Repression of RUNX1 Activity by EVI1: A New Role of EVI1 in Leukemogenesis

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Abstract

Recurring chromosomal translocations observed in human leukemia often result in the expression of fusion proteins that are DNA-binding transcription factors. These altered proteins acquire new dimerization properties that result in the assembly of inappropriate multimeric transcription complexes that deregulate hematopoietic programs and induce leukemogenesis. Recently, we reported that the fusion protein AML1/MDS1/EVI1 (AME), a product of a t(3;21)(q26;q22) associated with chronic myelogenous leukemia and acute myelogenous leukemia, displays a complex pattern of self-interaction. Here, we show that the 8th zinc finger motif of MDS1/EVI1 is an oligomerization domain involved not only in interaction of AME with itself but also in interactions with the parental proteins, RUNX1 and MDS1/EVI1, from which AME is generated. Because the 8th zinc finger motif is also present in the oncoprotein EVI1, we have evaluated the effects of the interaction between RUNX1 and EVI1 in vitro and in vivo. We found that in vitro, this interaction alters the ability of RUNX1 to bind to DNA and to regulate a reporter gene, whereas in vivo, the expression of the isolated 8th zinc finger motif of EVI1 is sufficient to block the granulocyte colony-stimulating factor–induced differentiation of 32Dcl3 cells, leading to cell death. As EVI1 is not detected in normal bone marrow cells, these data suggest that its inappropriate expression could contribute to hematopoietic transformation in part by a new mechanism that involves EVI1 association with key hematopoietic regulators, leading to their functional impairment.

Introduction

Cellular transformation is mediated by the accumulation of genetic mutations, which change the biochemical properties or the level of expression of key factors in a progenitor cell. Recurring chromosomal translocations are a group of such mutations in leukemia patients, and their location detected by cytogenetic analysis frequently points to the genes that are affected. Almost invariably, chromosomal translocations result in the generation of an abnormal fusion gene, and in myeloid leukemia, they often result in the expression of a fusion protein. In human leukemia, RUNX1, also known as AML1, is the gene most frequently targeted by point mutation, leading to haploinsufficiency, and by translocations, which consistently lead to fusion proteins. The t(3;21)(q26;q22) detected in chronic myelogenous leukemia (CML) and myelodysplastic syndrome (MDS) fuses in frame the two conserved genes RUNX1, a member of the RUNX family, and MDS1/EVI1 (ME), a member of the PR family (1, 2). This translocation is associated with very aggressive hematopoietic diseases and is characterized by the expression of the fusion gene AML1/MDS1/EVI1 (AME; refs. 3, 4). As for many members of the PR family, the locus that encodes ME also expresses another gene through an independently regulated promoter located about 500 kb downstream of the ME promoter (5). ME and the second gene, EVI1, have completely opposite properties (6, 7); however, because of the structure of the locus, the shorter protein EVI1 is entirely contained in ME. The ME locus is frequently disrupted by several rearrangements, leading to the inappropriate expression of EVI1 in hematopoietic cells in MDS and acute myelogenous leukemia (AML; refs. 8, 9). Whereas the combined effort of many investigators has provided considerable information on the pathways that are involved in hematopoietic transformation following the deregulation of the ME locus and the expression of EVI1, indeed, whereas there is no doubt that EVI1 is a nuclear protein that associates with several coactivators and corepressors, very little is known on the pathways by which it promotes leukemic transformation. Our recent work showed that AME interacts with itself in a highly complex pattern that involves several domains. One of them is a distal domain containing the last three zinc finger motifs of the protein (10). Here, we have further analyzed this domain and determined that the 8th zinc finger motif interacts with the DNA-binding domain of RUNX1. We found that the two proteins, EVI1 and RUNX1, interact and colocalize in the nucleus, and that their interaction leads to a reduction of DNA-bound RUNX1 and to the repression of a reporter gene regulated by RUNX1. In vivo, the isolated 8th zinc finger motif of EVI1 blocks by itself the differentiation of 32Dcl3 cells, leading to cell death. Taken together, these results suggest that a mechanism by which EVI1 could contribute to the disruption of hematopoietic programs associated with leukemia is by interaction with RUNX1, which weakens its DNA-binding activity, leading to overall loss of RUNX1 function.

