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Translocation Products in Acute Myeloid Leukemia Activate the Wnt Signaling Pathway in Hematopoietic Cells

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The acute myeloid leukemia (AML)-associated translocation products AML1-ETO, PML-retinoic acid receptor alpha (RARα), and PLZF-RARα encode aberrant transcription factors. Several lines of evidence suggest similar pathogenetic mechanisms for these fusion proteins. We used high-density oligonucleotide arrays to identify shared target genes in inducibly transfected U937 cells expressing AML1-ETO, PML-RARα, or PLZF-RARα. All three fusion proteins significantly repressed the expression of 38 genes and induced the expression of 14 genes. Several of the regulated genes were associated with Wnt signaling. One of these, plakoglobin (γ-catenin), was induced on the mRNA and protein level by all three fusion proteins. In addition, primary AML blasts carrying one of the fusion proteins significantly overexpressed plakoglobin. The plakoglobin promoter was cloned and shown to be induced by AML1-ETO, with promoter activation depending on the corepressor and histone deacetylase binding domains. The induction of plakoglobin by AML fusion proteins led to downstream signaling and transactivation of TCF- and LEF-dependent promoters, including the c-*myc* promoter, which was found to be bound by plakoglobin in vivo after AML1-ETO expression. β-Catenin protein levels and TCF and LEF target genes such as c-*myc* and cyclin D1 were found to be induced by the fusion proteins. On the functional level, a dominant negative TCF inhibited colony growth of AML1-ETO-positive Kasumi cells, whereas plakoglobin transfection into myeloid 32D cells enhanced proliferation and clonal growth. Injection of plakoglobin-expressing 32D cells into syngeneic mice accelerated the development of leukemia. Transduction of plakoglobin into primitive murine hematopoietic progenitor cells preserved the immature phenotype during colony growth, suggesting enhanced self-renewal. These data provide evidence that activation of Wnt signaling is a common feature of several balanced translocations in AML.

Balanced translocations are a hallmark of human leukemias (26). These translocations are often pathognomonic for a specific disease, and their occurrence can determine treatment options as well as the patient's prognosis. In acute myeloid leukemia (AML), the translocation t(8;21) is the most frequent balanced translocation and can be detected in up to 12% of patients. In recent years, important progress has been made regarding the role of t(8;21) and its resulting fusion protein, AML1-ETO, in the pathogenesis of AML (24). AML1-ETO recruits corepressor molecules such as N-Cor, mSin3A, SMRT, and histone deacetylase to AML1 DNA binding sites (1). Genes that are usually transactivated by AML1b are thus transcriptionally repressed (e.g., $p14^{ARF}$) (25). Translocations involving the retinoic acid receptor alpha (RARα), such as PML-RARα and PLZF-RARα, have also been shown to produce fusion proteins that recruit histone deacetylases to the promoters of target genes (12).

The aberrant recruitment of histone deacetylase activity is a common feature of AML1-ETO, PML-RARα, and PLZF-RARα. AML1-ETO interacts with transcription factors, including MEF, C/EBPα, other ets family members, and AP-1 (27, 57). The physical interaction between C/EBPα and AML1-ETO is thought to suppress C/EBPα (41). This repression is believed to contribute to the block in differentiation. Repression of C/EBPα activity by PML-RARα has also been proposed to be relevant for the pathogenesis of t(15;17)-associated acute promyelocytic leukemia (52). In addition, AML1-ETO forms a complex with PLZF and inhibits PLZF function (29). Thus, the pathogenetic mechanisms of PML-RARα, AML1-ETO, and PLZF-RARα appear to be linked.

The oncogenic activity of the three fusion genes is relatively weak. In murine models, secondary genetic events are required for the induction of full-blown leukemia (15, 58). However, all of these histone deacetylase-recruiting fusion proteins induce a block in hematopoietic differentiation. Ectopic expression of the fusion proteins in several cell models recapitulated the leukemic phenotype by inducing a state of refractoriness to inducers of differentiation (10, 11, 13, 32, 48, 57). In addition, PML-RARα-associated acute promyelocytic leukemia responds to all-*trans* retinoic acid (ATRA) by terminal differentiation of the leukemic promyelocytes (30). Despite knowledge about the shared biological effects of these fusion proteins,

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neither the shared target genes nor the common signal transduction pathways have been deciphered so far.

