EVI1 Interferes with Myeloid Maturation via Transcriptional Repression of Cebpa, via Binding to Two Far Downstream Regulatory Elements*

Michael Wilson§, Vasiliki Tsakklides§, Minh Tran§, Ying-Yi Xiao§, Yi Zhang†1, and Archibald S. Perkins§12

From the §Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester, New York 14642 and the †Department of Pathology, Yale University, New Haven, Connecticut 06520

One mechanism by which oncogenes work is through perturbation of cellular maturation; understanding the mechanisms by which this occurs can lead to the development of targeted therapies. EVI1 is a zinc finger oncoprotein involved in the development of acute myeloid leukemia; previous work has shown it to interfere with the maturation of granulocytes from immature precursors. Here we investigate the mechanism by which this occurs, using an immortalized hematopoietic progenitor cell line, EML–C1, as a model system. We document that overexpression of EVI1 abrogates retinoic acid-induced maturation of EML cells into committed myeloid cells, a process that can be documented by the down-regulation of stem cell anti-rat-phage colony-stimulating factor. We show that this requires DNA binding capacity of EVI1, suggesting that downstream target genes are involved. We identify the myeloid regulator Cebpα as a target gene and identify two EVI1 binding regions within evolutionarily conserved enhancer elements at +35 and +37 kb relative to the gene. EVI1 can strongly suppress Cebpα transcription, and add-back of Cebpα into EVI1-expressing EML cells partially corrects the block in maturation. We identify the DNA sequences to which EVI1 binds at +35 and +37 kb and show that mutation of one of these releases Cebpα from EVI1-induced suppression. We observe a more complex picture in primary bone marrow cells, where EVI1 suppresses Cebpα in stem cells but not in more committed progenitors. Our data thus identify a regulatory node by which EVI1 contributes to leukemia, and this represents a possible therapeutic target for treatment of EVI1-expressing leukemia.

The differentiation of myeloid progenitors from hematopoietic stem cells and their maturation into granulocytes is a complex process involving a number of key transcriptional regulators, including PU.1 and CEBPα; changes in DNA and histone methylation; and the expression of key lineage-specific cytokine receptors (1, 2). Recent work suggests that uncommitted progenitor cells exist in a metastable state with a fluctuating transcriptome, with commitment to a given lineage occurring by stochastic and directive factors (3). Deregulation of this complex regulatory process can lead to the development of myelodysplastic syndrome and acute myeloid leukemia (AML). 3

Ecotropic viral integration site 1 (Evi1) was first identified as a site of proviral insertion in murine myeloid leukemias (4, 5). The oncogene is expressed as three zinc finger protein isoforms via alternative splicing (6, 7). EVI1 is thought to function as a transcriptional regulator, binding DNA through two conserved zinc finger domains (ZF1, encompassing amino acids 1–249, and ZF2, harbored within amino acids 724–808) (4) in a sequence-specific manner (8, 9). Evi1 plays a critical role in maintaining the hematopoietic stem cell (HSC) compartment in normal bone marrow (10), whereas in the malignant setting, EVII is overexpressed in a subtype of AML characterized by an especially poor prognosis (11, 12). Leukemic cells overexpressing EVI1 display a block in myeloid maturation and resistance to apoptosis, both of which are reversed with EVI1 shRNA knockdown (13, 14). Recent studies have shown that the ability of EVI1 to bind DNA via zinc fingers 1–7 (ZF1 domain) is critical for malignant transformation and that a pyrrole-imidazole polyamide targeted against the canonical EVI1 binding motif partially inhibits the leukemic phenotype (16). However, very little is known about which EVI1 target genes are essential in generating disease.

In an effort to identify key EVI1 occupancy sites critical for disease, we have recently performed chromatin immunoprecipitation and sequencing (ChIP-Seq) along with whole transcriptome analysis (RNA-Seq) in two murine myeloid leukemia cell lines (14). The ChIP-Seq data revealed EVI1 occupancy of a binding site 35 and 37 kb downstream of the CCAAT/enhancer-binding protein α (Cebpα) gene in the murine leukemic cell line DA-1.

* This work was supported by National Institutes of Health Grants R01CA081216 (to A. S. P.), P01HL63357 (to A. S. P.), 5 U24 DK58776 (to Y. Z.), NYSTEM C029547 (to Y. Z.), and NYSTEM C026423 (to Y. Z.). The authors declare that they have no conflicts of interest with the contents of this article.

† To whom correspondence may be addressed: 601 Elmwood Ave., Rochester, NY 14642. Tel.: 585-275-1950 (to Y. Z.); E-mail: yi_zhang@urmc.rochester.edu.

‡ To whom correspondence may be addressed: 601 Elmwood Ave, Rochester, NY 14642. Tel.: 585-276-3399; E-mail: archibald_perkins@urmc.rochester.edu.

The abbreviations used are: AML, acute myeloid leukemia; DOX, doxycycline; HSC, hematopoietic stem cell; ChIP-Seq, chromatin immunoprecipitation and sequencing; RME, RUNX1-MDS1-EVI1; RA, all-trans-retinoic acid; TSS, transcription start site; qPCR, quantitative real-time PCR; Epo, erythropoietin; rtTA, reverse Tet repressor/transactivator; Dox, doxycycline; H3K27, histone H3 Lys27; H3K27ac and H3Kme3, acetylated and trimethylated H3K27, respectively.

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EVI1 Impedes Granulopoiesis via Suppression of Cebpa

A member of the leucine zipper family of transcription factors, C/EBPα is critical for the transition of a common myeloid progenitor to a granulocyte/monocyte progenitor during early hematopoiesis, and Cebpa<sup>−/−</sup> mice do not display terminally differentiated granulocytes in the peripheral blood (17). Additionally, Cebpa<sup>−/−</sup> HSCs exhibit enhanced repopulating capability, and the bone marrow of these mice is replete with myeloid blast cells, an observation that indicates a regulatory role in the early progenitor/stem cell compartment (18). Disruption of CEBPA function via DNA hypermethylation (19, 20), somatic mutation (21), and translational suppression (22) have all been described as contributory factors in human myeloid leukemia. EVI1 is a known regulator of CEBPA mRNA and protein function; the RUNX1-MDS1-EVI1 (RME) fusion protein (product of the AML-associated t(3;21) translocation) is known to suppress Cebpa translation through up-regulation of calreticulin (23); additionally, RME has been shown to bind C/EBPα protein and inhibit its ability to bind DNA and regulate its own transcription, most likely due to the recruitment of histone deacetylases via the CtBP region on the EVI1 portion of RME (24). No direct transcriptional regulation of the Cebpa gene by EVI1 has been described.