Materials and Methods

DNA cloning. Flag-EVI1 and hemagglutinin (HA)- and Flag-tagged RUNX1 were described previously (11, 12). HA-AME was described (13). To generate AME-related clones, HA or Flag epitopes were amplified by PCR from appropriate templates and were cloned into the NcoI/PstI site of the pCMV/myc/nuc vector (Invitrogen/Life Technologies). With appropriately...
designed primers, a BamHI site was inserted after the epitope tag. The cDNA fragments shown in Fig. 1A (lines 4–10) were amplified by PCR and cloned in frame to a nuclear localization signal (NLS) at the COOH terminus between the BamHI and the XhoI sites of the HA- or Flag-tagged vectors. The NLS was derived from pCMV/myc/nuc plasmid (Invitrogen/Life Technologies). The nuclear localization of AME or EVI1 fragments was confirmed by immunofluorescence (Supplementary Fig. S1). HA-EVI1 (Fig. 1A, line 11) was obtained by cloning the entire reading frame of EVI1 into the BamHI and XhoI sites of the HA-pCMV/myc/nuc vector. To generate HA-DZnF-EVI1 lacking the distal zinc finger domain (Fig. 1A, line 12), the HindIII-XhoI fragment of AME-Δ(1184–1246) (ref. 10) was used to substitute the homologous region in HA-EVI1. HA-EVI1-653 was described earlier (11). Flag-tagged RUNX1 fragments (Fig. 1A, lines 14–16) were created by PCR followed by cloning into pCMV-Flag-NLS vector (Invitrogen/Life Technologies). The coding region for CBFβ was PCR amplified and cloned into pGEX-KT vector at the EcoRI site to generate in-frame glutathione S-transferase (GST)-CBFβ fusion. All the clones were sequenced. The plasmid for CBFβ-MIGR1 was a generous gift from Dr. N. Speck (Dartmouth Medical School, Hanover, NH).

Cell culture. Adherent cell lines 293, 293T, and NIH-3T3 were maintained in DMEM supplemented with 10% newborn calf serum. The suspension cell line K562 was maintained in RPMI supplemented with 10% calf serum, whereas 32Dcl3 cells were maintained in the same medium additionally supplemented with 10% WEHI-3B conditioned medium as a source of interleukin-3 (IL-3).

Transfection. DNA transfection of adherent cells was done by the calcium phosphate precipitation method (14) or with NovaFector reagent (Venn Nova, Inc.) according to the manufacturer’s instructions. We used 10 μg plasmid per 10-cm plate for each transfection, unless otherwise indicated. For transfection of 32Dcl3 cells, we used the electroporator Nucleofector II (Amaxa Biosystems) according to the manufacturer’s protocol for this cell line.

Differentiation assay. 32Dcl3 cells were washed with PBS thrice and plated in RPMI supplemented with 10% calf serum and granulocyte colony-stimulating factor (G-CSF; 20 ng/mL). Manual cell count and cytoxin preparations were made every 24 h.

Immunofluorescence analysis. 293 cells were cultured on glass coverslips in 3.5-cm plates and were transfected with 1 μg of each plasmid. The procedure has already been described (11).

Figure 1. The 8th zinc finger motif of AME is an oligomerization domain. A, diagram of the proteins used in the present study. The first three lines show the domains of ME, RUNX1, and AME. The DNA-binding domain of RUNX1 (Runt) is maintained in the chimeric protein. The PR domain (PR) and the two zinc finger domains (ZnF) of ME. Line 11, diagram of EVI1. In the remaining lines, we show the diagrams of AME-, EVI1-, and RUNX1-related mutants in which the numbers on the right side indicate the amino acid boundaries. The full-length ME, RUNX1, AME, and EVI1 contain 1240, 453, 1499, and 1052 amino acids, respectively. B, Western blot. C, 293T cells were transiently cotransfected with Flag-AME-1184-1205 and the HA-tagged deletion mutants in lines 7 to 9 and analyzed as described above. Lanes 1 and 5, mock-transfected cells.
**Western blot analysis and coimmunoprecipitation assays.** Cells were harvested 48 h after transfection, and the assays were carried out as described (10). We used commercially available monoclonal mouse antibody M2 to the Flag epitope (Sigma-Aldrich), monoclonal rat antibody to the HA epitope (Roche), polyclonal rabbit antibody to EVI1 (Cell Signaling Technology), and C-19 polyclonal goat antibody or H-65 polyclonal rabbit antibody to RUNX1 (Santa Cruz Biotechnology).