Wnt signaling is an evolutionarily conserved signal transduction pathway that governs cell fate decisions during embryogenesis and on the stem cell level. Several molecules downstream of Wnt act as either tumor suppressors or proto-oncogenes in the pathogenesis of epithelial cancers (43). Recently, Wnt signaling has been implicated in the self-renewal and proliferation of hematopoietic progenitor cells (2, 40, 46). So far, the Wnt signaling pathway has not been shown to be involved in human leukemia development.

In the current study, we identified shared target genes of AML1-ETO, PML-RARα, and PLZF-RARα. We demonstrated activation of Wnt signaling and TCF- and LEF-dependent transcription as a specific consequence of the presence of each fusion protein. Plakoglobin was induced by all three fusion proteins and led to enhanced growth and proliferation of myeloid progenitor cells.

MATERIALS AND METHODS

Cell lines and experimental design. The U937, NB4, NB4-R2, 32D, and Kasumi cells were cultured in RPMI with L-glutamine and 10% fetal calf serum. For 32D cells, WEHI supernatant was added as a source of interleukin-3. The stably transfected U937 cell lines that express PML-RARα, PLZF-RARα, AML1-ETO, or empty vector control in a Zn^{2+}-inducible fashion have been described previously (10, 13, 48). For induction of the fusion genes in these cells, 0.1 mM $ZnSO_4$ was added to the culture medium for 12 h. For the microarray analyses, trichostatin A (TSA, 1 μM) was added for 2 h and vitamin D_3 was added at 250 ng/ml for 24 h where indicated. Adherent cell lines were cultured in Dulbecco's modified Eagle's medium with L-glutamine and 10% fetal calf serum. The tetracycline-regulated U937-AML1-ETO cell line was a kind gift of Dong-Er Zhang (5).

RNA extraction and hybridization. Total RNA was extracted from cells with Trizol reagent (Invitrogen), and 10 μg of total RNA was reverse transcribed into cDNA with an oligo(dT)-T7 polymerase primer. Subsequently, T7 polymerase was used for in vitro transcription. During transcription, cRNA was labeled with biotinylated oligonucleotides. The resulting labeled cRNAs were fragmented and hybridized to HuGeneFL oligonucleotide microarrays, containing probes for approximately 6,800 independent transcripts (Affymetrix, Santa Clara, Calif.). Arrays were scanned after being stained with streptavidin-phycoerythrin, signal amplification with biotinylated antistreptavidin antibodies, and subsequent staining with streptavidin-phycoerythrin. Raw data were normalized and scaled to an average level of 2,500.

Analyses of microarray data and bioinformatics. Bioinformatical analyses were performed with Genespring analysis software (Genespring). A two-class procedure of the Significance Analysis of Microarrays software (Stanford) was used to identify differentially regulated genes (55). The delta value was set at 0.59. The SPSS 10.0 software was used to evaluate the statistical significance of gene expression differences. For a gene to be classified as repressed, its expression had to be lower than 50% in each of the three fusion protein samples compared to wild-type control-transfected U937 cells. The expression levels (average intensity) as indicated by the Affymetrix software had to be above 150 units in U937-control vector cells. Finally, gene candidates that were repressed more than twofold by vitamin D_3 in either mock- or fusion protein-transfected U937 cells were excluded. For a gene to be called induced, its expression had to be increased at least twofold in each sample compared to the U937 control vector and the expression level in each of the three fusion protein samples had to be above 150 units (Affymetrix average intensity). In addition, the ratio of expression of the three U937 fusion protein-expressing samples in the presence and absence of vitamin D_3 compared to the U937 control in the presence and absence of vitamin D_3 had to be higher than 2. The microarray data set is available for download on our website, http://medweb.uni-muenster.de/institute/meda/research/.