Here, we show that EVI1 transduction into immortalized hematopoietic progenitor cell line (EML C1 (25)) can interfere with its all-trans-retinoic acid (RA)-induced myeloid differentiation program; furthermore, EVI1 causes strong suppression of Cebpa transcription, concomitant with decreased RNA polymerase II and p300 occupancy of the Cebpa promoter. We further show that EVI1 binds to and occupies two evolutionarily conserved enhancer elements located 35 and 37 kb downstream of the Cebpa transcriptional start site (TSS) in murine leukemic cells and hematopoietic progenitors. Finally, we show that Cas-9-mediated disruption of the EVI1 binding site is able to restore Cebpa transcription in EVI1-transduced hematopoietic progenitor cells.

**Materials and Methods**

**Cell Lines and Cell Culture**—EML and BHK-MKL cells (25) were provided by S. Tsai. Growth factors and cytokines were gifts of Amgen. DA-1 cells (26) were obtained from J.N. Ihle. Erythropoietin (PROCRIT) was obtained from Ortho Biotech Products, L.P. EML cells were cultured and induced as described (25). For [3H]thymidine incorporation, cells were seeded (3 × 10<sup>5</sup> cells in 0.2 ml) in wells of a 96-well plate with the appropriate growth factor. After 4–6 h, one μCi of [3H]thymidine (ICN) was added in 10 μl to each well, and the cells were incubated for an additional 18–30 h. Cells were transferred onto a glass fiber filter with a Tomtec harvester. Filters were dried, saturated with scintillation fluid in a sealed plastic bag, and counted on a β-counter. All cells were assayed in triplicate. After background subtraction, values were averaged and expressed as a ratio relative to [3H]thymidine incorporation with no factor. For the Cebpa add-back experiment, EML cells were cultured in 1 μM 4-hydroxytamoxifen tamoxifen for 48 h before flow cytometry analysis or concurrently with RA and IL-3 as described previously (25).

**Plasmid Construction**—The pBabe-puro-Evi1HA was constructed by insertion of a BamHI fragment of pBS-Evi1HA(Bam) into the BamHI site of the retroviral vector pBabe-puro (27). The construction of pBS-Evi1HA(Bam) was as follows. The 4.5-kb EcoRI fragment of p58.2-1 (4) was inserted into the EcoRI site of pEFneo (28), as modified by S. Orkin.5 A hemagglutinin tag was added to the C terminus of EVI1 by amplifying bp 3467–3603 of Evi1 with oligonucleotides 5’-CACAGGCTATGCTATGATG-3’ and 5’-GGCCGCTT-AGAGGCTAGCGTAATCCGGAACATCGTATGGGTATA- CATTGGCTATGGACCTGAT-3’. This 192-bp fragment extends from an NdeI site at bp 3474 to the C-terminal end of Evi1 at bp 3603, on a hemagglutinin epitope tag with sequence YPYVPDYASL, and terminates with a NotI site. After digestion with NotI and Ndel, the 176-bp fragment was inserted into Ndel-NotI-cut pEFneo-Evi1, and from there, Evi1HA was transferred into the EcoRI site of plBluescript as a blunt-ended 3.6-kb EcoRI-NotI fragment. Within this plasmid, the ClaI site was converted to BamHI by the addition of BamHI linkers. Evi1HA was transferred as a BamHI fragment to pBluescript to create pBS-Evi1HA(Bam). The pMYs-muEvi1HA-GFP plasmid was a generous gift from Dr. Mineo Kurokawa. The pMYs-Evi1HA-GFP vector was created by excision of the EVI1HA cDNA, followed by blunting of the overhanging ends and blunt end ligation. The pMYs-Evi1HA-GFP plasmid was created by excision of the EVI1HA cDNA, followed by blunting of the overhanging ends and blunt end ligation. The pMYs-Evi1HA-GFP plasmid was created by excision of the EVI1HA cDNA, followed by blunting of the overhanging ends and blunt end ligation.

**Creation of Evi1HA Retrovirus and Infection of EML Cells**—BOSC cells (30) were transfected (31) with plasmid (10 μg/10-cm plate) with chloroquine (25 μM). Medium was changed 8 h later. Viral supernatants were harvested at 24–48 h post-transfection and frozen at −80 °C. EML cells were infected in the presence of 8 μg/ml Polybrene. Selection was performed in 2 μg/ml puromycin.

**RNA Analysis, cDNA Synthesis, and qPCR**—Total RNA was isolated from cells using either TRIzol (Life Technologies) or an RNeasy kit (Qiagen) according to the manufacturer’s instructions. Isolated RNA was quantified by UV spectroscopy using a NanoDrop instrument (Thermo Scientific). cDNA was synthesized using a high capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer’s instructions. Quantitative real-time PCRs (qPCRs) were carried out using a Bio-Rad instrument and Power SYBR Green Master Mix (Applied Biosystems) with protocols and annealing temperatures set according to the manufacturer’s instructions.

**Flow Cytometry and Cell Sorting**—FACSCalibur, LSR II, and FACSscan instruments (BD Biosciences) were used for flow cytometric analysis and cell sorting. Analyses of all flow cytometry data were done using FlowJo version 8.5.3 software (TreeStar). All antibodies were purchased from BD Biosciences. Sample preparation was carried out as described (32). Briefly, cells were stained with antibodies targeted against the appropriate hematopoietic marker for 30 min on ice in the dark. Cells were then rinsed once with PBS and resuspended in 1× PBS, 2% FBS before analysis.

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5 S. Orkin, personal communication.
Creation of Evi1T0 Allele—Allele construction is described in a concurrent paper6 and resulted in the insertion of a Neo-Stop-Tet operon cassette (34) insertion into the first exon of Evi1 (Mus musculus Chr3:29,911,738 NCBI37/mm9). Insertion of the cassette was done via homologous recombination into embryonic stem cells at the University of Rochester Transgenic Core, and proper integration was assessed via Southern blotting with both 3’ and 5’ probes. The allele was deemed nondeleterious to viability via allelism testing. The Evi1T0 allele was combined with the Rosa26rtTA allele (35) (obtained from Jackson Laboratories), and induction was performed by placing the mice on doxycycline (DOX) chow (BioServ, Flemington, NJ).

shRNA Knockdown of Evi1 in DA-1 Leukemic Cells—shRNA expression constructs for Evi1 were made using RNAi-Ready SIREN-RetroQ vector (BD Bioscience), which harvests a puromycin resistance marker and a human U6 promoter for expressing short hairpin sequences. Two short hairpin sequences for Evi1 were utilized in these studies (displayed in Fig. 1B): sh11 (5’-GATCCGAGCGCCTGCATCCACAGGAAATCTCAAGAGATGTTCTTCGGTGATGTCAGGCGCGCtttttACCGGTG-3’), corresponding to bp 511–532 of GenBankTM sequence M21829) and sh54 (5’-GATCCGAGTTGTTGGATGAGGAGGAGATGTTCTTCGGTGATGTCAGGCGCGCtttttACCGGTG-3’), corresponding to bp 3139–3167 of M21829). These sequences were chosen using software at the RNAi OligoRetriever Database. These complementary strands were annealed and inserted into BamHI/EcoRI-cut pSIREN-RetroQ.

