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EVI1 is critical for the pathogenesis of a subset of *MLL-AF9*–rearranged AMLs

Eric M. J. Bindels,[1] Marije Havermans,[1] Sanne Lugthart,[1] Claudia Erpelinck,[1] Elizabeth Wocjtowicz,[2] Andrei V. Krivtsov,[3] Elwin Rombouts,[1] Scott A. Armstrong,[3] Erdogan Taskesen,[1] Jurgen R. Haanstra,[1] H. Berna Beverloo,[4] Hartmut Döhner,[5] Wendy A. Hudson,[6] John H. Kersey,[6] Ruud Delwel,[1] and Ashish R. Kumar[2,6]

[1]Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands; [2]Cincinnati Children's Hospital Medical Center, Cincinnati, OH; [3]Division of Hematology/Oncology, Children's Hospital, Department of Pediatric Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA; [4]Department of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands; [5]Department of Internal Medicine III, University of Ulm, Ulm, Germany; and [6]Masonic Cancer Center, University of Minnesota, Minneapolis, MN

The proto-oncogene *EVI1* (ecotropic viral integration site-1), located on chromosome band 3q26, is aberrantly expressed in human acute myeloid leukemia (AML) with 3q26 rearrangements. In the current study, we showed, in a large AML cohort carrying 11q23 translocations, that ~ 43% of all mixed lineage leukemia (*MLL*)–rearranged leukemias are *EVI1*^{pos}. High *EVI1* expression occurs in AMLs expressing the *MLL-AF6*, *-AF9*, *-AF10*, *-ENL*, or *-ELL* fusion genes. In addition, we present evidence that *EVI1*^{pos} *MLL*-rearranged AMLs differ molecularly, morphologically, and immunophenotypically from *EVI1*^{neg} *MLL*-rearranged leukemias. In mouse bone marrow cells transduced with *MLL-AF9*, we show that MLL-AF9 fusion protein maintains *Evi1* expression on transformation of *Evi1*^{pos} HSCs. MLL-AF9 does not activate *Evi1* expression in *MLL-AF9*–transformed granulocyte macrophage progenitors (GMPs) that were initially *Evi1*^{neg}. Moreover, shRNA-mediated knockdown of *Evi1* in an *Evi1*^{pos} *MLL-AF9* mouse model inhibits leukemia growth both in vitro and in vivo, suggesting that *Evi1* provides a growth-promoting signal. Using the *Evi1*^{pos} *MLL-AF9* mouse leukemia model, we demonstrate increased sensitivity to chemotherapeutic agents on reduction of *Evi1* expression. We conclude that *EVI1* is a critical player in tumor growth in a subset of *MLL*-rearranged AMLs. (*Blood*. 2012;119(24):5838-5849)

Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease, which can be classified based on cytogenetic aberrations, molecular abnormalities, and gene expression and methylation signatures.[1-3] One group of AML patients is particularly characterized by the overexpression of the proto-oncogene *EVI1* (ecotropic viral integration site-1), which was first identified as a common proviral integration site in retrovirally induced murine myeloid leukemias.[4] *EVI1* encodes a nuclear zinc finger protein, capable of DNA binding in a sequence-specific manner.[5,6] EVI1 recruits a variety of transcriptional and epigenetic regulators, such as CTBP (C-terminal–binding protein), CBP (CREB-binding protein), P/CAF (P300/CBP-associated factor), HDAC (histone deacetylase), DNMT (DNA-methyltransferase), MBD3, or histone methyltransferases, suggesting a role in the control of gene expression.[7-13] Human *EVI1*-mediated disease can partly be recapitulated using in vitro and in vivo models. Aberrant expression of *EVI1* in mouse BM precursors in vivo causes a disease which resembles myelodysplastic syndrome (MDS).[14,15] In vitro overexpression of *EVI1* in transformed myeloid progenitors blocks myeloid differentiation, and affects survival and proliferation of these progenitors.[16,17]

Aberrant expression of *EVI1* occurs in ~ 8%-10% of human adult AML patients and is associated with a poor outcome.[18-20] In approximately one-third of those, the *EVI1* gene is highly expressed as the result of rearrangements of chromosome 3q26, the locus where the gene resides. However, high expression of *EVI1* was also found in leukemias with chromosomal abnormalities, other than the ones affecting the *EVI1* locus.[19,21] Strikingly, ~ 20% of *EVI1*-overexpressing AMLs have a concurrent mixed lineage leukemia (*MLL*) gene rearrangement,[20] but the prevalence within the specific *MLL* rearrangements remains to be investigated. In addition, in pediatric AMLs, in 27% of MLL rearranged cases, *EVI1* overexpression was detected.[22] In a knockin *MLL-AF9* mouse model mimicking human AML development, high *Evi1* expression was detected in preleukemic stem and progenitor cells compared with corresponding wild-type cells.[23] Importantly, among the various *MLL-AF9* hematopoietic stem and progenitor cells, a direct correlation between *Evi1* expression and the level of transformation was observed, where the Lin$^-$/Sca1$^+$/c-Kit$^+$ (LSK) cells exhibiting highest *Evi1* expression induced leukemias with the highest efficiency in recipients that received transplants.[23] Furthermore, conditional ablation of *Evi1* expression significantly reduces the colony-forming capacity of *MLL-ENL*–transformed BM progenitors.[24] These studies suggest that, at least in murine model systems, a causal relationship between *EVI1* and *MLL* rearrangements exists.

In the present study, we investigated the frequency and the prevalence of *EVI1* overexpression in a large cohort of *MLL*-rearranged human AMLs. No prevalence was observed for high *EVI1* expression in any of the most commonly detected MLL rearrangements in human AML, that is, *MLL-AF6*, *MLL-AF9*, and *MLL-ENL*. We also show that *EVI1*^{pos} *MLL*-rearranged AMLs are different subtypes compared with *EVI*^{neg} AMLs, based on their

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gene expression signature, morphology, and immunophenotype. The role of *EVI1* in *MLL*-rearranged AMLs was studied in *MLL-AF9*–transformed mouse BM cells and in their human AML counterparts. We show that *EVI1* expression is uncoupled from normal myeloid differentiation and is regulated by MLL-AF9, only in $EVI1^{pos}$-transformed cells, in agreement with data reported by Arai and colleagues.[25] The critical contribution of *Evi1* in these model systems was shown by applying lentiviral knockdown strategies. *Evi1* knockdown resulted in reduced survival in vitro and in vivo of *MLL-AF9*–transformed cells. Furthermore, *Evi1* knockdown enhanced sensitivity of *MLL-AF9*–transformed cells to chemotherapeutic drugs. Our findings suggest a critical role for *EVI1* in the pathogenesis of a subset of *MLL*-rearranged leukemias, and targeting of *EVI1* could be beneficial for patients with $EVI1^{pos}$ MLL-rearranged AMLs.

Methods

For primers, Abs, culture conditions, and additional experimental methods, please refer to supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Patient material

Leukemic blast cells were purified from BM or blood of patients presenting with AML as previously reported.[3,20] Patients were recruited from the Dutch-Belgian Cooperative Trial Group for Hematology (HOVON) and the AML Study Group (AMLSG) trials. All trails have been approved by the Institutional Review Board of the Erasmus University Medical Center and the University of Ulm.

Cytogenetic and molecular analysis

AML samples were routinely checked for cytogenetic abnormalities using a combination of standard chromosome-banding analysis and FISH. Additional RT-PCR was performed to verify the most common *MLL* fusions (primers are described in supplemental Table 1, and Balgobind et al[26] and Jansen[27]). The karyotype of each patient according to the International System for Human Cytogenetic Nomenclature,[28] French-American-British classification (FAB) type morphology, and WHO classifications, and if applicable, the *MLL*-fusion gene are depicted in supplemental Table 2.

Flow cytometric cell sorting

BM cells from healthy donors and of AML patients were isolated using Ficoll-Hypaque gradients and viably frozen in liquid nitrogen until use. The leukemic and normal $CD34^+/CD38^-$ or $CD34^+/CD38^+$ subpopulations were obtained by flow cytometric cell sorting using double staining with anti-CD34 and anti-CD38 mAbs, with an exclusion of at least 500 channels between the $CD38^+$ and $CD38^-$ subpopulations. In all cases, the purity of the preparation (99%) was verified by flow cytometry of separated cells. The sorting strategy is shown in supplemental Figure 2A.