**GST fusion pull-down assay.** The assay was carried out as described (15). Briefly, GST-AML1 or GST was incubated for 2 h at 4°C with in vitro translated EVI1 or with the lysate of 293T cells transiently transfected with EVI1. Glutathione-Sepharose 4B beads (Pharmacia Biotech) were added and incubated for 30 min at 4°C. The beads were washed thrice with *Escherichia coli* lysis buffer [25 mmol/L Tris-HCl (pH 7.5) with 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100] and subjected to SDS-PAGE.

**Reporter gene studies.** We used the RUNX1-dependent minimal macrophage colony-stimulating factor receptor (M-CSFR) promoter (16, 17). NIH-3T3 cells were transiently cotransfected with a M-CSFR reporter gene in the presence of the effector plasmids. For normalization of the efficiency of transfections, we used the *Renilla* luciferase pRL-TK plasmid (Promega). All measurements were done in triplicate.

**Electrophoresis mobility shift assay.** RUNX1, EVI1, EVI1-DZnF, and the 8th zinc finger of EVI1 were generated by in vitro translation using the TNT-coupled reticulocyte lysate system (Promega) according to the manufacturer's instruction. CBFβ was obtained as a GST fusion. RUNX1 was mixed

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**Figure 2.** AME interacts with the parental proteins through the 8th zinc finger motif. A, 293T cells were cotransfected with HA-AME (lanes 2 and 7) or with HA-AME and Flag-RUNX1 (lanes 3 and 8). Flag-ME (lanes 4 and 9), or Flag-EVI1 (lanes 5 and 10). Lanes 1 and 6, mock-transfected cells. The proteins were analyzed as described in Fig. 1B legend. B, AME colocalizes in the nucleus with RUNX1 and ME. 293 cells were cotransfected with differently tagged AME and RUNX1 or AME and ME. After treatment of the slides as described in Materials and Methods, the proteins were visualized with immunofluorescence microscopy. Top, localization pattern of AME. Middle, localization patterns of RUNX1 and ME. Bottom, overlap of the images. C, 293T cells were cotransfected with HA-RUNX1 (lanes 2 and 6) or with HA-RUNX1 and Flag-AME-1184-1205 (lanes 3 and 6). Lanes 1 and 4, mock-transfected cells. The proteins were analyzed as described in Fig. 1B legend. A band is observed in lane 3, confirming that the proteins interact. D, 293T cells were cotransfected with HA-EVI1 (lanes 2 and 5) or with HA-EVI1 and Flag-AME-1184-1205 (lanes 3 and 6). Lanes 1 and 4, mock-transfected cells. The proteins were analyzed as described in Fig. 1B legend. The presence of the band in lane 3 indicates that the zinc finger motif interacts with the full-length protein.
EVI1 makes multiple contacts with RUNX1. A, 293T cells were cotransfected with HA-EVI1-ΔZnF alone (lanes 1 and 5) or with Flag-RUNX1 (lanes 2 and 6) and with HA-EVI1 alone (lanes 3 and 7) or with Flag-RUNX1 (lanes 4 and 8) and analyzed as described in Fig. 1B legend. Analysis of the immunoprecipitation complexes with anti-HA antibody identifies RUNX1 with the full-length EVI1 and the internal deletion mutant (lanes 2 and 4), indicating that there are multiple contact sites between EVI1 and RUNX1. B, 293T cells were cotransfected with HA-Proximal zinc finger domain alone (lanes 1 and 7) or with Flag-RUNX1 (lanes 2 and 8), HA-C-terminal domain alone (lanes 3 and 9) or with Flag-RUNX1 (lanes 4 and 10), HA-EVI1-653 alone (lanes 5 and 11) or with Flag-RUNX1 (lanes 6 and 12). C, 293T cells were transiently cotransfected with HA-EVI1-653 alone (lanes 1 and 5) or with the Flag-tagged RUNX1 deletion mutants shown in lines 14 to 16 in Fig. 1A. D, purified GST and GST-RUNX1 conjugated to Glutathione-Sepharose beads were incubated with in vitro translated EVI1 (top) or with total cell lysates of mock-transfected or HA-EVI1–transfected 293T cells (middle). After extensive washing, the beads were subjected to SDS-PAGE, and the separated proteins were analyzed by autoradiography (top) or by Western blot (middle) or by Coomassie staining (bottom). Lanes 1 and 2 or 4 to 7, pull-down results; lanes 3 and 8 to 11, input proteins.
with EVI1 or EVI1-ΔZnF or 8th zinc finger or with in vitro translated vector as a control in the presence or absence of GST-CBFβ and incubated on ice for 60 min. The probe was a 33-bp DNA fragment corresponding to the RUNX1 binding site in the M-CSFR promoter: 5'-TCGACAGATTTC-CAAAACTCTGTGGTTGCCTTG-3'. The binding assay was carried out at 4°C in a total volume of 15 μL [10 mmol/L Tris-HCl (pH 7.5) with 150 mmol/L NaCl, 12.5% glycerol, 5 mmol/L EDTA, 5 mmol/L DTT, and 1 μg poly[d(Deoxyinosinic-deoxycytidylic acid)] per reaction]. 32P end-labeled probe (0.2 pmol/L per reaction) was added after 10 min, and incubation was continued for additional 20 min. The samples were loaded on 0.5 × Tris-Borate-EDTA nondenaturing 6% polyacrylamide gel, electrophoresed at 100 V for 3 h at 4°C, and autoradiographed overnight. To evaluate the translation efficiency and input level, the in vitro translation reaction was done separately in the presence of 35S-methionine.