Microarray Analysis—Microarray studies were performed as competitive hybridizations (shRNA-mediated knockdown of Evi1 versus control shRNA) of fluorescently labeled cDNAs on glass slide-based 70-mer oligonucleotide arrays of mouse cDNA sequences as described previously (6). The pixel intensity data were background-subtracted, converted to log2 ratios, normalized to a mean of zero, and expressed as a ratio to the S.D.

Chromatin Immunoprecipitation and qPCR—Procedures were conducted in a manner similar to that described previously (36), with the following exceptions. EML C1 cells (2–20 × 106 cells) were fixed in cell culture medium, 1% formaldehyde for 10 min at 37 °C. The fixation reaction was stopped by the addition of 2.5 M glycine (0.26 M final) and incubation for 5 min at room temperature followed by 15 min on ice. This sample was used for the immunoprecipitation using rabbit polyclonal antibodies and Dynabeads (ThermoFisher Scientific). The negative control for the precipitation was Dynabeads alone. Fold enrichment (or “occupancy”) was calculated as the ratio of Ct values (the ChIP sample (or beads alone) versus input sample). The results were considered valid if enrichment was obtained with the specific antibody and not with beads alone.

Cas9/CRISPR Modification of Cebpa +35—The plentiCRISPR vector (Addgene number 52961) was created by Feng Zhang’s laboratory (37) and purchased from Addgene. A DNA oligonucleotide was designed containing the Cebpa +35 EVI1 binding motif and appropriate 5’ and 3’ overhangs (top, 5’-CACCAGACCGTACATGACGTGA-3’; bottom, 5’-AAAGCTCACTGTCTATGCCTGAGTTC-3’). For cloning into the plentiCRISPR vector, according to instructions from the source laboratory. The oligonucleotide was annealed and cloned into BsmBI-digested plentiCRISPR backbone. Ampicillin-resistant colonies were selected and screened by Sanger sequencing to identify positive clones. Lentivirus was generated via transduction of 293TN cells, and EML C1 cells expressing either pMYs-muEVI1-GFP (29) or empty vector were infected with plentiCRISPR +35 or WT vector at a 1:1 ratio of viral supernatant/cell culture medium. Infections were carried out by centrifuging virus/cell mixtures for 1 h at 700 relative centrifugal force, 25 °C. At 48 h postinfection, cells were selected in 1 μg/ml puromycin for 72 h. Clones were generated by single-cell sorting into round-bottom 96-well plates. Clones were picked approximately 2 weeks later, and stable clones were analyzed for Cebpa expression and Cas9 modification of the +35 kb enhancer.

Recombinant Protein Purification—An N-terminal portion of the EVI1 protein containing ZF1 was cloned into pET-15b. The EVI1 C-terminal portion, containing ZF2, was cloned into pGEX. Recombinant protein purification was carried out in a manner similar to that described previously (16) using nickel-nitrolotriacetic acid-agarose (Qiagen) and glutathione-agarose (Thermo Scientific) for the purification of ZF1 and ZF2 fragments, respectively, according to the manufacturer’s instructions.

EMSA—EMSA studies were carried out as described previously (38). For oligonucleotide mutagenesis binding assays, 20-μl binding reactions containing increasing amounts of cold competitor DNA (300, 600, and 900 fmol) were assayed in triplicate. For each concentration of cold competitor assayed, y values were calculated as the percentage of binding relative to binding reactions in the absence of cold competitor. These data were replotted as (1–y)/y, and binding affinity of each mutant oligonucleotide relative to the wild type Cebpa +35 sequence was calculated as the slope of the trend line as described previously (38).

DNase I Footprinting, Methylation Interference, and Missing Base Contact Probe Analysis—Methylation interference studies were similar to published studies (38). ImageQuant software (Molecular Dynamics) was used to quantify the differences in pixel intensity between bound and free bands.

For DNase I footprinting, a 535-bp DNA fragment containing the Cebpa +37 enhancer element was cloned into the pGEM vector, radiolabeled, and purified as described above. Approximately 20,000 cpm of probe was incubated with increasing amounts of recombinant EVI1 C-terminal protein containing the entire second set of zinc fingers. DNase I cleavage, inactivation, and probe purification were carried out as described previously (16). Samples were fractionated on a 7 M urea sequencing gel and imaged as described above.

Chromosome Conformation Capture (3C and 4C)—3C libraries were generated as described (39). Briefly, 5 × 107 cells were fixed in formaldehyde, and nuclei were isolated using a Dounce homogenizer (large clearance pestle). Nuclei were briefly heated to 65 °C to inactivate native proteases before being digested overnight with SacI (New England BioLabs). Samples

6 M. Wilson, X. Cui, S. Husain, M. Tran, Y. Zhang, and A. Perkins, manuscript in preparation.
were heated to 65°C and then diluted to 7.45 ml and treated with T4 DNA ligase for 2 h at 16°C. Cross-links were reversed by incubation overnight at 65°C, and 3C DNA libraries were purified by several rounds of phenol extraction and ethanol precipitation.

3C library DNA was verified on an agarose gel. To generate a 4C DNA library, the 3C DNA was digested overnight with Msel (New England BioLabs) and purified by phenol/chloroform extraction and ethanol precipitation. Digested DNA was then ligated for 4 h at 16°C and extracted/purified again. Inverse PCR was conducted using PCR primers directed outward from the Cebpα +35 distal enhancer. 1 μl of the primary inverse PCR was loaded as template into a secondary 50-μl PCR and run on the same protocol. This reaction generated multiple distinct bands on an agarose gel, which were subsequently gel-purified and subjected to Sanger sequencing to align the sequences to specific genomic sequences. Inverse PCR products were also gel-purified in bulk and TA-cloned (Promega) before Sanger sequencing.

**Results**

**EVI1 Expression in Hematopoietic Progenitors Interferes with Both Myeloid and Erythroid Differentiation**—To investigate the role of EVI1 expression on hematopoietic differentiation, we employed the pluripotent hematopoietic progenitor cell line EML-C1 (25), a murine bone marrow-derived cell line immortalized with a dominant negative retinoic acid receptor α. It has the potential to differentiate into erythroid or myeloid cells upon induction with erythropoietin or RA, respectively (25). Untreated EML cells are actually heterogeneous, having both myeloid- and erythroid-poised subpopulations, with cells fluctuating between the two states; the myeloid-poised cells show high Sca-1 (stem cell antigen-1) expression as well as PU.1 (purine-rich box-binding protein 1) expression, whereas the erythroid-poised cells have low Sca-1 and high GATA-1 levels (3).