Mononucleated murine normal BM cells were isolated on Ficoll-Hypaque gradients and stained with the BD Pharmingen Biotin-conjugated Mouse Lineage Panel (containing Abs against CD3e, CD11b, CD45R, Gr-1, and Ter119) and fluorophore-labeled Abs against c-kit (allophycocyanin), Sca-1 (PE-cy7), CD16/32 (PE), and CD34 (Pacific Blue). Biotin was targeted with streptavidin conjugated to allophycocyanin-Cy7. Subsequently, the BM cells were sorted for LSK (lineage marker negative [lin^-], $Sca1^+$, $c\text{-}kit^+$), common myeloid precursors (CMPs; $lin^-/ckit^+/CD34^+/CD16/CD32^{low}$), granulocyte macrophage progenitors (GMPs; $lin^-/ckit^+/CD34^+/CD16/CD32^{high}$), and megakaryocyte-erythroid progenitor (MEP; $lin^-/ckit^+/CD34^-/CD16/CD32^{low}$) cells using a FACSAria flow cytometer (BD Biosciences). Dead cells were excluded by staining with 7-AAD. Subsequently, for the analysis of single *MLL-AF9* colonies, a similar procedure was followed without the Ficoll separation. Cells were then analyzed on an LSR II (BD Biosciences).

Chromatin immunoprecipitation

ChIP experiments were performed on the knockin *MLL-AF9* cell line 4166,[29] *MLL-AF9* mBM clones, and primary AML samples of patients with known *MLL-AF9* rearrangements, according to the ChIP protocol (available on the Millipore Web site, http://www.millipore.com/userguides/tech1/mcproto407). Immunoprecipitation of cross-linked chromatin was performed with Abs against Histone H3 (trimethyl K4), Histone H3 (dimethyl K79; both Abcam; Ab ab8580, respectively, ab3594), and anti–trimethyl-Histone H3 (Lys27; Millipore) or an equal amount of isotype IgG (Cell Signaling Technology) as a background control. Three independent experiments were performed and the amount of immunoprecipitated DNA is represented as signal relative to cross-linked input DNA. Q-PCR was performed using primers (supplemental Table 1) directed against promoter regions of *EVI1*, *HoxA9*, and *β-actin*.

Transplantation experiments

For in vivo experiments, 4166 cells were transduced with shRNA-bearing or control vector at an MOI of 100. Transduced cells were grown in 4166 medium overnight without puromycin selection. Transduced or untreated 4166 cells combined with wild-type BM cells were transplanted via tail vein injection into 8-week-old lethally irradiated (8 Gy) C57BJ/6 mice (1×10^5 4166 cells + 5×10^5 wild-type normal BM cells/mouse; 9 mice in each of the *Evi1* shRNA, control virus, and untreated groups). All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Cell cycle, apoptosis, and in vitro drug-resistance assay after *Evi1* knockdown

Evi1 knockdown in 4166 cells was performed as described in the previous paragraph, and cells were selected for 3 days on 1.5 μg/mL puromycin before conducting experiments. Analyses for cell cycle and apoptosis were performed as previously described.[29]

In vitro drug resistance was assessed with the MTT assay.[30] Briefly, 96-well microculture plates contained 1.5×10^4 4166 cells suspended in 100 μL with 6 duplicate concentration ranges of each drug. The following drugs and range of concentrations were used: cytarabine (0-2μM); idarubicin (0-0.16μM). Untreated 4166 were used as a control. Cells were incubated for 3 days in the presence of each drug, and cell viability was measured by adding 10 μL of MTT solution (5 mg/mL) and culturing for an additional 6 hours, followed by addition of 100 μL of acidified 2-propanol. Optical density (OD) was measured at 562 nm on a microplate reader (Victor 3; PerkinElmer). Pilot assays were conducted to secure that the OD is linearly related to the number of viable cells within the settings of our experiments. Cell survival was calculated by the following formula: (mean OD drug-treated wells)/(mean OD control wells) × 100%. Dose-response curves and assessment of LD50 (lethal dose for 50%) was performed with GraphPad Prism software.

Statistical analysis and gene expression profiling

Statistical analyses were performed using Mathworks (Matlab) with the statistical and bioinformatics toolbox. Differences in FAB classification for the $EVI1^{pos}$ and $EVI1^{neg}$ AMLs were assessed using the Fisher exact test. Clustering analyses of the gene expression profiles (Gene Expression Omnibus GSE6891) were performed as previously described.[31]

Results

$EVI1^{neg}$ *MLL*-rearranged AMLs represents a distinct subtype with different gene expression profiles compared with $EVI1^{pos}$ AMLs

Previously, we found frequent *EVI1* overexpression in AMLs with *MLL* rearrangements.[19,20] To investigate whether $EVI1^{pos}$ and

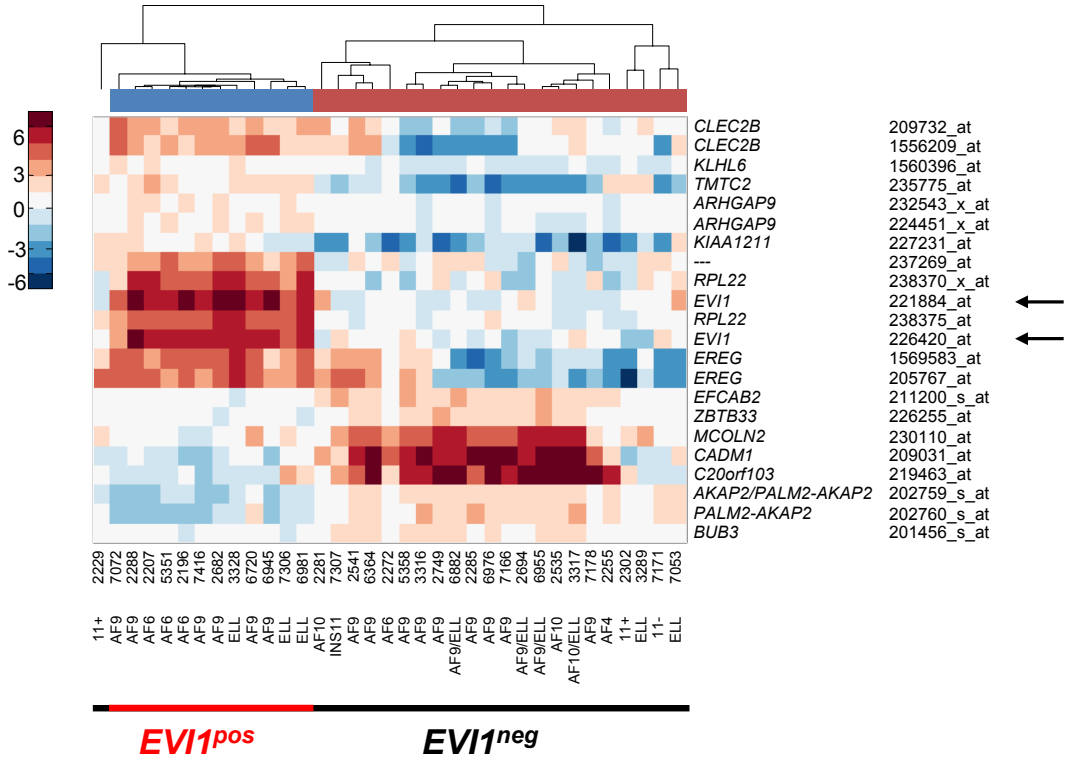

Figure 1. Supervised gene expression profiling of *MLL*-rearranged AMLs uncovers 2 different subgroups. Pearson correlation clustering of 35 *MLL*-rearranged leukemias defined 2 subgroups, an *EVI1*^{pos} group and an *EVI1*^{neg} group (supplemental Figure 1). Supervised analysis revealed 22 probe sets that were differentially expressed between the 2 *MLL*-rearranged subgroups. Red color corresponds to high correlation, whereas blue color corresponds to low correlation of mRNA expression of genes in patient samples. Note that arrows (←) point to probe sets for *EVI1* mRNA expression, which are part of the distinctive signature separating the 2 clusters.

EVI1^{neg} *MLL*-rearranged leukemias represent distinct molecular leukemia subtypes, we performed unsupervised clustering of gene expression profiling (GEP) data of 506 AMLs and identified a cluster that contains the majority of *EVI1*^{neg} AMLs. Pearson correlation distances of the 35 *MLL*-rearranged AMLs illustrate *EVI1*^{pos} being distinct from *EVI1*^{neg} AMLs (supplemental Figure 1). Comparison of mRNA gene expression data of *EVI1*^{neg} versus *EVI1*^{pos} revealed 17 differentially expressed genes (22 probe sets), which strikingly contain 2 probe sets for *EVI1* (Figure 1). *EVI1* positivity was validated by real-time quantitative PCR (RQ-PCR) (Figure 2A; supplemental Table 2). Thus, *EVI1*^{pos} *MLL*-rearranged AMLs represent a unique subtype that is different from *EVI1*^{neg} MLL-rearranged myeloid leukemias.

