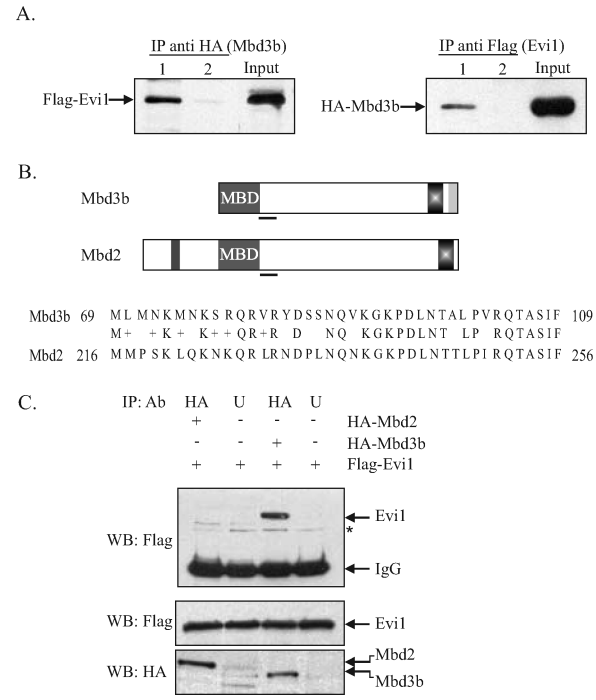

FIGURE 2: Evi1 interacts with Mbd3b but not with other MBD family members in mammalian cells. (A) Coexpressed FLAG-Evi1 and HA-Mbd3b in φE cells were immunoprecipitated with either anti-FLAG or anti-HA antibody. Following SDS−PAGE and Western blotting, the immunoprecipitant with HA antibody was stained with anti-FLAG antibody to analyze the presence of Evi1 (left blot). In a parallel experiment, anti-HA antibody was used to study the presence of Mbd3b following anti-FLAG immunoprecipitation (right blot). As a control (lane 2 in both experiments), lysates were exposed to unspecific antibody (anti-Myc) and beads. (B) Alignment of the Mbd3b binding region with the Mbd2 amino acid sequence. The 40 interacting amino acids of Mbd3b (positions 69−109) are 65% identical in sequence with Mbd2 (positions 216−256) and 82% similar (underlined region). (C) Immunoprecipitation of Evi1 from φE cells that were transiently cotransfected with FLAG-*Evi1* and either HA-*Mbd2* or HA-*Mbd3b*. Proteins were immunoprecipitated with the anti-HA antibody or anti-Myc as an unspecific antibody (U). Western blot detection was performed using anti-FLAG antibody. The bottom panels show a Western blot to demonstrate input Evi1, Mbd3b, and Mbd2 expression.

2/NuRD transcriptional repressor complex. Interaction between Evi1 and Mbd3b was confirmed on plates lacking Trp, Leu, His, and Ade (Figure 1A). Full-length Evi1 increases the level of Mdb3-mediated β-galactosidase reporter gene activation more than 5-fold in liquid culture assay (Figure 1B).

Mbd3b contains a methyl CpG binding domain (MBD) at the N-terminus and a coiled-coil (CC) domain as well as a glutamic acid repeat (EE) domain at the C-terminal part of the protein (Figure 1C) (*33*). To determine the critical regions for Evi1 interaction, we performed yeast two-hybrid analysis using Mbd3b mutants and full-length Evi1. The EE domain was neither required nor sufficient for binding Evi1. The deletion mutant lacking the MBD and the adjacent 40-amino acid region (ΔMBD−40) did not bind Evi1 (Figure 1D), while the mutant of MBD3 with MBD and 40 adjacent amino acids (MBD+40) was able to associate with Evi1. The MBD region alone without these 40 amino acids could not associate with Evi1 (Figure 1D). The data indicate that the 40 amino acids C-terminal to the MBD of Mbd3b are required for binding of Evi1 (Figure 1D).

Evi1 Interacts with Mbd3 but Not with Other MBD Family Members in Mammalian Cells. HA immunoprecipitation of HA-Mbd3b in lysates from HA-*Mbd3b*/FLAG-*Evi1* cotransfected Phoenix E (φE) cells revealed the coprecipitation of FLAG-Evi1 as determined by Western blot analysis using an anti-FLAG antibody (Figure 2A). HA-Mbd3b could be coprecipitated from the same lysates when an anti-FLAG antibody was used to immunoprecipitate FLAG-Evi1 (Figure 2A). The high degree of homology within the MBD family members prompted us to investigate the possibility of interaction of Evi1 with other members of the family. Whereas the homology between Mbd3b and Mbd1, Mbd4, or MeCP2 is restricted to the MBD only, Mbd2 shows