Native EML cells do not express detectable EVI1 mRNA (Fig. 1A) or protein (data not shown). To assess the effect of expression of the 135-kDa isoform of EVI1 on EML differentiation, we created overexpressing clones (Fig. 1B) and assessed the ability of EVI1 and vector clones to differentiate into GM-CSF-responsive cells upon RA/IL-3 induction. There was marked proliferative response to GM-CSF in the RA/IL-3/SCF-treated vector clones but not EVI1 clones, as assessed by either [3H]thymidine incorporation (Fig. 1C) or growth in liquid culture supplemented with GM-CSF (data not shown). In fact, whereas vector-transduced cells showed survival and expansion, EVI1-transduced cells underwent cell death, even during the 96-h RA treatment (data not shown), consistent with previous studies that observed apoptosis from forced EVI1 expression in differentiating myeloid cells (40, 41). This massive apoptotic response precluded biochemical assessment of EVI1-transduced cells following RA/IL-3 induction.

We also assessed the effect of EVI1 overexpression on Sca-1, a cell surface marker that is highly expressed in both stem cells and myeloid-biased EML cells and that is down-regulated upon RA-induced myeloid maturation (3). We found that in vector-transduced EMLs, RA causes marked suppression of Sca-1 expression (Fig. 1D). EVI1 strongly up-regulates Sca-1 in uninhibited EML cells relative to vector controls (Fig. 1D), and although RA treatment of these cells does cause Sca-1 down-regulation, the level is still markedly above that observed in vector control cells (Fig. 1D).
EML cells are able to undergo erythroid differentiation when induced with erythropoietin (Epo) (25). To determine whether EVI1 overexpression influenced the Epo-induced erythroid differentiation of EML cells, we cultured vector- and EVI1-transduced EML cells with 8 units/ml Epo and quantitated the percentage of benzidine-positive cells over time. EVI1-expressing cells display a markedly slower response rate to Epo treatment, similar to previous studies describing myeloid-poised EML cells (Fig. 1E).

In other cell types, EVI1 overexpression has been noted to have a stimulatory effect on the cell cycle (42, 43). We analyzed cell growth of uninduced EVI1-expressing and control EML cells by [3H]thymidine incorporation and found no consistent or significant change (data not shown). Furthermore, we examined doubling time under conditions of normal exponential growth by cell counting and found no significant difference in doubling time between EVI1-expressing cells and controls (doubling time in vector-transduced cells, 17.9 h; doubling time for EVI1-transduced cells, 17.6 h). We conclude from this that in EML cells, the 135-kDa isoform does not have a dramatic effect on cell cycle progression.

In summary, these studies uncover several effects of EVI1 on the EML cell line: marked up-regulation of Sca-1 and delay in Epo-induced erythroid maturation, consistent with skewing toward the myeloid lineage, but at the same time disruption in the RA-induced acquisition of responsiveness to GM-CSF accompanied by cell death.

Induction of EVI1 in Hematopoietic Progenitors Reveals Marked Up-regulation of Sca-1—To confirm that the effect of EVI1 on Sca-1 was not a peculiarity of the EML cell system and to learn more about the conditions under which this physiologic link is maintained, we assessed Sca-1 expression in hematopoietic cells in which EVI1 is induced to high levels versus control (native) levels of expression. To control EVI1 expression in the in vivo setting, we introduced seven copies of the tetracycline operon (TO) into the first exon of the gene. To show that this allele (termed Evi1TO) is not deleterious to the function of Evi1, we intercrossed Evi1TO mice to create Evi1TO/TO mice, which were viable and healthy, without a discernable phenotype (data not shown). Because homozygous loss-of-function allele status is lethal (44), we considered it likely that the Evi1TO allele functions as a normal allele in the absence of induction.

We combined the Evi1TO allele with ubiquitously expressed reverse Tet repressor/transactivator (rtTA; under control of the ubiquitous Rosa26 promoter (35)) to create Evi1TO/TO/Rosa26T2A/rtTA mice, which were viable and healthy. Via treatment with DOX, we were able to induce Evi1 expression to levels similar to those seen in DA-1 leukemic cells that have a provirally activated Evi1 allele (Fig. 1F). We then harvested lineage-negative marrow from uninduced and induced mice (3 days postinduction) and showed marked up-regulation of Sca-1 (Fig. 1G). These data confirm that up-regulation of EVI1 is linked to Sca-1 up-regulation and that this is not a peculiarity of one cell line.

Up-regulation of Sca-1 and Disruption of Myeloid Maturation by EVI1 Require DNA Binding—To understand the mechanism of both the myeloid skewing and the disruption of RA-induced myeloid differentiation, we asked whether these functions depended on the involvement of downstream target genes of EVI1. We used up-regulation of Sca-1 in RA-untreated (uninduced) cells as a marker of myeloid skewing and, following RA treatment, Sca-1 down-regulation as a marker of myeloid maturation. We used EVI1 mutants defective in DNA binding to determine the involvement of downstream genes. We transduced EML cells with two mutants that were defective in DNA binding: R205N, deficient in ZF1 DNA binding (6), and R769C, which precludes binding via ZF2 (16). In terms of myeloid skewing (as indicated by the Sca-1 up-regulation in uninduced cells), there was a clear difference between the groups; whereas 82% of EVI1-expressing cells were Sca-1high, <2% of vector-transduced were Sca-1high (Fig. 2). Both the R205N mutant and the R769C mutant behaved as the vector did; 3% of the mutant cells were Sca-1high. Similarly, RA induction led to a similar disparity, with the vector- and R205N-induced cells behaving comparably and distinct from the EVI1-expressing cells. <0.5% of vector- and R205N-transduced cells were Sca-1high after 4 days of RA, whereas 4% of viable EVI1-transduced cells were Sca-1high. In this instance, however, the R769C-expressing cells behaved more like the WT EVI1-transduced cells; over 4% of the R769C-transformed cells were Sca-1high at 96 h post-RA treatment (Fig. 2). In addition, the cell death found in RA-treated EVI1-transduced cells was not observed in the vector, R205N, and R769C groups; after RA induction, <1% of the EVI1-expressing cells were alive (0.98% DAPI-negative cells), whereas the vector, R205N, and R769C were all >60% viable (64.2, 63.7, and 62.0% for vector-, R205N-, and R769C-transduced EML cells, respectively, following RA induction).

EVI1 Suppresses Cebpa Expression in EML Cells—The data above illustrate the need for EVI1 to bind DNA via its zinc finger regions to induce up-regulation of Sca-1, suggesting that downstream target genes are involved. Because EVI1-transduced EML cells fail to respond to GM-CSF upon RA treatment (despite the presence of the receptor for GM-CSF; data not shown), we hypothesized that EVI1 causes changes in gene expression that block its responsiveness to myeloid-specific cytokines, such as GM-CSF. Cebpa is known to regulate responsiveness to several myeloid-specific cytokine receptors, including the GM-CSF receptor α (45). We assayed Cebpa expression by RT-PCR in both EVI1-transduced and mutant-expressing EML cells and compared this with vector control cells. We found that Cebpa was robustly suppressed (~40-fold) in the EVI1-expressing Sca-1high compartment (Fig. 3A). Importantly, there was no suppression of Cebpa transcripts when either ZF1 or ZF2 was mutated (R205N and R769C, respectively), indicating that this suppression requires DNA binding. To see what effect EVI1 has on Cebpa expression upon treatment with RA, we monitored Cebpa RNA levels by Northern blotting (Fig. 3B) and RT-PCR (Fig. 3C) analysis in vector- and EVI1-transduced cells for 72 h after commencing RA treatment; we found that whereas control EML cells up-regulate Cebpa during myeloid differentiation, EVI1-transduced cells continue to exhibit low levels of Cebpa mRNA. We also tested whether the R205N mutation affected this suppression, and we found that Cebpa transcripts rose in a similar manner to the transcripts seen in the
EVI1 Impedes Granulopoiesis via Suppression of Cebpα