EVI1 is expressed in 11q23-rearranged AMLs with distinct *MLL*-fusion partners

To study the relationship between *EVI1* expression and distinct MLL-fusion genes, we determined the relative *EVI1* expression by RQ-PCR in a larger cohort of patients with *MLL*-rearranged leukemias (54 cases from the HOVON cohort and 48 AMLs from the AMLSG cohort). *EVI1* expression (*EVI1*^{pos}) was found in 44 of 102 *MLL*-rearranged cases. Patient characteristics and the relative *EVI1* expression of the 102 *MLL*-rearranged AMLs included in this study are shown in supplemental Table 2. EVI1 positivity was found in 0 of 3 *MLL-AF4*, 12 of 15 *MLL-AF6*, 20 of 50 *MLL*-AF9, 2 of 8 *MLL-AF10*, 5 of 12 *MLL-ENL* and 3 of 5 *MLL-ELL* cases, and in 2 of 9 cases with other MLL rearrangements (Figure 2A). Western blot analysis on protein lysates of selected *EVI1*^{pos} AML samples with *MLL-AF6*, *MLL-AF9*, and *MLL-ENL* rearrangements and a control AML with a 3q26 rearrangement (3q) revealed high EVI1 protein expression (Figure 2B). An AML sample that did not express *EVI1* mRNA (*EVI1*^{neg}) showed no EVI1 protein on the same Western blot (Figure 2B).

EVI1^{neg} *MLL-AF9* AMLs are morphologically different from the *EVI1*^{pos} cases

MLL-AF6, *MLL-AF9*, and *MLL-ENL* are among the most frequently occurring MLL rearrangements in AML.[32,33] We found that 31 of 36 *EVI1*^{neg} *MLL-AF6*, *MLL-AF9*, and *MLL-ENL* cases for which morphologic data were available had FAB-M5 monoblastic morphology. On the other hand, only 5 of 28 *EVI1*^{pos} *MLL-AF6*, *MLL-AF9*, and *MLL-ENL* cases were of the FAB-M5 subtype. In fact, *EVI1*^{pos} *MLL*-rearranged cases were found within all FAB classes (Fisher exact test $P < .0001$; supplemental Table 2). Because FAB-M5 cases mainly consist of monoblasts, we sought to determine whether the stem and progenitor cell–enriched CD34$^+$/CD38$^-$ and CD34$^+$/CD38$^+$ populations in the *EVI1*^{neg} AMLs were *EVI1*^{neg} as well. CD34$^+$/CD38$^-$ and CD34$^+$/CD38$^+$ fractions from 2 *EVI1*^{neg} *MLL-AF9* cases were FACS-sorted (supplemental Figure 2a, supplemental Table 3) and studied for *EVI1* expression. The phenotypically immature fractions in these AMLs did not show *EVI1* expression (supplemental Figure 2b), emphasizing that these FAB-M5 *MLL-AF9* AMLs are genuinely *EVI1*^{neg}.

EVI1 expression pattern in *EVI1*^{pos} *MLL*-rearranged AMLs is aberrant

EVI1 mRNA expression is inversely correlated with differentiation in normal human BM samples, that is, *EVI1* is high in primitive CD34$^+$/CD38$^-$ fractions and markedly lower in the more differentiated CD34$^+$/CD38$^+$, CD34$^+$/CD38^{++}, or in CD34$^-$ cells (Figure

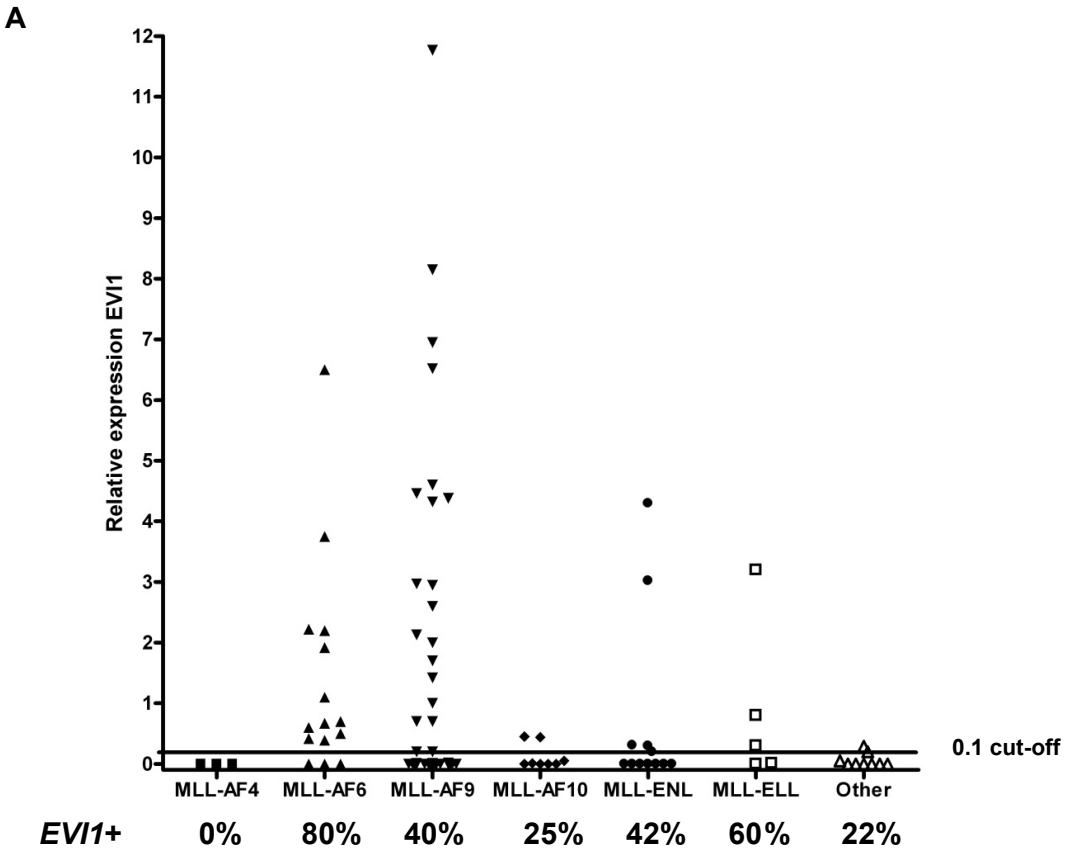

Figure 2. *EVI1* is frequently expressed in MLL-rearranged leukemias. (A) Relative *EVI1* expression of patients with different 11q23 aberrations, corresponding to *MLL-AF4*, *MLL-AF6*, *MLL-AF9*, *MLL-AF10*, *MLL-ENL*, and other *MLL*-fusions. A cutoff value of 0.1 relative to the calibrator ovarian carcinoma cell line SKOV3 was chosen to classify *EVI1*^{pos} from *EVI1*^{neg} cases.[19] Per *MLL* translocation, the percentage (%) of *EVI1*^{pos} patients is indicated. (B) EVI1 protein expression is shown in selected patient samples with MLL-rearranged leukemias. *For the *MLL-AF6* sample, we observed EVI1 protein expression at longer exposure time. A 3q26/*EVI1*-rearranged AML and an EVI1-negative case served as, respectively, positive and negative control for EVI1 protein expression. Actin staining shows equal protein loading. MW marker indicates molecular weight marker; marking bands at, respectively 170, 130, 95 kDa on EVI1 Western and 55, 43 kDa on actin Western blot.

3A). To investigate whether the *EVI1* expression patterns in defined subfractions of *MLL*-rearranged leukemias were abnormal, we determined *EVI1* mRNA levels in sorted fractions of *EVI1*^{pos} *MLL*-rearranged AMLs. In the *MLL-AF6* (n = 2) and *MLL-AF9* (n = 2) cases, high *EVI1* mRNA expression levels were observed in sorted CD34$^+$/CD38$^-$, CD34$^+$/CD38$^+$, CD34$^-$/CD38$^+$, and CD34$^-$/CD38$^-$ fractions (Figure 3B, supplemental Figure 3). Thus, in contrast to normal human BM-sorted fractions, *EVI1*^{pos} *MLL*-rearranged AMLs show ubiquitous expression of *EVI1* in the distinct stem and progenitor fractions.