Semiquantitative reverse transcription-PCR. Total cellular RNA was extracted from 32Dc13 cells using Trizol reagent according to the manufacturer’s instruction (Invitrogen/Life Technologies), and the cDNA was prepared according to the First-Strand cDNA synthesis kit protocol (Fermentas) using 2 μg of total RNA. Semiquantitative PCR was done in 90 μl reaction mixtures containing 2.5 μl of cDNA, 1 μl of Taq Red polymerase (CLP), and 300 nmol/L forward (5'-TACCGTTGTACCGAGCTTGCATAG-3') and reverse (5'-ATTCCATGTTGTGTC-CTGCAC-3') primers. Fifteen microliters of the PCR products were collected after 28, 32, 36, 40, and 44 cycles.

Results

The 8th zinc finger motif of AME is an oligomerization domain. AME is a fusion protein characterized by several structural motifs of the parental proteins, including the DNA-binding domain Runt of RUNX1 at the NH2 terminus; the PR domain of ME; the proximal zinc finger domain, containing seven motifs; and the distal zinc finger domain of ME formed by three motifs at the COOH terminus (Fig. 1A, line 3). Fusion proteins generated by chromosomal translocations often acquire the ability to oligomerize. Earlier, we reported that the fusion protein AME interacts with itself through the distal zinc finger domain containing zinc finger motifs 8, 9, and 10 (10). To identify the motif that mediates the self-interaction, we separately cloned several small regions that overlap the domain (diagrammed in Fig. 1A, lines 4–6) and used transient transfection and coimmunoprecipitation assays to evaluate the interaction between the cloned short peptides and the full-length protein. Exponentially growing 293T cells were cotransfected with HA-AME either alone or in combination with one of the Flag-tagged protein regions. Two days after transfection, the cells were harvested and lysed, and the cellular proteins were coimmunoprecipitated with anti-Flag antibody. The immunoprecipitated proteins were separated by electrophoresis, transferred to a membrane, and analyzed by Western blotting with anti-HA antibody. As shown in Fig. 1B, the small regions AME-1184–1306 (lane 2) and AME-1184–1205 (lane 4) efficiently interact with full-length AME. In contrast, AME-1218–1499, which includes the 9th and 10th zinc finger motifs (lane 3), is unable to interact with AME, suggesting that the 8th zinc finger contains the interacting surface. Lane 1 represents the reaction with only the antibody used as negative control. Lanes 5 to 8 show the expression of HA-AME (top) and Flag-tagged fragments (bottom).