Quantitative real-time RT-PCR. Quantitation of mRNA levels was carried out with real-time fluorescence detection methods as described previously (25, 35, 37). Primer and probe information will be provided upon request. Quantitative real-time reverse transcription (RT)-PCR data were evaluated with the nonparametric Mann-Whitney *U* test. All tests were performed two-sided, and a *P* value of <0.05 was considered significant.

Transfections and luciferase assays. Primary $CD34^+$ hematopoietic progenitors were kindly provided by U. Cassens (Department of Transfusion Medicine, University of Münster). The $CD34^+$ cells were enriched to >95% purity by standard immunomagnetic techniques and frozen in liquid nitrogen until used. After thawing, the cells were cultured for 48 h in Iscover's modified Dulbecco's medium containing 20% fetal calf serum, 1% human serum albumin, 20 ng of stem cell factor per ml, and 20 ng of FLT3 ligand per ml. $CD34^+$ cells (4×10^5) were electroporated with a total of 20 μg of plasmid with the indicated combinations of luciferase reporter constructs, cytomegalovirus-driven expression vectors and a *Renilla* luciferase vector (SV40-pRL) used for normalization purposes. Electroporation was performed by a single pulse of 250 V and 0.975 μF. For luciferase assays, cells were harvested after 16 h ($CD34^+$ cells). The effects of AML1-ETO on the Topflash/Fopflash system were analyzed in LoVo colon carcinoma cells. The adherent cells were transfected in six-well plates with 2 μg of DNA and lipofectamine (Invitrogen) as the transfection agent. The cells were harvested and analyzed after 48 h. Luciferase assays were performed with the dual reporter luciferase assay kit (Promega) as described before (38).

Cloning and characterization of the human plakoglobin promoter. The plakoglobin promoter was PCR amplified from genomic DNA with *Pfu* and cloned into the pGL3-Basic vector. The 5′ deletions of the promoter were generated by restriction digestion and religation and by *Pfu* PCR amplification and subsequent cloning in pGL3-Basic. All constructs were verified by DNA sequencing. The plakoglobin promoter constructs were transfected into HeLa and 32D cells and analyzed for luciferase activity in the presence of either pCMV-AML1-ETO or the pCMV empty vector control. Several pCMV-AML1-ETO mutants were expressed to identify the relevant domains leading to plakoglobin promoter induction. These vectors have been described (25). Luciferase assays were performed as described above.

LEF-1 coimmunoprecipitation. U937 leukemia cell lines were electroporated with an expression vector coding for a hemagglutinin (HA)-tagged LEF-1 in the presence of either pcDNA3.1 (control) or one of the fusion proteins (AML1-ETO or PML-RARα). Forty-eight hours after transfection, cells were lysed in radioimmunoprecipitation buffer. Equal amounts of protein in the different samples were used to immunoprecipitate HA-LEF-1 with an HA affinity matrix (Roche). Western blotting analyses for plakoglobin were performed to determine the presence of catenin-LEF complexes. The same blot was then analyzed for HA-LEF expression, which served as a control for transfection efficiency and loading. The experiments regarding the response of PML-RARα and AML1-ETO to the dose of LEF-1-bound catenins were carried out in HeLa cells to increase the transfection efficiency. The transfections were performed as described before (36).

NB4 cells were electroporated with the HA-LEF-1 expression vector only. After transfection, the cells were split into two aliquots, and one of them was exposed to ATRA (10^{-6} M). Detection of LEF-plakoglobin complexes was performed as for the experiments with U937 cells.

Chromatin immunoprecipitation assay. Tetracycline-regulated U937-AML1-ETO or control cells were subjected to chromatin immunoprecipitation analysis essentially as described previously (56) with the exception that an isotype monoclonal antibody was used as the negative control. PCRs were performed with previously published primer sequences (21) and visualized on a 1.5% agarose gel stained with ethidium bromide.

Colony growth of Kasumi-1 and U937 cells transfected with dnTCF4. Kasumi-1 or U937 cells were electroporated with 10 μg of either the pcDNA3.1 or pcDNA3.1-dnTCF4 expression vector (53). The following day, the cells were seeded in triplicate in methylcellulose colony assays in the presence of neomycin as a selection marker. Colonies were counted after 14 days.