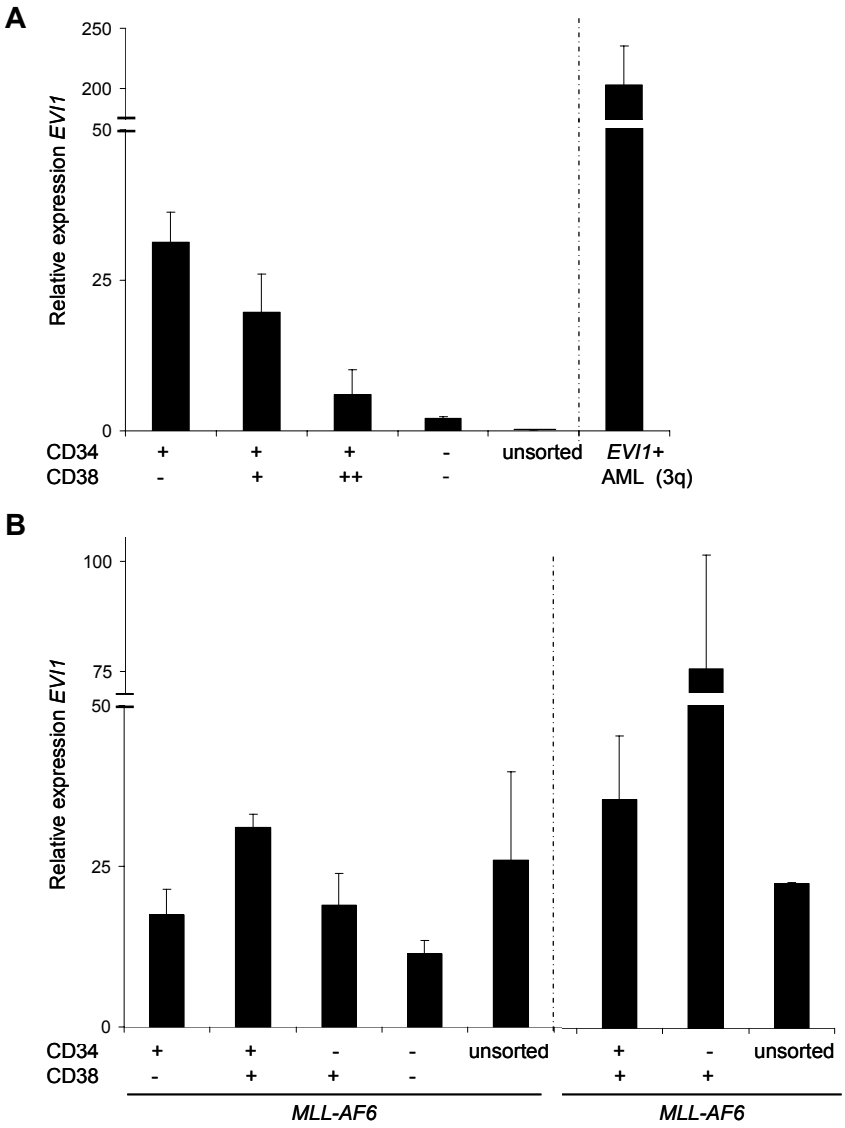

Figure 3. Aberrant expression of *EVI1* mRNA in sorted CD34/CD38 fractions of *MLL-AF6*–rearranged AMLs. BM cells from (B) 2 *MLL-AF6* rearranged AMLs and (A) normal BM were FACS sorted for the distinct CD34/CD38 populations, with subsequent isolation of mRNA. Relative expression was normalized against the reference gene *PBGD*. Each measurement was carried out in triplicate and SD is shown per measurement.

Evi1 is expressed in *MLL-AF9*–transformed murine BM cells

We next wished to investigate whether there was a causal relationship between the expression of MLL-fusion proteins and *EVI1* in myeloid precursor cells. Because *MLL-AF9* cases formed the major fraction of *MLL*-rearranged AMLs (49%) and *EVI1*^{pos} *MLL-AF9*–rearranged MLLs show a clinical behavior that is identical to the whole *EVI1*^{pos} *MLL*-rearranged AML cohort (Gröschel et al[20] and Gröschel et al[34]), we focused for the remainder of our study on the effects of MLL-AF9. Ficoll-separated murine BM (mBM) cells were transduced with retrovirus containing *MLL-AF9*, *E2A-PBX*, or empty vector (EV) and subsequently cultured in an in vitro clonogenic assay (schematic outline of the procedure in supplemental Figure 4A). In agreement with the literature, *MLL-AF9* and *E2A-PBX*–transformed cells provided colonies that could be serially replated, whereas EV control colonies could not (Figure 4A). Both *MLL-AF9* and *E2A-PBX*–transformed mBM colony cells showed elevated *Meis1* transcripts, whereas sustained, high *Evi1* mRNA was primarily found in *MLL-AF9*–transformed colonies (Figure 4B). We also detected up-regulation of *Evi1* mRNA in mBM cells transduced with other *MLL*-fusion constructs (supplemental Figure 4B-C). In accordance with the mRNA expression data, lysates of collected colonies of *MLL-AF9*–transduced mBM revealed EVI1 protein expression as detected by Western blotting, whereas no EVI1 protein was detected in *E2A-PBX*–transformed cells (Figure 4C). Together, these experiments demonstrate *Evi1* expression in mBM cells on transformation by *MLL* fusion genes as observed in human *MLL*-rearranged AMLs.

Evi1^{pos} and *Evi1*^{neg} replatable colonies are generated on MLL-AF9 transformation of mBM cells

The above experiments show that *Evi1* expression is high in pooled fractions of myeloid progenitors after transduction with *MLL-AF9*; however, they do not answer the question of whether *MLL-AF9* transduction leads to *Evi1* induction in all or in a subset of transformed progenitor cell. To address this question, we generated clonal *MLL-AF9* mBM cell lines by picking and expanding single primary *MLL-AF9*–transduced colonies (strategy outlined in supplemental Figure 5A). Seventy-five of those picked colonies could be indefinitely replated. In 26 (35%) of 75 clones, *Evi1* mRNA expression was detected, with half of them (13 of 75) expressing high *Evi1* mRNA levels (supplemental Figure 5B). *Evi1* mRNA expression levels remained stable after serial rounds of replating (supplemental Figure 5D). *Evi1*^{neg} clones remained negative on replating. We did not observe a difference in growth capacity