We previously reported that the DNA-binding region of RUNX1 (Runt) is involved in AME self-interaction through the distal zinc finger domain (10). To determine whether Runt interacts with the 8th zinc finger motif, 293T cells were cotransfected with Flag-AME-1184–1205 and with one of the HA-tagged clones shown in Fig. 1A (lines 7–9) and analyzed as described above. As shown in Fig. 1C, both Runt and the proximal zinc finger domain interact with AME-1184–1205 (lanes 2 and 3). In contrast, the PR domain does not interact with the 8th zinc finger (lane 4). These results suggest that the short 21-amino-acid sequence 1184–1205 containing the 8th zinc finger motif is sufficient for interaction with Runt.

AME forms hetero-oligomers. Based on these results, we hypothesized that RUNX1, ME, and EVI1 should be able to interact with each other and with AME. Therefore, we used transient
Figure 5. EVI1 represses RUNX1-dependent transactivation and RUNX1 binding to DNA. A. NIH-3T3 cells were transiently cotransfected with the M-CSFR-luciferase reporter gene in the presence of RUNX1 and EVI1 as shown. RUNX1 alone activates the M-CSFR promoter (column 2), but the activation of this promoter is reduced with increasing amounts of EVI1 (columns 3–5). The activation of the reporter in the absence of RUNX1 (column 1) was arbitrarily taken as 1. Expression of the transfected proteins (bottom).

B. NIH-3T3 cells were transiently cotransfected with the M-CSFR-luciferase reporter gene in the presence of RUNX1 and full-length EVI1 or the isolated 8th zinc finger motif or EVI1-ΔZnF as shown. Both EVI1 and 8th zinc finger (columns 4 and 6, respectively) abrogate RUNX1 activation of the reporter gene, whereas EVI1-ΔZnF represses the promoter at intermediate level (column 8). The activation of the reporter in the absence of RUNX1 (columns 1, 3, 5, and 7) was arbitrarily taken as 1. Protein expression (bottom).

C. EVI1 represses expression of the M-CSFR in 32Dcl3 cells. Semiquantitative reverse transcription-PCR was done as described in Materials and Methods.

D. Electrophoretic mobility shift assay (EMSA) was done as described in Materials and Methods in the absence (lanes 1–5) and in the presence (lanes 6–9) of CBFβ. As expected, there is a significant RUNX1-probe band (lanes 2 and 6). The intensity of the band significantly decreases in the presence of EVI1 (lanes 3 and 7) or of the 8th zinc finger (lanes 5 and 9), whereas EVI1-ΔZnF has very limited effect on RUNX1-probe complex formation (bands 4 and 8). Bottom, input proteins.
transfection of 293T cells and coimmunoprecipitation assays as described earlier to evaluate the interaction of the full-length proteins. As shown in Fig. 2A, RUNX1 (lane 3), ME (lane 4), and EVI1 (lane 5) coprecipitate with AME. Anti-Flag antibody alone is unable to interact with the protein (lane 2). Lanes 7 to 10 show the expression level of the proteins. Lanes 1 and 6 are mock-transfected cells used as controls. To confirm these findings, we used immuno- florescence analysis. Earlier, we showed that AME is a nuclear protein and has mostly a diffused pattern, but in about 10% of the cells, AME was localized in large speckles (13). For immunofluo-
rescence assays, 293 cells were cotransfected with HA-AME and either Flag-RUNX1 or Flag-ME. As shown in Fig. 2B, in nuclei of the cells, AME (top, red) colocalizes with RUNX1 and ME (middle, green). We found that both proteins colocalize with AME in about 10% of cells.