EVI1 up-regulates Sca-1 expression in EML cells. Flow cytometric analysis of EML cells transduced with four different retroviral constructs, as indicated. Cells are analyzed for Sca-1 expression (y axis) and forward scatter (x axis). Top row, uninduced cells; bottom row, flow analysis of cells after 96 h of RA treatment. Top row, high levels of Sca-1 in uninduced EML cells transduced with WT EVI1, indicative of myeloid skewing and stem cell-like phenotype. In RA-treated cells, Sca-1hi phenotype is maintained in EVI1-expressing cells and, to a lesser extent, in R769C-transduced cells, while not in vector- or R205N-transduced cells.

EVI1 suppresses Cebpα transcription in hematopoietic progenitors and DA-1 myeloid leukemia cells but not in common myeloid progenitors (CMP) or granulocyte/monocyte progenitors (GMP). A, RT-qPCR showing Cebpα mRNA levels of EML cells transduced with vector, WT EVI1, or an EVI1 ZF1 mutant (R205N) or ZF2 mutant (R769C), as indicated. B, Northern blotting analysis of Cebpα and Ev1 expression in EML cells induced to differentiate with RA and IL-3 for 0–72 h, as indicated, with β-actin serving as a control. C, RT-qPCR data showing the increase in Cebpα expression in vector-transduced EML cells following RA-induced myeloid differentiation. In contrast, EVI1-expressing EMLs retain low levels of Cebpα and undergo massive cell death. D, Cebpα mRNA expression (quantitative RT-PCR) in bone marrow subsets, as indicated, isolated from Ev1TO/TO/Rosa26rtTA/rtTA mice, with or without 3 days of DOX induction, as indicated. E, microarray analysis of Ev1, Cebpα, and Hdc gene expression in DA-1 leukemic cells, with or without shRNA-mediated knockdown of Ev1. Hdc is a myeloid maturation marker that is strongly up-regulated upon knockdown of Ev1 expression. Results of three hybridizations are shown, each in a different shade of gray. The -fold change is normalized between experiments by dividing by the S.D. (error bars) for the log2 ratios for all genes. *, p < 0.05.

Evi1 Induction in HSCs in Vivo Results in Cebpα Suppression in HSCs but Not in Committed Progenitors—To confirm that these results were not unique to the EML cell system, we iso-

Vector control EML cells, indicating that the function of ZF1 was essential for Cebpα suppression in this setting (data not shown); the R769C mutation was not tested.
EVI1 Impedes Granulopoiesis via Suppression of Cebpa

Suppression of EVI1 in Leukemic Cells Leads to Up-regulation of Cebpa—To further investigate the relationship between EVI1 and Cebpa expression, we knocked down EVI1 transcripts in DA-1 leukemic cells with two different shRNAs for EVI1, which caused a reduction to 5–30% of the level of transcript seen in control cells (Fig. 3E). These were accompanied by a doubling of the level of Cebpa transcripts in two experiments and no change in one experiment (Fig. 3E). By χ² analysis, these data were found to be significant (p < 0.05). To determine whether EVI1 knockdown induces myeloid maturation, we examined the microarray data for myeloid-specific maturation markers, and many such genes were upregulated, including Hdc (encoding the histidine decarboxylase cluster; Fig. 3E) as well as Cpa3, Csf2rb2, Cst7, Cxcr4, Fgcr3, Mertk, Ms4a2, Lgals1, and Serpinb2 (data not shown). These data indicate that in the context of leukemic cells that overexpress EVI1, knockdown of EVI1 is associated with up-regulation of Cebpa, consistent with the notion that EVI1 helps to establish a cell state conducive to Cebpa suppression.

Our differentiation experiments show that EVI1-expressing EML cells are unable to undergo maturation and in fact undergo rapid cell death upon the induction of myeloid differentiation, and this correlates with strong suppression of Cebpa, an effect that is dependent on DNA binding via both ZF1 and ZF2. For this reason, we decided to focus on the molecular regulation of Cebpa by EVI1 as a potential mechanism for the EVI1-induced block in myeloid maturation.

The Effect of EVI1 on Cebpa Is at the Level of Transcript Initiation—The regulation of steady-state mRNA levels is complex, involving changes in the rate of initiation of transcription, transcriptional pausing, changes in rates of processing, and changes in the rate of mRNA decay. To determine the mechanism of EVI1-mediated suppression of Cebpa transcript levels in EML cells, we examined changes in transcriptional initiation and pausing as well as in mRNA decay. The first two were quantitated by ChIP-qPCR analysis of polymerase II loading at the Cebpa TSS and at the 3’-end of the coding region. This revealed a marked reduction to 50% in RNA polymerase II localization at the TSS as well as at the 3’-end of the coding region (Fig. 4A), indicating a suppression of initiation of transcription but no change in pausing. To examine mRNA decay, EML cells expressing either empty vector or EVI1 were treated with actinomycin D to arrest transcription. Total RNA was harvested at several time points, and Cebpa transcript was measured by qPCR. The results show no difference in the rate of Cebpa mRNA decay between vector and EVI1 groups (Fig. 4B). ChIP-qPCR also revealed a strong decrease in co-activator p300 localization to the Cebpa promoter with EVI1 transduction (Fig. 4C); similar to Cebpa suppression, this phenomenon was found to be dependent upon DNA binding by ZF1 but only partially dependent upon ZF2 binding. Thus, it appears that the major effect of EVI1 expression is a decrease in TSS loading for both RNA polymerase II and p300 at the Cebpa promoter.

EVI1 Occupies Two Binding Sites, 35 and 37 kb Downstream of Cebpa in Hematopoietic Progenitors and Leukemic Cells—In previous ChIP-Seq studies, we identified binding sites for EVI1 at 35 and 37 kb downstream of Cebpa (14) (Fig. 5A). Further analysis of the two binding sites using the UCSC Genome Browser revealed that both regions were strongly evolutionarily conserved throughout mammals and birds and to a higher degree than other conserved regions in the area (Fig. 5A). ChIP-Seq data available on the UCSC Genome Browser indicates histone H3 Lys⁴ monomethylation and histone H3 Lys⁹ acetylation, two marks of active enhancers, at both the +35 and +37 kb peaks; indeed, we confirmed these histone marks in EML cells, but there was no difference in the presence and absence of EVI1 (data not shown). Additionally, the +37 kb enhancer has been described as a binding locus for the RUNX1 transcription factor; indeed, RUNX1 binds to four conserved sites on the +37 kb enhancer and up-regulates Cebpa transcription, shifting early myeloid cells toward granulocytic differentiation (46). To test whether EVI1 binding to these distal enhancers is the mechanism by which EVI1 regulates Cebpa transcription, we first confirmed that EVI1 protein did in fact bind to these sites; we then fully characterized these sites at the base pair level, and

![FIGURE 4. EVI1 alters Cebpa transcript initiation](image-url)
finally, using the CRISPR system, we deleted these sites to determine their role in transcriptional regulation of Cebpα.