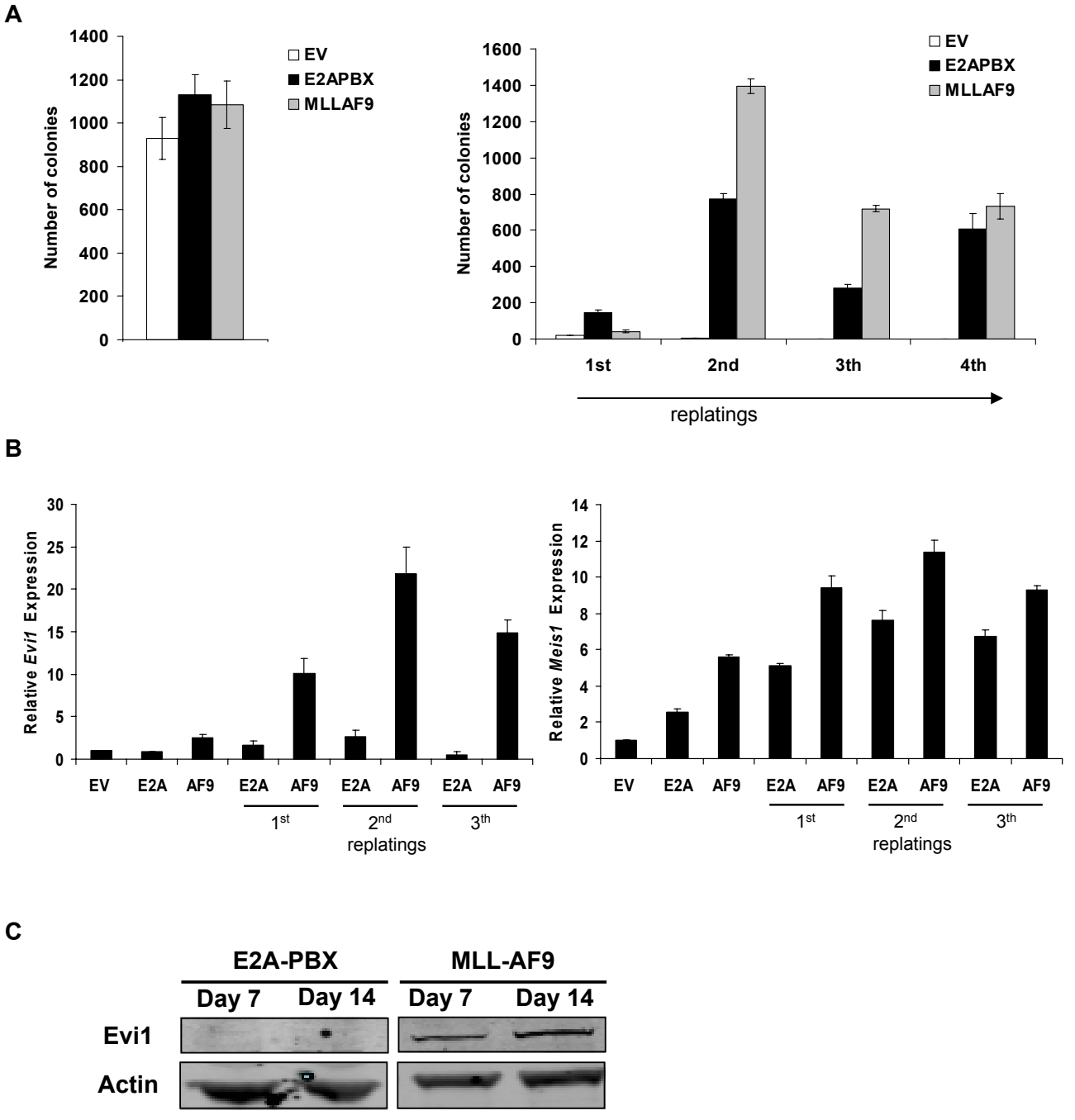

Figure 4. High *Evi1* expression in *MLL-AF9*–transformed mononucleated normal mouse BM. (A) Transduction of normal mBM with *MLL-AF9* leads to colony formation and sustained replating capacity compared with empty vector control (EV). Normal mBM transduced with *E2A-PBX* served as a positive control for transformation. Experiments were performed in triplicate and presented as average number of CFUs with SD. (B) *Evi1* and *Meis1* relative mRNA expression was determined from colonies at day 7, 14, or 21. An average of 3 measurements is depicted with SD. (C) Detection of Evi1 protein by Western blot analysis in lysates of pooled colonies from day 7 or 14. Blots were reprobed with an α-actin Ab to show equal protein loading.

between $Evi1^{pos}$ or $Evi1^{neg}$ clones as monitored by colony or liquid cell cultures (supplemental Figure 5C,E).

Evi1 expression pattern in $Evi1^{pos}$ *MLL-AF9*–transformed mouse BM cells is abnormal

Flow cytometric analysis of serially replated *MLL-AF9*–transformed BM cells revealed a remarkable difference between $Evi1^{pos}$ versus $Evi1^{neg}$ *MLL-AF9*–transformed clones. $Evi1^{pos}$ *MLL-AF9* cell fractions contained CMPs as well as GMPs, whereas in $Evi1^{neg}$ *MLL-AF9*–transformed cell fractions only GMPs were observed (Figure 5A-B). These data are in line with immunophenotyping and morphologic analysis of human *MLL*-rearranged AMLs which showed that $Evi1^{neg}$ cases were more mature than the $Evi1^{pos}$ leukemias.

Importantly, in normal mBM, *Evi1* is expressed primarily in the HSC fraction while CMP, GMP, and MEP fractions show significantly reduced levels (Figure 5C). To address the question of whether the *Evi1* expression pattern was aberrant in transformed mBM cells, we determined *Evi1* mRNA levels in mature Lin^+ cell fractions of $Evi1^{pos}$ clones. Q-PCR on lineage-positive cells sorted

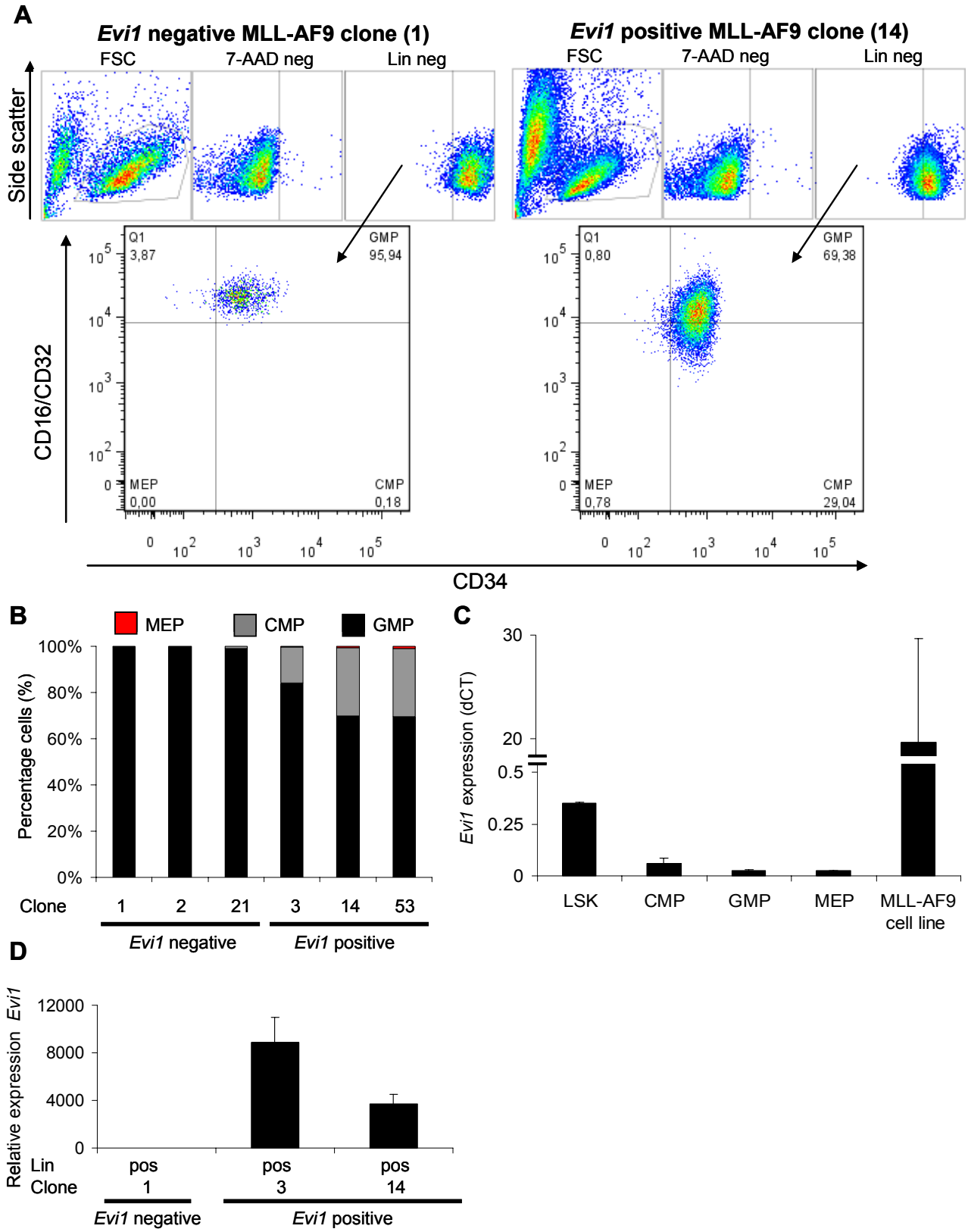

Figure 5. Enrichment of CMPs in *Evi1*^{pos} clonal *MLL-AF9*–transformed BM cell cultures. (A) Example of FACS-sorting strategy to select for CMP, GMP, and MEP derived from cultured *Evi1*^{pos} and *Evi1*^{neg} *MLL-AF9* clones. (B) Percentages of CMP, GMP, and MEP relative to the total number of progenitor cells are calculated for each clone. (C) *Evi1* expression in LSK, CMP, GMP, and MEP subpopulations of normal mononucleated mBM cells. The *MLL-AF9* cell line 4166 served as positive control for *Evi1* expression. (D) *Evi1* mRNA expression in lineage-positive subfractions of *MLL-AF9* clones. The expression of *Evi1* was calculated relative to the lineage-negative fraction of *MLL-AF9* clone #1. *Evi1* relative expression was normalized using *Hprt* as a reference gene. For the last 2 panels, the average of 3 experiments with its SD is shown.