Generation of transfected 32D cell lines and mouse experiments. Stable cell lines were generated as described previously (34). In brief, 32D cells were electroporated with 10 μg of pCGN-plakoglobin or empty vector as a control. For selection purposes, cells were cotransfected with 10 μg of pcDNA3.1. Transfected cells were seeded in neomycin-containing methylcellulose the following day, and single colonies were picked after 10 days. These were expanded and tested for plakoglobin expression by real-time RT-PCR and Western blotting. Two 32D-plakoglobin cell lines and two 32D-vector control cell lines were analyzed. The plakoglobin protein levels in both 32D-plakoglobin cell lines were similar to those observed in AML1-ETO-expressing U937 cells but lower than the levels found in Kasumi-1 cells. Mouse experiments were performed as described previously (34). In brief, the mice were injected retroorbitally with 2×10^5 32D cells expressing either plakoglobin ($n = 9$) or control vector ($n = 8$). Mice were monitored over time, sacrificed when moribund, and analyzed for signs of leukemia such as enlarged liver and spleen as well as bone marrow

infiltration. Plakoglobin expression in mouse organs was confirmed by real-time RT-PCR. Murine glyceraldehyde-3-phosphate dehydrogenase expression was used to verify cDNA quality. Kaplan-Meier plots visualized survival differences, and the log-rank test was used to test statistical significance.

Assays of murine hematopoietic progenitors in vitro. Murine hematopoietic progenitors were purified from the bone marrow of C57BL/6N mice on the basis of the presence of ScaI stem cell marker and the absence of lineage differentiation markers ($ScaI^+/lin^-$) by immunomagnetic beads with MACS cell separation columns (Miltenyi, Bergisch-Gladbach, Germany). Purified cells were stimulated for 2 days in medium containing minterleukin-3 (20 ng/ml), murine interleukin-6 (20 ng/ml), and murine stem cell factor (100 ng/ml) (all purchased from StemCell Technologies, Vancouver, Canada). The cells were incubated with supernatants from Phoenix packaging cells transfected with the retroviral PINCO and PINCO-plakoglobin constructs (14). Infection efficiency was 70 to 80% as assessed by fluorescence-activated cell sorting analysis of green fluorescent protein-positive cells. At day 5 after infection, the cells were seeded in methylcellulose plates supplemented as above. After 15 days, colonies were collected from the methylcellulose, and expression of ScaI and Mac1 of green fluorescent protein-positive cells was measured by fluorescence-activated cell sorting analysis.

Antibodies and Western blot analysis. Cells were lysed in radioimmunoprecipitation buffer, and Western blotting was performed as described previously (38). Antibodies against c-Myc and plakoglobin were obtained from Santa Cruz, and plakoglobin antibodies were also purchased from Transduction Laboratories; the cyclin D1 antibody was from Pharmingen.

Protein half-life of plakoglobin and β-catenin in fusion protein-expressing cells. U937-control, U937-PML-RARα, and U937-AML1-ETO cells (3×10^5 cells/ml) were induced with zinc (0.1 mM). Cycloheximide was added after 24 h at a final concentration of 50 μg/ml. Cells were harvested at the indicated time points in radioimmunoprecipitation buffer, and Western blotting was performed as described previously (38).

RNA extraction from paraffin-embedded mouse organs. Six sections of 10 μm were cut from paraffin-embedded organ blocks with a sterile microtome. Tissue samples were deparaffinized by incubation in 0.4 ml of xylene at 37°C for 15 min. After centrifugation, the samples were washed twice with 100% ethanol. After vacuum drying, the pellet was resuspended in 230 μl of lysis buffer (20 mmol of Tris-HCl [pH 7.5] per liter, 20 mmol of EDTA per liter, 1% sodium dodecyl sulfate, 20 μl of proteinase K [2%]). The samples were incubated at 56°C for 16 to 24 h. After addition of 1 ml of Trizol reagent (Invitrogen), total RNA was extracted with chloroform (15 min of centrifugation 12,000 × *g*, 4°C). The RNA pellet was redissolved in 40 μl of RNase-free water and stored at −80°C.