**EVI1 makes multiple contacts with RUNX1.** Because the 8th zinc finger motif of AME is also contained in EVI1, we asked whether this motif is also capable of interacting with RUNX1 and EVI1. Using coimmunoprecipitation and Western blotting, we confirmed that the 8th zinc finger motif by itself efficiently coprecipitates with RUNX1 (Fig. 2C, lane 3) or EVI1 (Fig. 2D, lane 3). To determine whether additional domains interact with RUNX1, we generated the mutant EVI1-ΔZnF (Fig. 1A, line 12) in which an internal deletion of the distal zinc finger domain was introduced. As shown in Fig. 3A, both EVI1-ΔZnF and EVI1 interact with RUNX1 (lanes 2 and 4, respectively), suggesting that EVI1 contains additional interacting region(s) that recognize RUNX1.

To identify and map the second interaction domain, we used the mutants shown in Fig. 1A (lines 9, 10, and 13–16). By using coimmunoprecipitation assays, we found that the central domain of EVI1 (amino acids 246–653) interacts with the central region of RUNX1 (amino acids 185–411; Fig. 3B, lane 6 and Fig. 3C, lane 3). We also found that the NH2 and COOH termini of EVI1 (amino acids 23–245 and 800–1052) do not interact with RUNX1 (Fig. 3B, lanes 2 and 4).

To confirm the interaction, we used GST-RUNX1 pull-down assays with either *in vitro* translated EVI1 or with EVI1 expressed in 293T cells. After mixing the proteins with the beads and extensive washing, the proteins were separated by electrophoresis and analyzed by autoradiography (Fig. 3D, top) or by Western blotting (Fig. 3D, middle). In both cases, GST-RUNX1 efficiently interacts with EVI1. In contrast, GST alone was not able to interact with EVI1. We further confirmed our results by Western blot analysis of endogenous proteins. For these studies, we used the cell line K562, a CML cell line that weakly expresses endogenous EVI1 and RUNX1. Total proteins were immunoprecipitated with anti-
RUNX1 antibody, and the proteins in the complex were identified by Western blotting. As shown in Fig. 4A, endogenous EVI1 interacts and coprecipitates with RUNX1, which can also be visualized with the appropriate antibody after stripping the membrane (see also Supplementary Figs. S2 and S3). Immunofluo-
rescence analysis showed that EVI1 and RUNX1 colocalize in the nucleus, confirming the immunoprecipitation results (Fig. 4B).

It has been reported that EVI1 forms discrete nuclear speckles in about 10% of cells (11). We observed colocalization of the two proteins in discrete nuclear speckles in about 10% of cells. In the remaining cells, EVI1 appeared in a nuclear diffused pattern as reported previously (11).

Taken together, these data provide an unexpected and important link between EVI1 and RUNX1. Because EVI1 is not expressed in normal hematopoietic cells, the inappropriate direct interaction between these two proteins could contribute in part to the deregulation of the myeloid programs controlled by RUNX1, leading to myeloid leukemia.

**The 8th zinc finger motif of EVI1 represses RUNX1 activity.** To determine whether the transactivating property of RUNX1 is altered in the presence of EVI1, we did reporter gene assays with the M-CSFR minimal promoter, which depends on RUNX1 for activation (17). NIH-3T3 cells were transiently cotransfected with the M-CSFR-luciferase reporter gene and the effector plasmid as shown in Fig. 5A. As expected, we found that RUNX1 alone activates the promoter (column 2). Coexpression of EVI1 suppresses the activation in a dose-dependent manner (columns 3–5). The expression of the transfected proteins is shown in Fig. 5A (bottom). To determine whether the isolated 8th zinc finger motif is sufficient to repress the RUNX1 activity, we repeated the reporter gene assays. The results indicate that indeed this small region is fully competent to repress by itself the promoter activation by RUNX1 (Fig. 5B, column 6). To determine whether the second interaction domain of EVI1 has an effect on RUNX1 transactivation, or whether the 8th zinc finger is the only domain involved in repression, we used an EVI1 mutant that lacks the 8th zinc finger motif. The reporter gene assay showed that this mutant represses RUNX1 but at an intermediate level (Fig. 5B, column 8), suggesting that EVI1 affects RUNX1 activity in multiple ways. The expression of the transfected proteins is shown in Fig. 5B (bottom). AME (13) and ME (Supplementary Fig. S4) repress the M-CSFR reporter activation at a level similar to that of EVI1.