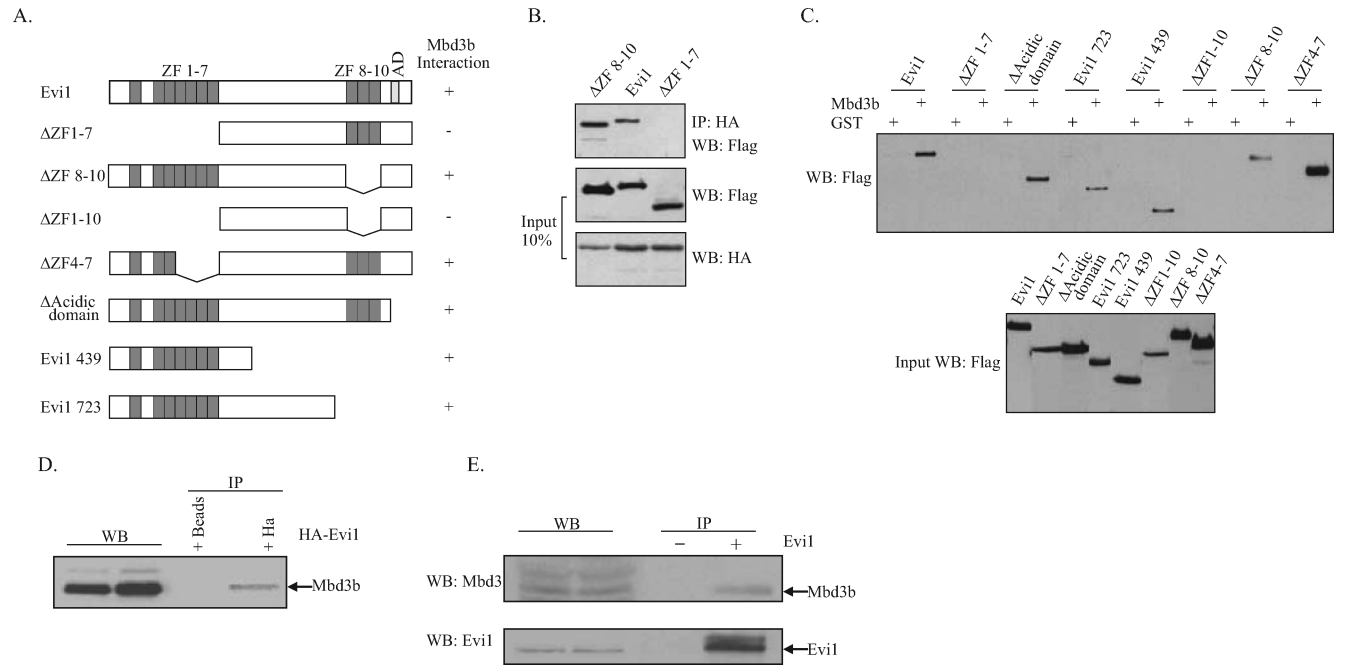

FIGURE 3: N-Terminal zinc finger domain of Evi1 is required for Mbd3 interaction. Full-length Evi1 interacts with endogenous Mbd3b in mammalian cells. (A) Diagram of full-length Evi1 and its mutants. Interaction with Mbd3b is indicated (+). (B) φE cells were transiently transfected with HA-*Mbd3b* and constructs of full-length or mutant *Evi1* (FLAG-tagged). Total cell lysates were immunoprecipitated (IP) with anti-HA antibody, and the proteins were analyzed by Western blotting with anti-FLAG antibody to detect FLAG-Evi1. In the bottom panels, 10% protein input of FLAG-Evi1 and Ha-Mbd3b is depicted. (C) Mapping of the interaction domain of Evi1 using the GST−Mbd3 protein in a pull-down assay. Full-length *Evi1* or *Evi1* mutants were transiently expressed in φE cells. Protein extracts were incubated with purified GST−Mbd3b protein or GST only. Blots were stained with FLAG antibody to detect FLAG-Evi1. Input levels of the wild type or mutants of FLAG-Evi1 are shown in the lower panel. (D) φE cells transiently transfected with HA-*Evi1* were lysed and incubated with anti-HA antibody (HA) or Sepharose G-protein beads (control sample). Immunoprecipitates (IP) were separated by electrophoresis and stained with anti-MBD3 antibody. The endogenous MBD3b band was specifically detected in the presence of HA-Evi1 but not in the control sample. A Western blot (WB) stained with anti-Mbd3 confirms the presence of the Mbd3b protein in both lysates. (E) Immunoprecipitation of Evi1 in lysates from the NFS 56 myeloid leukemia cell line, with Evi1 specific antibody. The top panel shows protein extract from the NFS 56 cell line was incubated with Evi1 antiserum (+) or Sepharose G-protein beads (−) and stained with anti-MBD3 antibody. The presence of endogenous Mbd3b in both lysates before immunoprecipitation was confirmed by Western blot (WB) analysis. The bottom panel shows the same Western blot stained with Evi1 antiserum. The presence of Evi1 protein was confirmed in the sample with Evi1 antiserum. The total cell lysate was used to detect the presence of endogenous Evi1 in the NFS 56 cell line.

homology with Mbd3 outside this region (*34*). In particular, the 40-amino acid region required for interaction between Mbd3b and Evi1 shows a high degree of homology (65%) with a comparable region in Mbd2 (Figure 2B).

Although FLAG-Evi1 coprecipitated with HA-Mbd3b using an anti-HA antibody, no Evi1−Mbd2 interaction was observed in an anti-HA co-immunoprecipitation experiment in FLAG-*Evi1*/HA-*Mbd2* transfected cells (Figure 2C). Moreover, no interaction has been observed between Evi1 and Mbd1, Mbd4, or MeCP2 using transfected φE cells (data not shown).