To assess EVI1 occupancy of the distal enhancers in the EML system, we performed quantitative ChIP-qPCR on transduced EML cells using antibodies against EVI1 protein; this showed prominent occupancy by WT EVI1 at both the +35 and +37 kb enhancers but no occupancy of either site by EVI1-R205N (Fig. 5B). The EVI1-R769C mutant displayed some occupancy of
Cebpa + 35 kb, but its occupancy at the +37 kb distal enhancer was comparable with that of vector control.

Next, we analyzed the DNA sequences in both the +35 and +37 kb regions for canonical binding sites for ZF1 protein (8) and ZF2 protein (9) using sequence analysis programs, but this failed to reveal any convincing motifs (data not shown). Therefore, we pursued identification of the binding sites using functional assays. Recombinant proteins harboring either the first set of zinc fingers (fingers 1–7, amino acids Met1–Gly249 (6), termed ZF1 protein) or the second set of zinc fingers (fingers 8–10, corresponding to amino acids Asn732–Glu821 (16), termed ZF2 protein) were purified and assayed for binding via EMSA to 150-bp PCR products encompassing either Cebpa +35 kb or +37 kb. The EMSA results indicated binding of ZF1 protein to the +35 kb probe and ZF2 protein binding to the +37 kb probe but not vice versa (data not shown). The two 150-bp fragments for the +35 and +37 kb sites were then divided into 30-bp oligonucleotides that overlapped at 15-bp intervals. These short DNA oligonucleotides were assayed by competitive EMSA; the results showed that the Cebpa +35 kb-derived oligonucleotides 1–6 and 1–7 displayed competitive binding, suggesting that the overlapping sequence ACATGACAGTGACGG constituted the binding site at the +35 kb (Fig. 5C). Sequence data available on the UCSC Genome Browser indicate that this EVI1 binding motif is strongly evolutionarily conserved, suggesting functional significance (Fig. 5D). Surprisingly, whereas ZF2 protein bound to the 150-bp probe encompassing the entire +37 kb region, none of the oligonucleotides derived therefrom were capable of effectively competing for this binding (data not shown), suggesting that the +37 kb region harbors a complex binding site for ZF2 protein that requires a larger context for binding than was contained on any individual 30-bp oligonucleotide.

To identify the base pairs that are essential for EVI1 binding at the +35 kb site, we characterized this further by methylation interference and missing base contact probe analysis (Fig. 5E) (47); such information is essential in determining which bases need to be mutated by Cas9 to disrupt the EVI1 binding motif. Quantitation of the autoradiogram allowed the overall strength of each contact to be assessed, which revealed that the 5′-GACGG-3′ had proportionately stronger base pair contacts than did the region to the 5′-side of this motif (Fig. 5F). As an orthogonal approach to the characterization of binding, we conducted a single bp mutagenesis study using short DNA oligonucleotides by competitive EMSA; the results revealed that, consistent with the methylation interference data, the vast majority of mutations within the 12-bp binding motif resulted in reduced affinity for ZF1 protein, whereas mutations in the 5′ and 3′ adjacent nucleotides had no effect (data not shown).

Next, we characterized EVI1 binding at the +37 kb site. EMSA studies were conducted with non-radioactive competitor DNA oligonucleotides containing the canonical binding motifs for either ZF1 protein (GACAAGATAA) (8) or ZF2 protein (GAAGATGAG) (9). The non-radioactive competitor for the ZF2 protein-binding reaction was able to completely outcompete the radiolabeled 150-bp probe but exhibited only partial competition for the 400-bp probe (Fig. 6A). In contrast, no competition was observed with the non-radioactive competitor corresponding to the binding site for ZF1 protein. Interestingly, the binding reactions containing the 400-bp probe displayed multiple shifted bands, possibly indicating the presence of multiple ZF2 protein binding sites across the +37 kb enhancer.

Because the EMSA studies indicated multiple EVI1 ZF2 protein binding sites at +37 kb, we conducted DNase I footprinting experiments to better define the EVI1 binding sites. A radiolabeled probe encompassing the entire +37 kb region was incubated with increased amounts of recombinant ZF2 protein before DNase I treatment and denaturing PAGE analysis. The results clearly indicated four ZF2 protein binding sites across the region (Fig. 6B), including sites that overlap with consensus RUNX1, ETS, and GATA motifs documented by others (48) (Fig. 6C). Sequence analysis indicates that all four of the EVI1 binding sites are highly evolutionarily conserved (Fig. 6D). Taken together, these data indicate that EVI1 binds to +37 kb via its second set of zinc fingers at sites alongside sites for other key hematopoietic transcription factors.

**Assessment of the Effect of EVI1 on H3K27 Trimethylation and Acetylation and p300 Localization at the +35 and +37 kb Enhancers**—We hypothesized that forced EVI1 expression would alter histone markings and create an epigenetic profile consistent with decreased transcription of Cebpa. We discussed above our finding that the coactivator p300 is markedly less abundant at the Cebpa promoter (Fig. 4C); here, we extend this analysis to the +35 kb and +37 kb sites in EML cells in the presence and absence of
As expected, this revealed decreased p300 localization at both distal enhancers when EVI1 was expressed (Fig. 7A). The decrease in p300 was not seen in the EVI1-R205N-transduced cells; EVI1-R769C-transduced EMLs showed partially decreased p300 binding. Overall, the p300 localization level correlated partially with the level of Cebpa expression (Fig. 3A).

Acetylation of lysine 27 of histone 3 (H3K27ac) often describes active enhancer elements, whereas trimethylation of lysine 27 of histone 3 (H3K27me3) often describes repressive enhancer elements (12). In our study, we observed that EVI1 binding sites are characterized by both H3K27ac and H3K27me3 marks, indicating a balance between activation and repression in the context of EVI1 binding.

In conclusion, our study demonstrates the regulatory role of EVI1 in granulopoiesis and identifies specific binding sites that contribute to the transcriptional activation of Cebpa. Further studies are needed to understand the dynamic interplay between EVI1 and its target genes in the context of hematopoiesis.