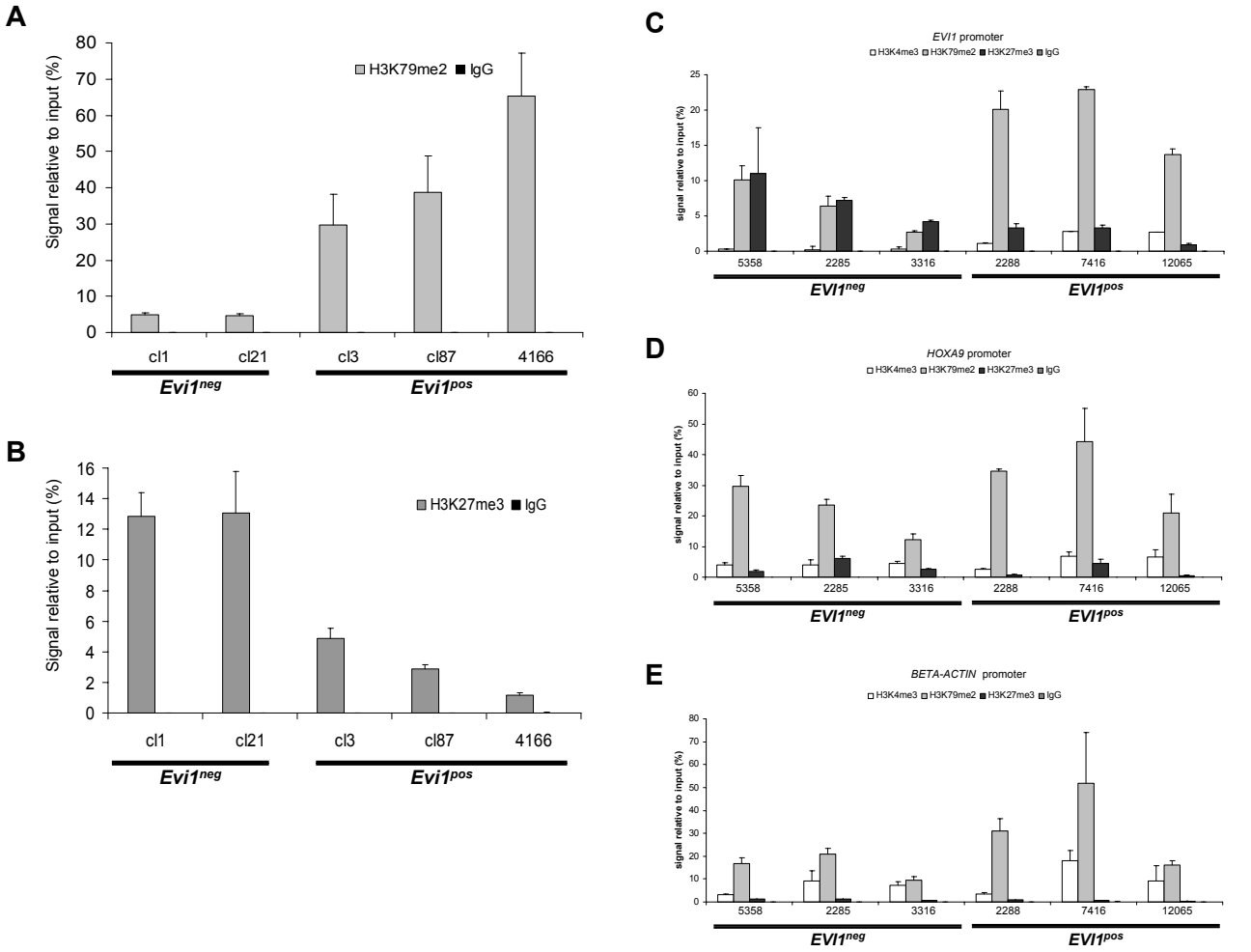

Figure 6. Enrichment of H3K79me2 on the *Evi1* promoter of *MLL-AF9*–transformed cells. ChIP using (A) H3K79me2 and (B) H3K27me3 Abs showing enrichment of the H3K79me2 mark on the *Evi1* promoter of *Evi1*^{pos} vs *Evi1*^{neg} clones. Conversely, the H3K27me3 mark is enriched on the *Evi1* promoter of *Evi1*^{neg} clones compared with *Evi1*^{pos} *MLL-AF9* clones. ChIP using H3K4me3, H3K79me2, and H3K27me3 Abs performed on 3 *EVI1*^{pos} and 3 *EVI1*^{neg} *MLL-AF9* rearranged leukemias showed higher H3K79me2 marks present on the EVI1 promoter of *EVI1*^{pos} *MLL-AF9*–rearranged human AMLs compared with EVI1neg cases. Q-PCRs were performed to monitor relative enrichment of each histone mark on the promoters of either (C) EVI1, (D) HoxA9 promoter, or (E) β-actin. Experiments show average relative enrichment with SD of either (A-B) 3 biologic replicates or (C-E) 2 independent ChIP pulldowns with triplicate Q-PCR measurements performed on the same cross-linked patient material.

from *Evi1*^{pos} *MLL-AF9*–transformed clones showed high *Evi1* levels (Figure 5D) in the Lin$^+$ cells. Because normal Lin$^+$ cells are *Evi1*^{neg}, these data point to an aberrant *Evi1* expression pattern in these *MLL-AF9*–transformed clones. In fact, these data are in full agreement with the abnormal expression patterns observed in human *EVI1*^{pos} *MLL*-rearranged AMLs (Figure 3, supplemental Figure 3) and are highly suggestive of a role for MLL-AF9 in aberrant *EVI1* expression in *MLL*-rearranged *EVI1*^{pos}-transformed cells.

Enrichment of H3K79me2 on the *EVI1* promoter in *EVI1*^{pos} *MLL-AF9*–transformed cells

MLL-AF9 rearrangements result in loss of the H3K4 methyl transferase domain of MLL, but create a chimeric protein that recruits the H3K79 histone methyl transferase DOT1L, leading to H3K79 dimethylation (H3K79me2) at target promoter regions.[35,36] To investigate whether the observed up-regulation of *Evi1* is mediated by MLL-AF9, we used ChIP to assess H3K79me2 of the *Evi1* promoter region. Clones with high *Evi1* expression showed significant H37K79me2 enrichment on the *Evi1* promoter (Figure 6A), whereas H3K27me3, a mark for repressed genes was low on the *Evi1* promoter (Figure 6B). On the contrary, *Evi1*^{neg} clones showed reduced levels of the H3K79me2 but high H3K27me3 at the *Evi1* promoter (Figure 6A-B). Importantly, in the *Evi1*^{neg} clones, we were able to detect expected enrichment of H3K79me2 on the putative MLL-AF9 target genes *HoxA9* and *Meis1* (supplemental Figure 6, and data not shown) indicating that the lack of H3K79me2 mark at the *Evi1* locus was specific. H3K79me2 enrichment was also detected on the *Evi1* promoter region in the *MLL-AF9* knock-in cell line 4166, compared with wild-type mBM cells (supplemental Figure 7). Specificity of the H3K79me2 ChIP in 4166 cells was shown by the clear H3K79me2 mark on the promoter region of the *MLL-AF9* target gene *HoxA9*, in contrast to the absence of an enrichment on the *HoxC8* promoter, which is not a target gene of MLL-AF9 (supplemental Figure 7). *Dot1L* knockdown carried out on the *Evi1*-expressing *MLL-AF9* knockin cell line 4166 showed a partial loss of *Dot1L* expression (supplemental Figure 8). *Evi1* levels decreased significantly on *Dot1L* knockdown. Moreover, this decrease was comparable with that of *HoxA9* and *Meis1* (supplemental Figure 8). These data suggest that the H3K79me2 mark at the promoter of *Evi1* is important for its expression in *MLL-AF9*–transformed cells.

We applied the same type of ChIP experiments for a panel of 6 human AML samples, that is, 3 with and 3 without *EVI1* expression. Again, we observed enrichment of H3K79me2, with corresponding low levels of H3K27me3 levels on the *EVI1* promoter of AML samples with high *EVI1* expression. Notably, AML samples with no *EVI1* expression exhibited lower levels of the H3K79me2 mark with a striking concomitant rise of equal levels of H3K27me3 on the *EVI1* promoter region (Figure 6C). This phenomenon was only observed on the *EVI1* promoter and not on the *HoxA9* or *β-actin* promoter of $EVI1^{neg}$ human AML samples (Figure 6D-E)

MLL-AF9 maintains *Evi1* expression in *Evi1*-expressing BM fractions

The flow cytometric difference between $Evi1^{pos}$ versus $Evi1^{neg}$ *MLL-AF9*–transformed clones led us to hypothesize that *MLL-AF9* may transform different progenitor fractions (Figure 5A). We therefore purified HSCs as $Lin^-CD34^-Sca1^+c\text{-}Kit^+$ and GMP as $Lin^-CD34^+Sca1^-c\text{-}Kit^+CD16/32^+$ from mouse BM, which we subsequently transduced with *MLL-AF9*. Both HSC- and GMP-produced colonies were replatable in semisolid media supplemented with IL-3, IL-6, and SCF. *MLL-AF9*–expressing HSC-derived colonies expressed *Evi1*, while *MLL-AF9*–expressing GMP-derived colonies expressed no detectable levels of *Evi1* mRNA (supplemental Figure 9). Thus, our data suggest that the difference between $Evi1^{pos}$ and $Evi1^{neg}$ MLL-AF9–transformed cells is the result of different progenitors that were initially transformed by the same *MLL*-fusion gene. In normal mouse BM fractions, *Evi1* expression is high in the LSK fraction that contains the HSCs and low in CMP, GMP, or MEP compartments (Figure 5C). This difference was also observed in the different human stem cell and progenitor fractions. It is therefore suggestive that *MLL-AF9* can transform *Evi1*-expressing primitive marrow precursors which subsequently maintain $Evi1^{pos}$ and the fusion gene is capable of transforming more mature $Evi1^{neg}$ progenitors that stay $Evi1^{neg}$.