Evi1 Requires the First Three Zinc Fingers of the N-Terminal Zinc Finger Domain To Interact with Mbd3b. We next investigated which domains of Evi1 are required for interaction with Mbd3b. HA-*Mbd3b* was cotransfected with FLAG-*Evi1* or FLAG-*Evi1* mutants lacking either zinc finger domain 1 (ΔZF1−7) or the second zinc finger domain (ΔZF8−10) (Figure 3A). Immunoprecipitation was performed with an anti-HA antibody against HA-Mbd3b, and Western blot analysis was carried out with an anti-FLAG epitope antibody to detect Evi1. Full-length Evi1 and the Evi1 mutant lacking the second zinc finger domain did bind to HA-Mbd3b, whereas the Evi1 mutant lacking the first seven zinc fingers (ΔZF1−7) did not (Figure 3B). These results were confirmed using GST-Mbd3b pull-down experiments (Figure 3C). FLAG-*Evi1* mutants (Figure 3A) were expressed in mammalian φE cells, and lysates were exposed to purified GST−Mbd3b protein bound to glutathione beads. Western blot analysis of captured proteins from the lysates revealed that FLAG-*Evi1* mutants lacking the first seven zinc fingers (mutants ΔZF1−7 and ΔZF1−10) did not bind to Mbd3b (Figure 3C). Mutants of FLAG-*Evi1* containing the ZF1−7 domain were all capable of binding to Mbd3b. A mutant lacking four zinc fingers in the first domain (ΔZF4−7) showed strong interaction with Mbd3b, indicating that the first three zinc fingers are important for protein interaction.

Endogenous Interaction of Evi1 and Mbd3. We next studied whether transiently overexpressed HA-Evi1 in φE cells could interact with endogenous Mbd3. HA-Evi1 immunoprecipitation and subsequent staining with an Mbd3 specific antibody demonstrated coprecipitation of endogenous Mbd3 (Figure 3D). No Mbd3 was detected when the same sample was incubated with Sepharose G-protein beads only. Due to a proviral integration within the *Evi1* locus, the gene is overexpressed in NFS 56 myeloid leukemia cells (*35*) (Figure 3E). Immunoprecipitation using an antibody specific for Evi1 and subsequent Western blot analysis using anti-Mbd3 antibodies revealed the presence of significant levels of protein with the size corresponding to that of Mbd3b (Figure 3E). No protein could be detected when the sample was incubated with Sepharose G-protein beads only.

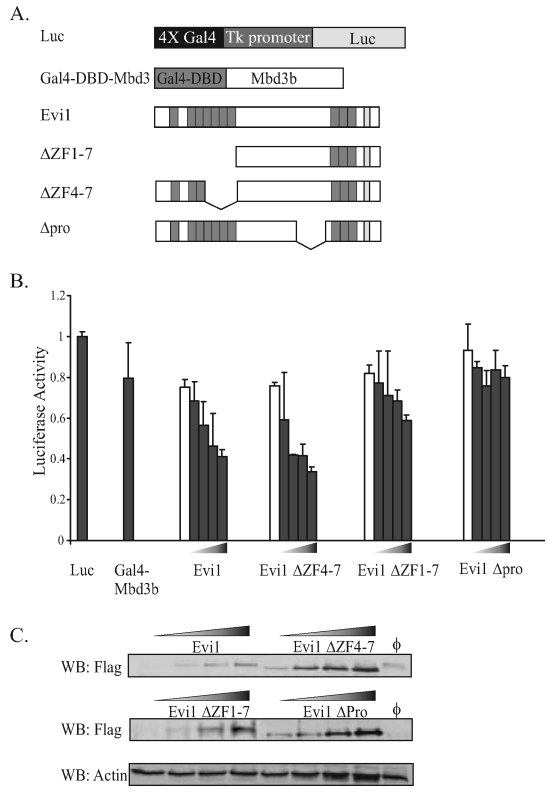

FIGURE 4: Evi1 and Mbd3b interact in vivo as determined in a mammalian two-hybrid luciferase reporter assay. (A) Schematic representation of the reporter construct that was used in the luciferase assay containing four repeats of the Gal4 binding sites upstream of a thymidine kinase promoter-driven luciferase marker gene. In the expression vector, a full-length Mbd3b cDNA was fused in frame with the Gal4 DNA binding domain (Gal4DBD). (B) Luciferase assay showing repression of Gal4-Mbd3b-mediated luciferase activity in a dose-dependent manner by Evi1. Phoenix cells were transfected with 0.1 μg of the 4xGal4-TK-Luc construct as a reporter. Increasing amounts (0.025, 0.050, 0.1, and 0.15 μg) of *Evi1*, *Evi1*Δ4−7, *Evi1*Δ1−7, and *Evi1*Δpro mutant expression vectors (black bars) were cotransfected with 20 ng of Gal4-*Mbd3b*. A white bar represents cells transfected with 0.15 μg of the different *Evi1* constructs and 4xGal4-TK-Luc, but no Gal4-Mbd3b. The expression level of the 4xGal4-TK-Luc luciferase vector alone was normalized to 1.0. (C) Western blot of protein samples isolated from cells used for the luciferase assay. The bottom blot shows anti-actin staining for equal loading.

Evi1 Interacts with Mbd3 in Vivo As Determined in a Mammalian Two-Hybrid Luciferase Reporter Assay. Evi1 fused in-frame to the DNA binding domain of Gal4 has previously been shown to repress luciferase activity of Gal4-TK-luciferase. Moreover, this repression depends on the C-terminal binding protein (CtBP) interacting proline rich region of Evi1 (*17, 18, 36, 37*). To assess the in vivo interaction between Evi1 and Mbd3, we decided to investigate whether the same repression activity could be achieved when *Evi1* was cotransfected with Gal4-TK-luciferase and *MBbd3* fused in-frame to the DNA binding domain of Gal4 (Figure 4A). Expression of full-length Evi1 decreases the luciferase activity in the presence of Gal4-Mbd3b in a dose-dependent manner but not in the control experiment without Gal4-Mbd3b (Figure 4B). Mutant Δpro Evi1 lost its ability to repress luciferase activity, indicating that the repression by full-length Evi1 required the CtBP interacting proline rich domain. The extent of this repression was also decreased when the first seven zinc fingers of Evi1 (ΔZF1−7) were deleted. A deletion mutant lacking zinc fingers 4−7 (ΔZF4−7), which could still interact with Mbd3, showed the same dose-dependent repression that was observed with the full-length Evi1 construct. Comparable levels of protein were detected in most samples by Western blot (Figure 4C). In fact, the level of full-length Evi1 protein was overall lower than Evi1 mutant levels.