To determine whether EVI1 can also repress M-CSFR transcription *in vivo*, we used semiquantitative reverse transcription-PCR of 32Dcl3 cells stably expressing EVI1 or EVI1-ΔZnF. As shown in Fig. 5C, the M-CSFR transcription was partially reduced in EVI1-
expressing cells, strongly suggesting a dominant role of the 8th zinc finger of EVI1 in repression of RUNX1 activity. This observation could also indicate that *in vivo* (32Dcl3 cells), the second interaction region of EVI1 has limited repression potential against the RUNX1 transactivity.

**EVI1 represses the DNA-binding property of RUNX1.** We used electrophoretic mobility shift assay to determine whether EVI1 has an effect on the ability of RUNX1 to bind to DNA. *In vitro* translated RUNX1 and EVI1 or EVI1-ΔZnF or the 8th zinc finger were mixed and incubated on ice for 60 min before adding the labeled probe. As shown in Fig. 5D, in the presence of EVI1, the intensity of the band corresponding to the RUNX1-probe complex significantly decreases, suggesting that the interaction with EVI1 reduces the ability of RUNX1 to bind to the DNA (lanes 2 and 3). A similar effect was observed with the 8th zinc finger (lane 5) but not with EVI1-ΔZnF (lane 4), which has lost the ability to interact with the DNA-binding domain of RUNX1. Inclusion of CBFβ in the electrophoretic mobility shift assay reactions does not abrogate the inhibitory effect of EVI1 or the 8th zinc finger on RUNX1-probe complex formation (lanes 7 and 9). To evaluate the efficiency of *in vitro* protein synthesis, the translation reactions were repeated in the presence of 35S-methionine and verified by autoradiography (bottom). We also found that RUNX1 has no effect on the DNA binding property of EVI1 (Supplementary Fig. S5).

**The 8th zinc finger motif inhibits the differentiation of 32Dcl3 cells.** To characterize the role of the isolated 8th zinc finger motif *in vivo*, we generated two separate 32Dcl3 cell lines that stably express this short motif. In the absence of G-CSF, the cells grew well in culture and were undistinguishable from the naive cell line K562.
32Dcl3 cells (data not shown). However, after the cells were removed from IL-3–containing medium and transferred to G-CSF–containing medium to induce their differentiation, we found that the expression of the isolated 8th zinc finger motif blocked granulopoiesis at very early stages of differentiation and induced massive cell death within 4 to 6 days of culture (Fig. 6A and B). All the cells expressing the isolated 8th zinc finger motif appeared to form clusters of 15 to 20 elements and were blocked at the myeloblasts stage characterized by a high nuclear/cytoplasm ratio, immature chromatin often with evident nucleoli. The pale cytoplasm was generally agranulated or, sometimes, with sparse immature granules.

Discussion

We report a novel interaction between RUNX1 and EVI1, two proteins that have been long associated with human leukemia. RUNX1, first identified as one of the genes involved in the recurring 8;21 chromosomal translocation (18), is the most frequent target of a variety of genetic lesions detected in human hematopoietic disorders. RUNX1, necessary for the establishment of the hematopoietic stem cell (HSC), controls the expression of several genes that participate to the execution of myeloid differentiation programs; therefore, it is generally thought that gene deregulation caused by RUNX1 point mutations and RUNX1 fusion proteins leads to deregulation of hematopoietic programs and results in leukemia. In contrast to RUNX1, EVI1 is not detected in normal hematopoietic cells and requires retroviral insertion (in the mouse) or a chromosomal rearrangement (in man) to be transcriptionally activated, and at this time, it is unclear whether it is required at all for primitive or definitive hematopoiesis. The consistent association of EVI1 with myeloid leukemia indicates that this protein disrupts preferentially myeloid programs. Because EVI1 interacts with several corepressors, it was suggested that inappropriately expressed EVI1 alters gene regulation by recruitment of corepressors. The molecular mechanisms by which EVI1 disrupts myelopoiesis are not clear yet, but the association of EVI1 with disruption of exclusively myeloid programs tends to suggest the involvement of alternative mechanisms that do not necessarily require the association of EVI1 to universally expressed corepressors. Rather, it is likely that EVI1 affects specifically the activity of transcription factors that regulate these lineages. A recent report seems to support this model by showing that the aggressive dyserythropoiesis that is a hallmark of a subset of EVI1-positive leukemia is caused by the interaction between EVI1 and GATA1, leading to the repression of GATA1 functions, rather than to the recruitment of corepressors to promoter sites (19). In that report, the association between the two proteins was mediated by the proximal zinc finger domain of EVI1 that completely inhibited GATA1 binding to the DNA.