Evi1 knockdown inhibits the growth of *MLL-AF9*–transformed cells in vitro and in vivo

To investigate the role of *EVI1* in MLL-AF9–directed leukomogenesis, we made use of lentiviral shRNA-mediated knockdown of *Evi1* in experimental *MLL-AF9* leukemia models. The *MLL-AF9* knock-in leukemia cell line 4166 closely mimics human disease because each cell contains only one copy of wt-*MLL* and one copy of the *MLL-AF9* fusion gene, under control of the endogeneous *MLL* promoter.[29] This 4166 cell line expressed high levels of *Evi1* mRNA and EVI1 protein, which could be down-regulated after *Evi1* knockdown (Figure 7A,D). *Evi1* knockdown resulted in reduced clonogenic survival in methylcellulose semisolid media (Figure 7B). Similarly, *Evi1* knockdown also reduced colony formation in $Evi1^{pos}$ *MLL-AF9* mBM clones. Importantly, $Evi1^{neg}$ cells were unresponsive to *Evi1* knockdown, showing that the observed effects on colony formation are not because of off-target effects of the applied shRNA for *Evi1* (supplemental Figure 10).

To discern by which mechanism *Evi1* knockdown leads to reduced cell growth, we performed cell cycle and apoptosis analysis on 4166 cells. Flow cytometric analysis showed that *Evi1* knockdown did not lead to significant changes in cell-cycle profile (Figure 7C top panel). On the other hand, we observed a significant increase in apoptosis in 4166 cells after *Evi1* knockdown compared with vector control transduced 4166 cells (Figure 7C bottom panel). The increase in apoptosis after *Evi1* knockdown was characterized by an induction of the apoptotic markers: cleaved forms of Caspase -3, 8, and -12 and PARP (Figure 7D). These experiments demonstrate that down-regulation of *EVI1* expression in *MLL-AF9*–transformed cells causes reduced cell growth through induction of apoptosis, without affecting cell-cycle progression.

We have shown that *MLL*-rearranged leukemia patients with high *EVI1* expression have an adverse prognosis with a corresponding poor response to current treatment modalities.[19,20] Therefore, we determined the cytotoxicity in 4166 cells of 2 cytostatic drugs, cytarabine and idarubicin, with a previously described in vitro toxicity test.[30] We treated cells with *Evi1* specific or control shRNA and observed that a reduction of Evi1 protein levels resulted in an increased sensitivity of 4166 cells to either idarubicin or cytarabine or the combination of the 2, as monitored by a left-shift of the dose-response curve and a reduced LD50 after *Evi1* knockdown (supplemental Figure 11). These results show that $Evi1^{pos}$ *MLL-AF9*–transformed cells become more sensitive to chemotherapeutic agents on *Evi1* down-regulation.

Previously, we have shown that transplantation of 4166 cells in irradiated mice recapitulates *MLL-AF9* leukemia.[29] To study the effect of *Evi1* knockdown on in vivo tumor formation, lethally irradiated mice were transplanted with a mixture of wild-type donor cells plus 4166 cells that were either untreated or transduced with *Evi1* shRNA or control vector. Mice were killed when becoming moribund. At necropsy, mice had signs of AML, displaying leukocytosis and splenomegaly. As depicted in Figure 7E, most of the control animals (transplanted with 4166 cells that were either untreated or transduced with control virus) died of leukemia within 170 days after transplantation. In contrast, leukemia development was significantly delayed in animals receiving 4166 cells transduced with *Evi1* shRNA ($P < .001$, log-rank test), with 44% of the animals being long-term survivors (Figure 7E). Mice that did not develop leukemia were killed at the end of the experiments and showed no signs of leukemogenesis at autopsy.

Discussion

In previous studies, we found that a subset of AMLs overexpressed *EVI1* and were associated with an adverse prognosis compared with $EVI1^{neg}$ AMLs that had similar molecular and karyotypic features. In particular, it was demonstrated that leukemias with *MLL* translocations showed frequent *EVI1* expression.[19,20] In the current study, we showed in a larger AML cohort carrying 11q23 translocations that ~ 43% of all *MLL*-rearranged leukemias are $EVI1^{pos}$, and that *EVI1* expression was independent of the fusion partner involved in the translocation with *MLL*. In addition, we present evidence that, using *MLL-AF9* AMLs as an example of the whole cohort, $EVI1^{pos}$ *MLL-AF9* AMLs differ molecularly, morphologically, and immunophenotypically from $EVI1^{neg}$ *MLL-AF9* leukemias.

Using mouse models for *MLL-AF9* fusion leukemia, we provide evidence that on transformation of $Evi1^{pos}$ HSCs, the presence of the MLL-AF9 fusion protein causes *Evi1* expression not to be turned off (supplemental Figure 9). Moreover, shRNA-mediated knockdown inhibits leukemia growth both in vitro and in vivo, suggesting that Evi1 is important for the growth of these $EVI1^{pos}$ *MLL*-fusion gene–transformed leukemias. These results are corroborated by recent reports showing that MLL-ENL up-regulates *Evi1*