Evi1 and Mbd3 Coexist in Large Molecular Structures, Particularly in the Mi2/NuRD Complex. Mbd3 is a member of the Mi-2/NuRD multiprotein complex (*24*). To study whether Evi1 and Mbd3 may be part of a larger complex, we first studied whether Mbd3 and Evi1 colocalize in nuclear structures. φE cells were transiently transfected with FLAG-*Evi1* and HA-*Mbd3b*. Cells were stained with specific anti-FLAG and anti-HA antibodies followed by secondary FITC and TRITC staining, respectively (Figure 5A). Confocal microscopy revealed that Evi1 was expressed in a speckled pattern in the nucleus, as described previously (*38*). HA-Mbd3b exhibited a similar staining pattern, and merging of both images revealed colocalization of Evi1 and Mbd3b in subnuclear speckles (Figure 5A). To demonstrate that Evi1 and Mbd3b could be found in the same large protein complex, as observed by confocal microscopy, we performed Sepharose 6 gel filtration analysis of whole-cell extracts derived from the stably transfected Ha-Evi1-expressing 32D myeloid cell line. Fractions were collected and subsequently analyzed with Western blotting using anti-HA or anti-Mbd3 antibodies. Evi1 as well as Mbd3b appeared to be present in closely related high-molecular mass fractions of approximately 1−1.5 MDa (*32*) (Figure 5B).

Mi-2/NuRD is a transcriptional repressor complex that contains several components, of which RbAp46/p48, HDAC1, and HDAC2 form the core complex that is mutual between Mi-2/NuRD and the Sin3-histone deacetylase complex. Factors that are restricted to Mi-2/NuRD only are MTA2, Mi-2, Mbd3a, and Mbd3b (*24*). To confirm that Evi1 is able to interact with Mbd3b and is found in the Mi-2/NuRD complex, we introduced HA-Evi1 in stable TAP-tagged Mbd3a-expressing 293T cells (*23*) and used an immunoprecipitation assay to isolate the Mi-2/NuRD complex bound to TAP-tagged Mbd3a protein. We demonstrated the presence of Evi1 in the TAP-Mbd3a sample (Figure 5C), and the positive staining using antibodies directed against HDAC2, MTA-2, and Rbp-46 revealed that we indeed purified the Mi-2/NuRD complex using this procedure (Figure 5C). The level of MTA-2 protein recovered was lower in the Evi1-containing sample. It would be interesting to study whether EVI1 interferes with the interaction of this protein with the Mi2/NuRD complex.

Evi1 Interferes with the Histone Deacetylase Activity of the Mi-2/NuRD Complex in Vitro. To study the putative role of Evi1 in Mi-2/NuRD, we performed an in vitro deacetylation assay on nucleosomal templates acetylated by the SAGA complex. Specific deacetylation was observed when the Mbd3-containing Mi-2/NuRD complex with mock infected 293T precipitates was added. Addition of Evi1 protein containing immunoprecipitate, obtained from FLAG-Evi1 cDNA-transfected 293 cells, to this mixture prohibited specific deacetylation (Figure 5D). A lysate containing the ΔZF1−7 mutant Evi1 protein, which does not interact with Mbd3, did not affect deacetylation by Mi-2/NuRD. Thus, Evi1, when associated with Mi-2/NuRD through Mbd3 interaction, interferes with the deacetylation activity of this