FIGURE 6. EVI1 ZF2 binds four conserved sites within the Cebpa +37 kb enhancer. A, EMSA showing specific binding of EVI1 ZF2 to two radiolabeled probes encompassing Cebpa +37 kb. Recombinant ZF2 displays one shifted band for the 150-bp +37 fragment and at least two shifted bands for the 400-bp +37 kb fragment. DNA cold competitors encompassing the canonical ZF1 motif were unable to disrupt binding to either probe, whereas the ZF2 cold competitor fully disrupted binding to the 150-bp probe and partially disrupted binding to the 400-bp probe. B, DNase I footprinting assay reveals four EVI1 ZF2 binding sites across the Cebpa +37 kb enhancer. A 500-bp radiolabeled probe was incubated with various concentrations of recombinant ZF2 protein before DNase I treatment. Denaturing PAGE analysis reveals the presence of four regions of DNase I protection. C, summary of DNase I footprinting analysis documenting four EVI1 ZF2 binding sites within the Cebpa +37 kb enhancer. A semi-log plot analysis of DNA standards and Maxam-Gilbert sequencing analysis allows for mapping of the ZF2 binding sites to specific regions of Cebpa +37 kb. EVI1 ZF2 binds in close proximity to or overlaps with consensus motifs for RUNX1, GATA, and ETS transcription factors involved in regulation of early hematopoiesis. D, UCSC genome browser sequencing data reveal that the four ZF2 binding sites contain evolutionarily conserved sequences. All four ZF2 binding sites contain sequences that are strongly conserved throughout mammals.
EV1 Impedes Granulopoiesis via Suppression of Cebpa

from the plentiCRISPR control pool, and five were analyzed from EML cellsexpressing EVI1, three clones were analyzed transduced clones had significantly higher levels (Fig. 8)

geted EML clones, 12 that were EVI1-expressing and 14 that harbored vector. All 12 of the EVI1-expressing clones had very low levels of Cebpa transcript, whereas all 14 of the vector-transduced clones had significantly higher levels (Fig. 8A). From EML cells expressing EVI1, three clones were analyzed from the plentiCRISPR control pool, and five were analyzed from the experimental plentiCRISPR +35 kb pool. This analysis revealed that whereas all of the control clones had very low levels of Cebpa transcript, five of the seven clones from the experimental pool showed levels of Cebpa transcript that were markedly increased, essentially to levels seen in control EML cells (Fig. 8B).

To confirm that the EML cells infected with the plentiCRISPR +35 kb construct actually harbored mutations at +35 kb, we PCR-amplified the genomic region surrounding the guide RNA binding site and performed Sanger sequencing. The results show significant insertions and deletions for the eight plentiCRISPR +35 clones but none for the plentiCRISPR vector clone (Fig. 8C). Interestingly, the E35-3 clone retained suppression of Cebpa, despite significant deletion and mutations of the EV11 binding site. This clone contains unique deletions of evolutionarily conserved residues (data not shown) 3’ of the EV11 binding site; this may account for the lack of Cebpa expression. Overall, these results indicate that deletion or mutation of the EV11 +35 kb motif can reverse the EV11-mediated suppression of Cebpa transcript in EML cells.

To examine the effects of deletion of the Cebpa +35 kb locus in an EV11-overexpressing leukemia, we repeated the plentiCRISPR infection and single-cell cloning in DA-1 murine leukemic cells. The results show that puromycin-resistant clones infected with plentiCRISPR +35 express the same low levels of Cebpa mRNA observed in vector controls (data not shown). This indicates that modification of the Cebpa +35 kb locus in EV11-overexpressing murine leukemic cells is unable to restore Cebpa expression, indicating more complex regulation of the gene, perhaps acquired in the process of malignant transformation.

The Cebpa +35 kb Enhancer Is Linked to Other Conserved Elements in the Context of Chromatin—In contrast to the Cebpa +37 kb enhancer, the role of the Cebpa +35 kb enhancer in regulating transcription remains unstudied. We hypothesized that EV11 may regulate Cebpa transcription via the +35 kb element by inducing changes in chromatin conformation, such as causing an uncoupling of the +35 kb element from the Cebpa promoter region. To pursue this idea, we performed a
chromatin conformation capture (3C) assay, using primers in the Cebpa promoter region and the /H11001 35 kb region. However, this failed to yield any products to indicate a physical interaction between the two regions (data not shown). We were concerned that this might be a false negative result due to the high GC content of the Cebpa promoter. Therefore, we performed open-ended chromosome conformation capture studies (4C assay); this was performed in both DA-1 leukemic cells and EML progenitor cells. We generated 4C libraries using primers targeting the Cebpa/H11001 35 kb region and performed inverse PCR; sequencing and cloning of amplification products revealed interactions with evolutionarily conserved regions located at Cebpa/H11001 19 kb in DA-1 cells and /H11001 31 kb in EML cells (Fig. 9).

However, we did not obtain experimental evidence indicating that the /H11001 35 kb motif directly interacts with the Cebpa promoter. Data deposited into the UCSC Genome Browser indicate the presence of many transcription factors and regulatory proteins critical for hematopoietic regulation at the /H11001 19 kb element, which are similar to those found at the +35 kb enhancer. In addition, ENCODE data from the UCSC Browser indicate that both the +19 and +35 kb loci share common histone modifications, open chromatin states, and transcription factor binding with the CEBPA promoter in a variety of cellular contexts (Fig. 9). These data suggest that both the Cebpa +19 and +35 kb elements serve important roles in the regulation of CEBPα function.

Overexpression of Cebpa Can Reverse the EVI1-induced Block to Maturation—Our data suggest a model in which EVI1 expression suppresses Cebpa transcription in EML cells, and loss of CEBPα, a critical regulator of myeloid maturation, leads to a block in this process. If this is the case, then overexpression of exogenous Cebpa should override the EVI1-induced block in maturation. To test whether decreased Cebpa was the reason for the decreased response to myeloid differentiation stimuli, we added back C/EBPα as a tamoxifen-inducible estrogen-receptor fusion protein. Activation of C/EBPα without RA treatment caused myeloid maturation, as evidenced by down-regulation of Sca-1, in both vector and EVI1-expressing groups; a similar observation was made following 72 h of RA/IL-3 treatment (Fig. 10, A and B). Many cells became adherent, indicative of mature myelomonocytic cells. However, EVI1-expressing cells displayed massive cell death with C/EBPα activation, comparable with earlier observations from our laboratory and others that EVI1 expression is not tolerated past a certain stage of differentiation (40, 41) and demonstrating that lack of Cebpa is not mechanistically connected to the cell death phenomenon.
These findings indicate that Cebpa can substitute for RA in driving myeloid maturation, even in EVI1-transduced EML cells, and implies that Cebpa up-regulation following RA treatment is an essential component in the maturation process. That Cebpa can induce these maturational changes in the presence of EVI1 strongly implies that we have identified an important regulatory node within the EVI1-mediated deregulation of myeloid differentiation and maturation.