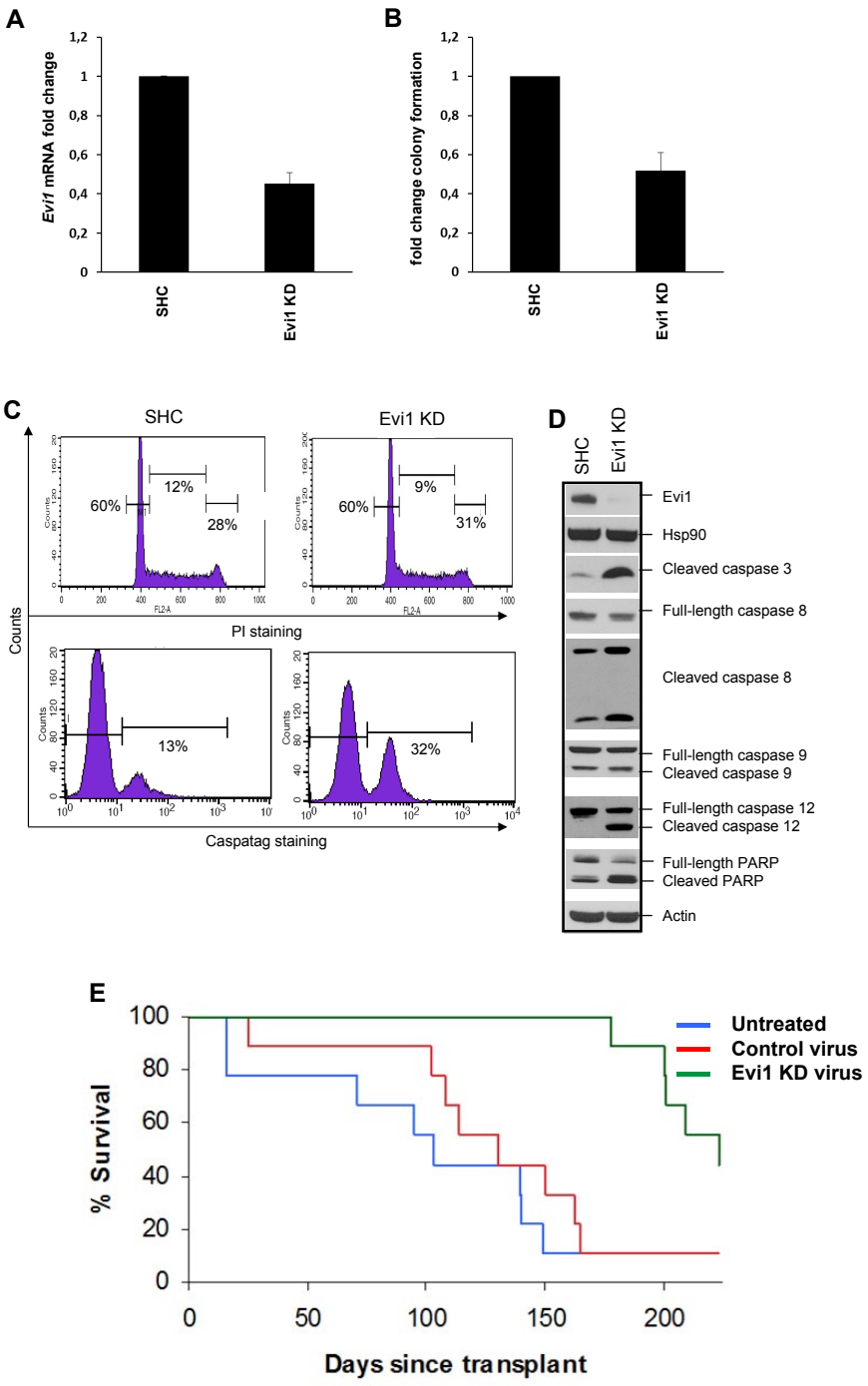

Figure 7. *Evi1* knockdown (KD) in *MLL-AF9* cell line 4166 leads to reduced growth in vitro and in vivo. Transduction of 4166 with lentiviral vectors containing shRNA against *Evi1* leads to reduced (A) *Evi1* mRNA and (D top) protein expression. (B) Reduced *Evi1* levels were accompanied with a significant reduction of colony formation. Fold change of 4 experiments (mean + SD) is shown for panels A and B. (C) *Evi1* shRNA-mediated inhibition of cell growth was attributed to (bottom panel) an induction of apoptosis, (top panel) with no changes in cell-cycle profile. (D) Western blotting shows an increase of (pro)apoptotic markers after knockdown of *Evi1*. HSP90 and actin were used to show equal cell viability and loading of protein lysates. SHC indicates short hairpin control. (E) Survival curve of lethally irradiated mice transplanted with 1×10^5 4166 cells, transduced with either *Evi1* shRNA or control virus supplemented with 5×10^5 normal mBM cells. As a control group, untreated 4166 cells were transplanted to monitor offset of normal 4166-mediated MLL-AF9 tumorigenesis. All 3 groups contained 9 mice each. By posttransplantation day 170, the majority of the mice in the untreated (8 of 9) or control virus (8 of 9) transduced group had succumbed to leukemia, while all mice receiving *Evi1* shRNA-transduced cells were still alive. Leukemia development was significantly delayed in animals receiving *Evi1* shRNA-transduced 4166 cells (log-rank $P < .001$) with 44% of the mice being long-term survivors.

expression in a mouse leukemia model, in which leukemic transformation was dependent on *Evi1*.[24,25]

We noted that although *MLL-AF9* is readily able to transform normal mouse BM cells, only a fraction of colonies were *Evi1*^{pos} (supplementary figure S5B). Similar to Arai et al, we found that in these retroviral models of *MLL*-fusion gene leukemia, *Evi1* overexpression was predominantly associated with the stem cell–enriched LSK raction of normal BM compared with the more differentiated myeloid progenitors CMP, GMP, and MEP.[25] Notably, several studies have shown that *MLL*-fusion gene–induced leukomogenesis is more efficient in LSKs compared with the more mature myeloid progenitors GMPs.[23,37] Because *Evi1* expression is normally high in these LSK cells, our results suggest that coexpression of *Evi1* is at least partly responsible for the observed transformation of LSKs. This is in line with our observations in human AMLs, which show that *EVI1*^{neg} *MLL*-rearranged leukemias are predominantly of the monoblastic subtype whereas the *EVI1*^{pos} leukemias were not restricted to one morphologic subtype. Furthermore, we show that while *EVI1* expression in normal hematopoiesis is primarily restricted to the stem cell–enriched CD34$^+$/CD38$^-$ fraction, expression of *EVI1* is aberrantly extended into the more differentiated CD34$^+$/CD38$^+$, CD34$^-$/CD38$^+$, and CD34$^-$/CD38$^-$ fractions in *EVI1*^{pos} *MLL*-rearranged leukemias. Recently, Eppert et al reported that

EVI1 is part of the gene signature associated with HSCs and leukemia stem cells (LSCs).[38] Moreover, they show that high expression of these HSC- and LSC-associated signatures is correlated with poor survival.[37] Collectively, these results underline the prognostic significance of high *EVI1* expression in AML and suggest that aberrant transcriptional regulation of *EVI1* might impart aberrant self-renewal to LSCs. In *EVI1*^{neg} *MLL*-rearranged leukemias, defects in other genes may have taken over this role.[26,39]

We further investigated what could be the mechanism leading to up-regulation of *Evi1* in a subset of *MLL-AF9*–transformed normal mBM cells. It is well established that the *Evi1* locus contains a hot spot for retroviral insertions.[4] Strikingly, a significant number of retroviral-induced leukemias contain mutually exclusive retroviral insertions in *Evi1* or its family member *Prdm16*.[40] Retroviral insertions in *Evi1* locus are predominantly between the *Mds* and *Evi1* gene leading to activation of *Evi1* and not *MdsEvi1*.[4,40] Because we detected concomitant up-regulation of *MdsEvi1* and *Evi1* in our *Evi1*^{pos} *MLL-AF9*–transduced mBM cells, and no up-regulation of *Prdm16* transcript in *Evi1*^{neg} *MLL-AF*9–transduced cells (supplemental Figure 12), we postulated that *Evi1* up-regulation is unlikely the result of retroviral insertion into the *Evi1* locus.

MLL fusions can transactivate the *EVI1* promoter in luciferase reporter assays (supplemental Figure 13).[25] Although others reported binding of MLL-ENL to the *Evi1* promoter, we were not able to show direct interaction between MLL-AF9 and *Evi1*, possibly because of the lack of suitable ChIP-grade Abs. However, we observed clear differences between *Evi1*^{pos} and *Evi1*^{neg} *MLL-AF9*–transformed cells (both human or mouse) with regard to enrichments of H3K79/H3K4 versus H3K27 on the *EVI1* promoter. We hypothesize, based on our ChIP data, that MLL-AF9 up-regulates *EVI1* transcription via H3K79 methylation, which is known to be a major gene regulatory mechanism used by some MLL-fusion proteins in leukemia.[35,36] In accordance, we witnessed that knockdown of *Dot1L* knockdown in 4166 resulted in decreased mRNA expression levels of *Evi1* (supplemental Figure 8). The absence of increased H3K79 methylation at the *EVI1* promoter of *EVI1*^{neg} *MLL-AF9* tumors was locus specific, because *HoxA9*, a known MLL-AF9 target gene, displayed a clear enrichment of the H3K79 mark in those cells. Thus, our findings may be explained by direct regulation of the *Evi1* promoter by MLL-AF9 recruiting the DOT1L enzyme to the locus. ChIP experiments using Abs that recognize MLL-fusion proteins will be essential to adequately address this issue.

We suspect that the relatively silent *Evi1* gene in GMPs was inaccessible for the MLL-AF9 protein complex and hence was not up-regulated. Our findings are in line with a recently described epigenetic profiling of L-GMPs, which showed no H3K79 methylation on the *Evi1* locus in these transformed cells.[41] The MLL-AF9 protein complex contains components of the PAFc and super elongation complex required for MLL-fusion protein function.[42-46] At least for PAFc it is well established that its expression decreases during normal hematopoietic differentiation[47] providing a potential additional layer of regulation of *EVI1* expression by MLL-AF9. The above might explain why we detect *MLL*-fusion leukemias that are *EVI1*^{neg}, because they might originate in cells that are more differentiated (GMP-like, with low *EVI1* expression). In contrast, we propose that the *EVI1*^{pos} *MLL*–rearranged leukemias might arise from cells that are more immature (HSC-like, with higher *EVI1* expression).

Two recent reports describe the development of therapeutic interventions in *MLL*-fusion leukemias. They showed efficacy of 2 small molecule inhibitors, I-BET151 or EPZ004777, in which the first leads to displacement of the MLL-fusion protein complex from chromatin and the second selectively inhibits DOT1L.[48,49] Because we have shown evidence for qualitative differences between *EVI1*^{pos} and *EVI1*^{neg} *MLL*-rearranged AMLs, we suggest that future research is needed to investigate whether EVI1 expression status would predict treatment response with inhibitors for *MLL*-fusion leukemias.

Here we showed evidence that *MLL*-rearranged leukemias can be classified on the basis of their relative *EVI1* expression, separating these leukemias into *EVI1*^{pos} and *EVI1*^{neg} leukemias with clear distinct morphologic, molecular, and mechanistic differences. We provide indirect evidence for a role of MLL-fusion proteins in the regulation of *EVI1* expression in those *EVI1*^{pos} MLL-rearranged AMLs. Furthermore, we suggest a critical role for *EVI1* in the pathogenesis of a subset of *MLL*-rearranged leukemias, and that targeting of *EVI1* in combination with chemotherapeutic agents could be beneficial for patients with *EVI1*^{pos} *MLL*-rearranged AMLs.

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Authorship

Contribution: E.M.J.B., M.H., S.L., C.E., E.W., A.V.K., E.R., E.T., J.R.H., and W.A.H. performed experiments; E.M.J.B., M.H., S.L., S.A.A., J.R.H., R.D., and A.R.K. analyzed the results and made the figures; E.M.J.B., S.L., J.H.K., R.D., and A.R.K. designed the research and wrote the manuscript; H.B.B. performed karyotyping and provided additional patient samples; and H.D. provided biologic materials.

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Correspondence: Ruud Delwel, Department of Hematology, Erasmus University Medical Center, Dr Molewaterplein 50, 3015 GE Rotterdam, The Netherlands; e-mail: h.delwel@erasmusmc.nl.

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