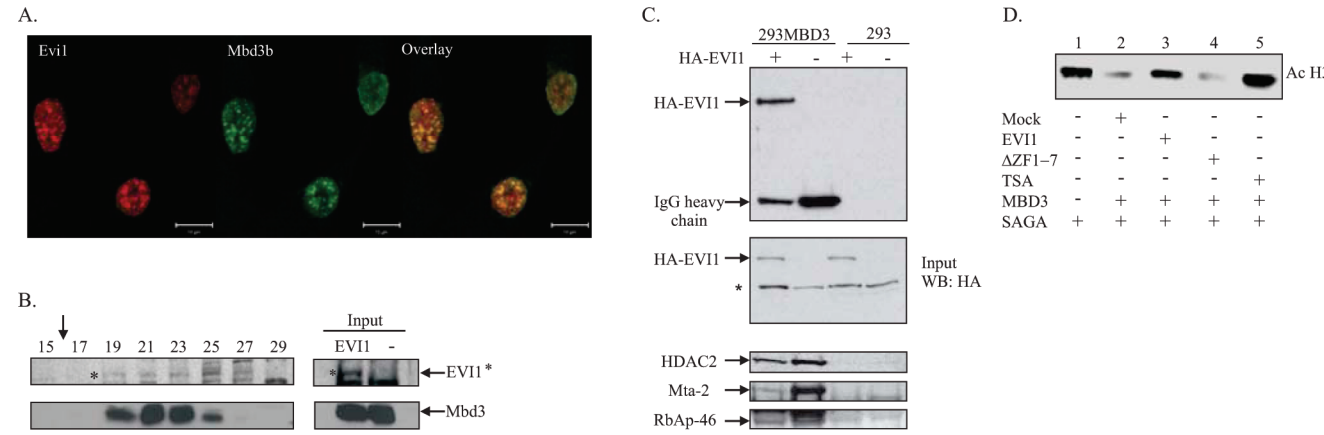

FIGURE 5: Evi1 associates with Mbd3 in the Mi2/NuRD complex and interferes with histone deacetylation. (A) Confocal microscopy of φE cells transiently transfected with FLAG-Evi and HA-Mbd3b. FLAG-Evi was stained with anti-FLAG antibody followed by secondary TRITC staining (red). HA-Mbd3 was stained with anti-HA antibody followed by secondary FITC staining (green). Both proteins form nuclear speckles, and the merged slide shows colocalization between the two proteins (yellow). (B) Sepharose 6 gel filtration of whole-cell extracts derived from HA-Evi1-transduced 32D cells. Fractions were analyzed by Western blotting using an anti-HA (HA-Evi1) and an Mbd3 specific antibody. The void of the column was between fractions 15 and 17 (arrow). (C) 293T cells with or without stably expressed TAP-MBD3a were transfected with HA-Evi1 (+) or an empty vector (−). TAP-MBD3a was immunoprecipitated from the total cell lysate using Sepharose IgG beads. Proteins were stained for the presence of Evi1 (anti-HA staining, top blot). The arrow indicates HA-Evi1. The presence of Mi-2/NuRD proteins in the IgG immunoprecipitation experiment was confirmed using anti-HDAC2, anti-MTA-2, and anti-RbAp-46 antibody (bottom blot). (D) Nucleosomal templates reconstituted with recombinant histones were acetylated by the *S. cerevisiae* SAGA or NuA4 complex and subsequently incubated with the TAP−MBD3 complex in the absence or presence of TSA (Trichostatin-A) (*32*) or with lysates from mock, Evi, or mutant ΔZF1−7 Evi1 transduced φE cells. H3 acetylation was assessed by Western blotting using antibodies against diacetylated histone H3 Lys-9,14.

enzyme complex and may consequently deregulate the normal transcriptional repression function of the Mi-2/NuRD complex in vitro.

DISCUSSION

Evi1 is a nuclear zinc finger protein involved in myeloid leukemia development, which has been reported to act as a transcriptional repressor (*17–19, 39*). However, recently published data have also suggested that Evi1 may function as a transcriptional activator (*20*). We demonstrate a direct interaction between Evi1 and methyl-binding domain 3 protein b (Mbd3b), a member of the Mi-2/NuRD complex (*24, 33*). Six independent full-length Mbd3b-expressing clones were identified in the yeast two-hybrid assay, and the interaction between Mbd3b and Evi1 in mammalian cells was demonstrated using various biochemical approaches. The in vitro experiments presented here (Figure 5D) suggest that Evi1 may counteract repression of gene expression evoked by the histone deacetylase transcriptional Mi-2/NuRD repressor complex.

Mbd3b is a member of a family of proteins which possesses a conserved methyl binding domain (MBD) (*34*). This motif has been shown to recognize methylated CpG islands, although Mbd3 appears to have lost this function (*21, 22*). In contrast to Mbd3, other family members such as Mbd1, Mbd2, and MeCP2 repress transcription through direct interaction with methylated cytosines in CpG islands (*40*). We demonstrate here that Evi1 is capable of interacting only with Mbd3 and not with any of the other family members, although Mbd3 is highly homologous to the closely related protein Mbd2. We conclude that we identified a distinctive region C-terminal of the MBD that is fully required for this unique interaction between Evi1 and Mbd3. Experiments reported by Saito and Ishikawa demonstrated how a minimal modification in the amino acid sequence of the MBD of Mbd3 alters its function. In fact, one amino acid change, i.e., Phe-34 to Tyr-34, within the MBD changed Mbd3 into a methylated CpG binding protein. Moreover, it appeared that the unaltered Mbd3 was capable of specifically interacting with HDAC1 and MTA2, two components of the Mi-2/NuRD complex.

Mbd3 and Mbd2 have been shown to assemble into mutually exclusive distinct Mi-2/NuRD-like complexes, called Mbd2/NuRD and Mbd3/NuRD (*23*). Although the exact difference between the two complexes needs further elucidation, the major difference between the two complex structures was that a number of proteins were present in Mbd2/NuRD which were absent in Mbd3/NuRD. In contrast, no unique Mbd3/NuRD proteins were reported that were absent in Mbd2/NuRD. As shown in our study, Evi1 appears to uniquely assemble in Mbd3/NuRD and not in Mbd2/NuRD. The fact that Evi1 was previously not identified in complex purification by protein tagging and mass spectrometry in HEK293 cells (*23*) is simply explained by the fact that Evi1 is not expressed in those cells. Consequently, in leukemia cells that aberrantly express Evi1, an interaction between those two nuclear proteins does occur (Figure 3E), and as such, a unique Evi1/Mi-2/NuRD complex may exist. Specific Evi1 pull-down experiments and subsequent mass spectrometry should reveal which other unique proteins may be present in such a complex.

Our data also suggest that although at least two unique Mbd2- or Mbd3-containing Mi-2/NuRD complexes exist, these large protein structures may also be different in distinct cell types. Unique nuclear proteins, particularly nucleotide specific transcription regulators, may specifically direct the repressor function of the Mi-2/NuRD complexes to specific regions in the DNA. Transcription factors with well-defined nucleotide recognition sites, such as Ikaros, Rorγ, and SATB1, have been demonstrated to interact with the Mi-2/

NuRD complex (*41–43*). Recently, it has been demonstrated that Gata1 interacts in erythroid cells via Fog1 with NuRD complexes (*44, 45*). Interestingly, since aberrant Evi1 expression has been demonstrated to cause erythroid abnormalities (*7, 10*), and Evi1 has recently been demonstrated to interact with Gata1 (*15*), it will be of great interest to investigate whether Evi1−NuRD interaction affects Gata1 or Fog1 function in erythroid precursors.