EVI1 is also involved in the development/progress of other more primitive types of leukemia characterized by arrest at an earlier stage of differentiation at which GATA1 is not yet required. The occurrence of these types of early myeloid leukemia suggests that EVI1 could block the function of other factors expressed further upstream along the myeloid program. The results that we present here support this model and show that EVI1 interacts with and inhibits RUNX1. As for GATA1, EVI1 seems to repress RUNX1 function by interacting specifically with its DNA-binding domain Runt, leading to destabilization and dissolution of the DNA-RUNX1 complex. RUNX1 activates several genes required for myelopoiesis

![Figure 6](https://example.com/figure6.png)

**Figure 6.** The 8th zinc finger motif blocks granulocytic differentiation of 32Dcl3 cells and induces their death. A, naive 32Dcl3 cells and two clones of 32Dcl3 cells expressing the isolated 8th zinc finger motif were cultured in IL-3. The differentiation of the cells in response to G-CSF was done as described in Materials and Methods. Cytospin preparations of the cells at consecutive days of differentiation were stained with Wright-Giemsa stain. After 5 to 6 days in culture, all the cells expressing the 8th zinc finger motif were dead without signs of differentiation. Expression of the isolated 8th zinc finger motif (bottom). B, number of live cells in 1 mL of culture (×1,000). The 32Dcl3 cells were cultured in the presence of G-CSF.
of 32Dcl3 cells. It has been known for several years that EVI1 blocks the differentiation of these cells (20), although the mechanism of such inhibition is still not known. The finding that the short zinc finger motif of EVI1 is by itself sufficient to block myeloid differentiation of 32Dcl3 cells as efficiently as the full-length EVI1 suggests that perhaps the interaction with RUNX1 and the functional repression of RUNX1 activity are at least in part the cause of the differentiation block. It is likely that EVI1 could act in a similar fashion to reach functional disruption of other DNA-binding transcription factors that regulate myelopoiesis.

Given that RUNX1 is expressed in HSC all the way to terminal myeloid, megakaryocytic, and erythroid differentiation, a difficult question that remains to be answered is why we do not see the same phenotype in all the patients who inappropriately express EVI1. Perhaps, the answer rests in the fact that EVI1 expression is rarely seen as the first abnormality in a neoplastic cell. In fact, this gene is associated mostly with myelodysplastic syndrome in which, as shown by cytogenetic studies, EVI1 rearrangements appear in a pre-existing clone that already contains unrelated chromosomal abnormalities (deletion of chromosome 5 and/or 7), and in chronic myelogenous leukemia, in which the t(9;22) and the expression of BCR/ABL are thought to be the first transforming event. Therefore, the variability of EVI1-positive leukemia could be a consequence of other mutations with which EVI1 cooperating.

In conclusion, this study supports the possibility that EVI1 could induce the progression of leukemia by specific inhibition of key regulatory transcription factors that are the gatekeepers of hematopoietic lineages. The finding that a very small amino acid region with well-defined structure and geometry inhibits differentiation provides a tempting opportunity to test whether the disruption of the interaction with RUNX1 could remove the differentiation block. Positive results would offer new goals to design or identify drugs that inhibit one of the most aggressive oncoproteins in human leukemia.

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Repression of RUNX1 Activity by EVI1: A New Role of EVI1 in Leukemogenesis

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