**Discussion**

The mechanisms by which EVI1 induces AML are complex and incompletely understood, but there is experimental support for several leukemogenic effects, including suppressing apoptosis, promoting cell cycle, and blocking differentiation (reviewed in Ref. 7). In this paper, we have sought to gain new insights into the leukemogenic role of EVI1 by studying the biological effects of the protein on a cultured progenitor cell line and trying to probe the molecular mechanistic underpinnings of these effects. We have uncovered three independent mechanisms by which EVI1 may contribute to AML. First, we found that EVI1 overexpression in the EML progenitor cell line causes the heterogeneous EML population to shift away from an equally erythroid/myeloid-poised population toward a myeloid-skewed population and away from the erythroid. This may well explain the tight association between EVI1 and myeloid neoplasms that has long been noted but poorly understood. At the same time, the cells fail to respond appropriately to RA-induced maturation, as evidenced by their failure to mature to GM-CSF-responsive progenitors; instead, many EVI1-expressing EML cells undergo cell death upon RA treatment. Concomitant with this, there is active suppression of transcription of Cebpa, which encodes a key myeloid regulatory transcription factor that is essential for the common myeloid progenitor to granulocyte/monocyte progenitor step in myeloid maturation. This suppression depends at least in part on the binding of EVI1 to at least one if not both of two regulatory elements situated in the genomic DNA at +35 and +37 kb relative to the TSS of Cebpa.

In Fig. 10, we show that overexpression of Cebpa in EVI1-expressing cells results in myeloid maturation, as evidenced by marked down-regulation of Sca-1. These findings indicate that Cebpa can substitute for RA in driving myeloid maturation, even in EVI1-transduced EML cells, and imply that Cebpa up-regulation following RA treatment is an essential component in the maturation process. That Cebpa can induce these maturational changes in the presence of EVI1 strongly implies that we have identified an important regulatory node within the EVI1-mediated deregulation of myeloid differentiation and maturation.

What is abundantly clear is that Cebpa overexpression cannot rescue EVI1-transduced cells from the “toxicity” that is observed when EVI1 expression is forced in mature myeloid cells; this aspect of EVI1 function remains largely unexplained.

Previous studies have identified a block to differentiation as a major effect of EVI1 (reviewed in Ref. 7). Although the exact mechanism remains unclear, evidence for several possibilities has been presented, including binding to and inactivation of the
key regulator, PU.1 (51), and suppression of the myeloid differentiation gene, Cebpe (14). Consistent with our results, previous studies have also shown EVI1 expression to have an inhibitory effect on erythroid differentiation (52, 53). Other studies noted that EVI1 might down-regulate the EpoR gene and hypothesized that EVI1 protein may interfere with this gene’s expression via interaction with GATA1 protein (54). In all of these studies, it is not clear how much these phenomena contribute to myeloid leukemogenesis or myelodysplasia in humans or in animal models.

The +37 kb enhancer has been the subject of several studies (55); these have documented not only a plethora of binding sites for known regulatory factors but also functional importance for several of these, most notably RUNX1 (46). Functional studies have demonstrated that RUNX1 up-regulates Cebpa mRNA via the +37 kb enhancer. This led us to hypothesize that EVI1 may regulate Cebpα transcription by dislodging RUNX1 from the +37 kb enhancer, thereby removing a potent and essential positive regulatory factor. To test this hypothesis, we performed ChIP-qPCR and did obtain some evidence of RUNX1 displacement from the +37 enhancer with EVI1 overexpression, but this phenomenon was inconsistent (data not shown); the exact role of EVI1 at the +37 enhancer will be the focus of further investigation.

Past studies detailing EVI1 ZF2 function indicate that ZF2 is capable of loosely binding to the canonical ZF1 motif, indicating a degree of heterogeneity in motif recognition. The same study found that binding of the middle zinc finger to the central GAT of its canonical GAAGATGAG motif is critical for the other two zinc fingers to bind. Indeed, whereas the entire
canonical motif is absent from all four ZF2 occupancy sites at Cebp\(a\) +37, the sequence data indicates conserved GAT sequences at each of the four loci (9).

The acetylation of H3K27 results in changes to chromosome conformation and is usually carried out by the histone acetyltransferase p300 (56). Previous studies have described p300 localization at the Cebp\(a\) +37 kb element in myeloid cells (46), characteristic of many mammalian enhancers. Additionally, ChIP-Seq data show that the +37 kb enhancer progressively becomes enriched for H3K27ac as LT-HSCs differentiate into later stage myeloid progenitors and eventually to mature granulocytes. We detected decreases in H3K27ac at the +37 kb enhancer with EVII overexpression in EML cells, but the results did not reach statistical significance. This result may be due to the differences in H3K27ac between HSCs and later stage progenitors, documented by Cooper et al. (48), being small enough to avoid detection by the lower resolution of ChIP-qPCR. However, the strong decrease in p300 localization indicates that the Cebp\(a\) locus is less poised for the histone acetylation and up-regulation of Cebp\(a\) that is observed during myeloid differentiation. We found no change in H3K27me3 levels at the +37 kb enhancer, but our results show that EVII increases H3K27me3 at the +35 kb enhancer. The increase in H3K27me3 could indicate that the +35 kb enhancer is held in a poised state by EVII and is one mechanism by which EVII maintains low levels of Cebp\(a\) mRNA in HSCs.

Recent studies show Mecom and Cebp\(a\) as part of the top 10% of genes that exhibit decreased and increased expression, respectively, during the transition from early stage, long term HSCs to lineage-committed progenitor cells (33), and Cebp\(a\) has a documented role in maintaining hematopoietic stem cells (15, 18). Together, these published data and our data indicate that the function of EVII in maintaining HSCs may include maintaining Cebp\(a\) mRNA levels at a low level to prevent premature myeloid maturation (i.e. to maintain some degree of lineage plasticity). Our data indicate that overexpression of EVII can induce a Sca-1\(^{high}\), myeloid-poised phenotype concomitant with suppression of erythropoiesis and a suppression of Cebp\(a\). This degree of dissonant signaling within cells may indeed be the trigger for apoptosis, which we and others routinely observe when EVII is overexpressed in mature myeloid cells (40, 41).

While our data identify a key node of regulation that unite EVII overexpression with a block in myeloid maturation, the exact mechanism downstream of Cebp\(a\) remains unclear; does CEBP\(a\) regulate the GM-CSF receptor and its signaling directly, or are other factors involved? And why does EVII overexpression in mature myeloid cells cause cell death, whereas in leukemic cells, it promotes cell survival? These questions may allow for the development of novel targeted therapies for AML and are the focus of current investigation.

Author Contributions—M. W. helped design and perform experiments in Figs. 2–6, analyzed data, and wrote the paper. V. T. performed Northern blotting experiments. Y.-Y. X. performed the shRNA experiments. M. T. designed the retroviral constructs. Y. Z. helped design experiments and write the paper. A. S. P. designed experiments, revised figures, and wrote the paper.

Acknowledgments—We thank Shickwann Tsai for providing the EML, BHK-MKL, and BHK-HMS cell lines; Dr. Mineo Kurakawa for the pMYS-EVII vector; Alan Friedman for supplying Cebp\(a\) expression constructs; and Syed Tupur Husain and Olga Mironenko for technical assistance.

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