We also identified a specific domain within Evi1 that interacts with Mbd3b. In fact, many proteins have been found to interact with Evi1 (*16–19*). Several of those have been shown to bind to the first zinc finger domain. These reported diverse interactions between Evi1 and a number of nuclear proteins accentuate the structural and functional complexity of this oncogenic nuclear protein. Consequently, it is at the moment difficult to fully understand the meaning of each of those interactions and the putative role in leukemic transformation.

The luciferase experiment demonstrates that Evi1 may also act as a repressor itself when recruited to a promoter site. Since Mbd3 can be found in only an Mi-2/NuRD complex and not as a single protein, one should realize that the luciferase experiment should only be considered as evidence that Evi1 and Mbd3 can interact in vivo in mammalian cells. The interaction between Evi1 and Mbd3 may in fact have different consequences for the function of Evi1, Mbd3, and Mi-2/NuRD. As suggested above, Evi1 may recruit Mbd3 and consequently Mi-2/NuRD to specific sites in the genome either due to direct DNA interaction (*11–13*) or through binding to other previously reported Evi1 binders (*11–13*), resulting in transcriptional repression. This latter function has been reported by many investigators before and may involve several transcription repressors, of which Mbd3, including the Mi-2/NuRD complex, may play a role. Although these possibilities may occur in the living cell, the most surprising finding of our study is the observation that Evi1 is attracted to the Mi-2/NuRD complex and counteracts deacetylation, which abolishes the repressor function of this multiprotein complex. The specificity of this interaction and interference of the repressor function is demonstrated by the fact that a mutant that does not bind Mbd3 and is also unable to interfere with Mi-2/NuRD-directed histone deacetylation. Although at present we have no evidence that Evi1-mediated interference with histone deacetylation is the main explanation for the transcriptional activation of particular Evi1 target genes, the results do confirm that Evi1 is a protein that is capable of evoking various effects depending on the context in which it is being expressed. The fact that Evi1 binds Mbd3 in NFS56 leukemia cells may suggest that this interaction has been important in Evi1-mediated leukemic transformation.

The role of Evi1 in malignant transformation and leukemia progression is well-recognized, and different in vitro and in vivo models have indicated a dominant role of Evi1 in leukemogenesis. One of the indisputable functions of Evi1 is its capability to repress transcription, by interaction with distinct factors (*46*). In recent years, it has become evident that transcriptional repressors are able to associate with multiprotein complexes that contain repressor subunits (*47*). The interaction is not always direct but frequently requires adaptor proteins (*48*) . Although the exact mechanism is not clear yet, our results add more evidence supporting the notion that Evi1 plays an important role in transcriptional control by interacting with transcription repression complexes. Insight into the exact interactions between Evi1 and these complexes as well as the effects thereof will provide invaluable information required for the development of molecules that may interfere with these complexes.

ACKNOWLEDGMENT

We thank Dr. R. Deplus from the Laboratory of Molecular Virology, Free University of Brussels, for luciferase assay constructs and Dr. B. Aarts for technical assistance with confocal microscopy. We are grateful to Dr. G. Follows for his suggestions and reading of the manuscript.

REFERENCES

1. Suzukawa, K., Parganas, E., Gajjar, A., Abe, T., Takahashi, S., Tani, K., Asano, S., Asou, H., Kamada, N., and Yokota, J. (1994) Identification of a breakpoint cluster region 3′ of the ribophorin I gene at 3q21 associated with the transcriptional activation of the EVI1 gene in acute myelogenous leukemias with inv(3)(q21q26). *Blood 84*, 2681–2688.
2. Morishita, K., Parganas, E., William, C. L., Whittaker, M. H., Drabkin, H., Oval, J., Taetle, R., Valentine, M. B., and Ihle, J. N. (1992) Activation of EVI1 gene expression in human acute myelogenous leukemias by translocations spanning 300−400 kilobases on chromosome band 3q26. *Proc. Natl. Acad. Sci. U.S.A. 89*, 3937–3941.
3. Barjesteh van Waalwijk van Doorn-Khosrovani, S., Erpelinck, C., van Putten, W. L., Valk, P. J., van der Poel-van de Luytgaarde, S., Hack, R., Slater, R., Smit, E. M., Beverloo, H. B., Verhoef, G., Verdonck, L. F., Ossenkoppele, G. J., Sonneveld, P., de Greef, G. E., Lowenberg, B., and Delwel, R. (2003) High EVI1 expression predicts poor survival in acute myeloid leukemia: A study of 319 de novo AML patients. *Blood 101*, 837–845.
4. Lugthart, S., van Drunen, E., van Norden, Y., van Hoven, A., Erpelinck, C. A., Valk, P. J., Beverloo, H. B., Lowenberg, B., and Delwel, R. (2008) High EVI1 levels predict adverse outcome in acute myeloid leukemia: Prevalence of EVI1 overexpression and chromosome 3q26 abnormalities underestimated. *Blood 111*, 4329–4337.
5. Morishita, K., Parker, D. S., Mucenski, M. L., Jenkins, N. A., Copeland, N. G., and Ihle, J. N. (1988) Retroviral activation of a novel gene encoding a zinc finger protein in IL-3-dependent myeloid leukemia cell lines. *Cell 54*, 831–840.
6. Morishita, K., Parganas, E., Matsugi, T., and Ihle, J. N. (1992) Expression of the Evi-1 zinc finger gene in 32Dc13 myeloid cells blocks granulocytic differentiation in response to granulocyte colony-stimulating factor. *Mol. Cell. Biol. 12*, 183–189.
7. Kreider, B. L., Orkin, S. H., and Ihle, J. N. (1993) Loss of erythropoietin responsiveness in erythroid progenitors due to expression of the Evi-1 myeloid-transforming gene. *Proc. Natl. Acad. Sci. U.S.A. 90*, 6454–6458.
8. Shimizu, S., Nagasawa, T., Katoh, O., Komatsu, N., Yokota, J., and Morishita, K. (2002) EVI1 is expressed in megakaryocyte cell lineage and enforced expression of EVI1 in UT-7/GM cells induces megakaryocyte differentiation. *Biochem. Biophys. Res. Commun. 292*, 609–616.
9. Louz, D., van den Broek, M., Verbakel, S., Vankan, Y., van Lom, K., Joosten, M., Meijer, D., Lowenberg, B., and Delwel, R. (2000) Erythroid defects and increased retrovirally-induced tumor formation in Evi1 transgenic mice. *Leukemia 14*, 1876–1884.
10. Buonamici, S., Li, D., Chi, Y., Zhao, R., Wang, X., Brace, L., Ni, H., Saunthararajah, Y., and Nucifora, G. (2004) EVI1 induces myelodysplastic syndrome in mice. *J. Clin. Invest. 114*, 713–719.
11. Delwel, R., Funabiki, T., Kreider, B. L., Morishita, K., and Ihle, J. N. (1993) Four of the seven zinc fingers of the Evi-1 myeloid-transforming gene are required for sequence-specific binding to GA(C/T)AAGA(T/C)AAGATAA. *Mol. Cell. Biol. 13*, 4291–4300.
12. Perkins, A. S., Fishel, R., Jenkins, N. A., and Copeland, N. G. (1991) Evi-1, a murine zinc finger proto-oncogene, encodes a sequence-specific DNA-binding protein. *Mol. Cell. Biol. 11*, 2665–2674.
13. Matsugi, T., Kreider, B. L., Delwel, R., Cleveland, J. L., Askew, D. S., and Ihle, J. N. (1995) The Evi-1 zinc finger myeloid

transforming protein binds to genomic fragments containing $(GATA)_n$ sequences. *Oncogene 11*, 191–198.
14. Funabiki, T., Kreider, B. L., and Ihle, J. N. (1994) The carboxyl domain of zinc fingers of the Evi-1 myeloid transforming gene binds a consensus sequence of GAAGATGAG. *Oncogene 9*, 1575–1581.
15. Laricchia-Robbio, L., Fazzina, R., Li, D., Rinaldi, C. R., Sinha, K. K., Chakraborty, S., and Nucifora, G. (2006) Point mutations in two EVI1 Zn fingers abolish EVI1-GATA1 interaction and allow erythroid differentiation of murine bone marrow cells. *Mol. Cell. Biol. 26*, 7658–7666.
16. Kurokawa, M., Mitani, K., Irie, K., Matsuyama, T., Takahashi, T., Chiba, S., Yazaki, Y., Matsumoto, K., and Hirai, H. (1998) The oncoprotein Evi-1 represses TGF-β signalling by inhibiting Smad3. *Nature 394*, 92–96.
17. Palmer, S., Brouillet, J. P., Kilbey, A., Fulton, R., Walker, M., Crossley, M., and Bartholomew, C. (2001) Evi-1 transforming and repressor activities are mediated by CtBP co-repressor proteins. *J. Biol. Chem. 276*, 25834–25840.
18. Izutsu, K., Kurokawa, M., Imai, Y., Maki, K., Mitani, K., and Hirai, H. (2001) The corepressor CtBP interacts with Evi-1 to repress transforming growth factor β signaling. *Blood 97*, 2815–2822.
19. Vinatzer, U., Taplick, J., Seiser, C., Fonatsch, C., and Wieser, R. (2001) The leukaemia-associated transcription factors EVI-1 and MDS1/EVI1 repress transcription and interact with histone deacetylase. *Br. J. Haematol. 114*, 566–573.
20. Yuasa, H., Oike, Y., Iwama, A., Nishikata, I., Sugiyama, D., Perkins, A., Mucenski, M. L., Suda, T., and Morishita, K. (2005) Oncogenic transcription factor Evi1 regulates hematopoietic stem cell proliferation through GATA-2 expression. *EMBO J. 24*, 1976–1987.
21. Saito, M., and Ishikawa, F. (2002) The mCpG-binding domain of human MBD3 does not bind to mCpG but interacts with NuRD/Mi2 components HDAC1 and MTA2. *J. Biol. Chem. 277*, 35434–35439.
22. Wade, P. A., Gegonne, A., Jones, P. L., Ballestar, E., Aubry, F., and Wolffe, A. P. (1999) Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. *Nat. Genet. 23*, 62–66.
23. Le Guezennec, X., Vermeulen, M., Brinkman, A. B., Hoeijmakers, W. A., Cohen, A., Lasonder, E., and Stunnenberg, H. G. (2006) MBD2/NuRD and MBD3/NuRD, two distinct complexes with different biochemical and functional properties. *Mol. Cell. Biol. 26*, 843–851.
24. Zhang, Y., Ng, H. H., Erdjument-Bromage, H., Tempst, P., Bird, A., and Reinberg, D. (1999) Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. *Genes Dev. 13*, 1924–1935.
25. Yu, J., Angelin-Duclos, C., Greenwood, J., Liao, J., and Calame, K. (2000) Transcriptional repression by blimp-1 (PRDI-BF1) involves recruitment of histone deacetylase. *Mol. Cell. Biol. 20*, 2592–2603.
26. Deplus, R., Brenner, C., Burgers, W. A., Putmans, P., Kouzarides, T., de Launoit, Y., and Fuks, F. (2002) Dnmt3L is a transcriptional repressor that recruits histone deacetylase. *Nucleic Acids Res. 30*, 3831–3838.
27. Gietz, D., St Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res. 20*, 1425.
28. James, P., Halladay, J., and Craig, E. A. (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics 144*, 1425–1436.
29. Graham, F. L., and van der Eb, A. J. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology 52*, 456–467.
30. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
31. Santos, F., Zakhartchenko, V., Stojkovic, M., Peters, A., Jenuwein, T., Wolf, E., Reik, W., and Dean, W. (2003) Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. *Curr. Biol. 13*, 1116–1121.
32. Vermeulen, M., Carrozza, M. J., Lasonder, E., Workman, J. L., Logie, C., and Stunnenberg, H. G. (2004) In vitro targeting reveals intrinsic histone tail specificity of the Sin3/histone deacetylase and N-CoR/SMRT corepressor complexes. *Mol. Cell. Biol. 24*, 2364–2372.
33. Zhang, Y., LeRoy, G., Seelig, H. P., Lane, W. S., and Reinberg, D. (1998) The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. *Cell 95*, 279–289.
34. Hendrich, B., and Bird, A. (1998) Identification and characterization of a family of mammalian methyl-CpG binding proteins. *Mol. Cell. Biol. 18*, 6538–6547.
35. Joosten, M., Valk, P. J., Vankan, Y., de Both, N., Lowenberg, B., and Delwel, R. (2000) Phenotyping of Evi1, Evi11/Cb2, and Evi12 transformed leukemias isolated from a novel panel of cas-Br-M murine leukemia virus-infected mice. *Virology 268*, 308–318.
36. Turner, J., and Crossley, M. (1998) Cloning and characterization of mCtBP2, a co-repressor that associates with basic Kruppel-like factor and other mammalian transcriptional regulators. *EMBO J. 17*, 5129–5140.
37. Chinnadurai, G. (2002) CtBP, an unconventional transcriptional corepressor in development and oncogenesis. *Mol. Cell 9*, 213–224.
38. Chakraborty, S., Senyuk, V., Sitailo, S., Chi, Y., and Nucifora, G. (2001) Interaction of EVI1 with cAMP-responsive element-binding protein-binding protein (CBP) and p300/CBP-associated factor (P/CAF) results in reversible acetylation of EVI1 and in co-localization in nuclear speckles. *J. Biol. Chem. 276*, 44936–44943.
39. Bartholomew, C., Kilbey, A., Clark, A. M., and Walker, M. (1997) The Evi-1 proto-oncogene encodes a transcriptional repressor activity associated with transformation. *Oncogene 14*, 569–577.
40. Wade, P. A. (2001) Methyl CpG-binding proteins and transcriptional repression. *BioEssays 23*, 1131–1137.
41. Johnson, D. R., Lovett, J. M., Hirsch, M., Xia, F., and Chen, J. D. (2004) NuRD complex component Mi-2β binds to and represses RORγ-mediated transcriptional activation. *Biochem. Biophys. Res. Commun. 318*, 714–718.
42. O'Neill, D. W., Schoetz, S. S., Lopez, R. A., Castle, M., Rabinowitz, L., Shor, E., Krawchuk, D., Goll, M. G., Renz, M., Seelig, H. P., Han, S., Seong, R. H., Park, S. D., Agalioti, T., Munshi, N., Thanos, D., Erdjument-Bromage, H., Tempst, P., and Bank, A. (2000) An ikaros-containing chromatin-remodeling complex in adult-type erythroid cells. *Mol. Cell. Biol. 20*, 7572–7582.
43. Yasui, D., Miyano, M., Cai, S., Varga-Weisz, P., and Kohwi-Shigematsu, T. (2002) SATB1 targets chromatin remodelling to regulate genes over long distances. *Nature 419*, 641–645.
44. Hong, W., Nakazawa, M., Chen, Y. Y., Kori, R., Vakoc, C. R., Rakowski, C., and Blobel, G. A. (2005) FOG-1 recruits the NuRD repressor complex to mediate transcriptional repression by GATA-1. *EMBO J. 24*, 2367–2378.
45. Rodriguez, P., Bonte, E., Krijgsveld, J., Kolodziej, K. E., Guyot, B., Heck, A. J., Vyas, P., de Boer, E., Grosveld, F., and Strouboulis, J. (2005) GATA-1 forms distinct activating and repressive complexes in erythroid cells. *EMBO J. 24*, 2354–2366.
46. Buonamici, S., Chakraborty, S., Senyuk, V., and Nucifora, G. (2003) The role of EVI1 in normal and leukemic cells. *Blood Cells Mol. Dis. 31*, 206–212.
47. Li, J., Lin, Q., Wang, W., Wade, P., and Wong, J. (2002) Specific targeting and constitutive association of histone deacetylase complexes during transcriptional repression. *Genes Dev. 16*, 687–692.
48. Sekimata, M., Takahashi, A., Murakami-Sekimata, A., and Homma, Y. (2001) Involvement of a novel zinc finger protein, MIZF, in transcriptional repression by interacting with a methyl-CpG-binding protein, MBD2. *J. Biol. Chem. 276*, 42632–42638.

BI800267F