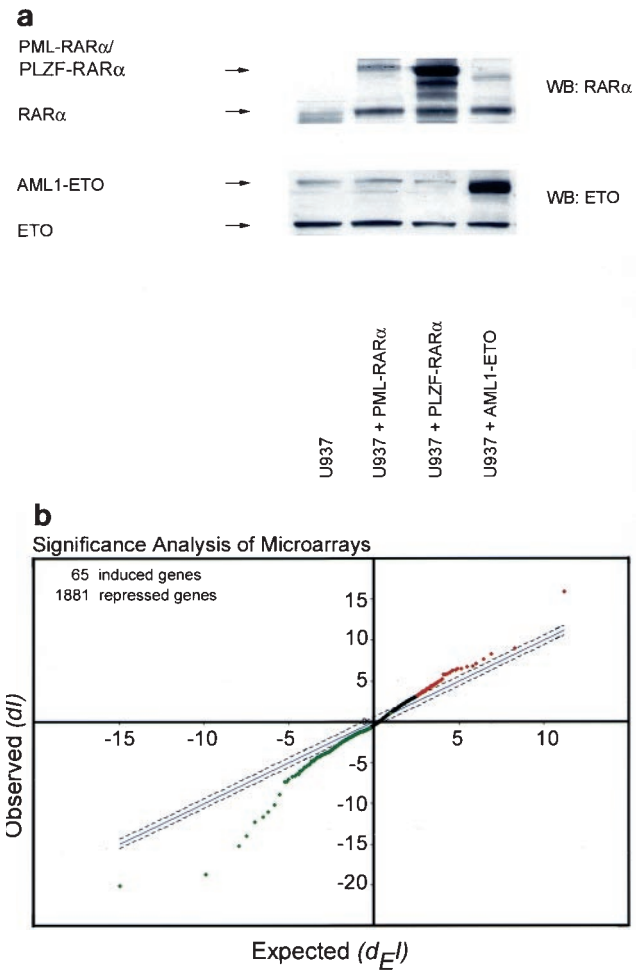

FIG. 1. Fusion protein induction and microarray analyses. (a) Western blot analysis of PML-RARα and PLZF-RARα expression with anti-RARα antibodies. Expression of AML1-ETO in transfected U937 cells was demonstrated by Western blotting with anti-ETO antibodies. Similar amounts of proteins (10 μg) of the same protein lysates were used in both experiments. The reason for the lower expression of RARα in the U937-control cells is unclear. (b) Significance Analysis of Microarray softwared was used as a paired analysis to identify genes that were differentially regulated between fusion protein-positive (U937-AML1-ETO, U937-PML-RARα, and U937-PLZF-RARα) and fusion protein-negative cells (U937-vector control plus zinc and U937-PML-RARα without zinc). The graph indicates the expected as well as the observed variability. Induced and repressed genes are indicated in red and green, respectively.

RESULTS

Microarray analyses of inducibly transfected U937 cell lines. We used high-density oligonucleotide microarray analyses to identify target genes of the histone deacetylase-associated fusion proteins AML1-ETO, PML-RARα, and PLZF-RARα. U937 cell lines inducibly transfected with one of the fusion proteins were analyzed to provide a common genetic background for all experiments. Expression of each of the fusion genes in U937 cells induced a block in differentiation in vitamin D_3-exposed U937 cells (data not shown). Global gene expression profiles of the cell lines under three different experimental conditions were analyzed. In these experiments, the cell lines (U937-AML1-ETO, U937-PML-RARα, U937-PLZF-RARα, and U937-empty vector) were zinc induced for 12 h. Expression of the transgenes was verified by Western blot analysis (Fig. 1a). Cells were either harvested after 12 h or exposed to vitamin D_3 for another 24 h. A third group of zinc-induced cells was exposed to the histone deacetylase inhibitor TSA for 2 h. The 2-h exposure to TSA has previously been shown to lead to maximum induction of histone deacetylase-repressed genes (28). Zinc-induced U937 cells transfected with an empty vector and PML-RARα-transfected U937 cells in the absence of zinc were used as controls. Genome-wide gene expression profiles were also obtained for AML1-ETO-positive Kasumi-1 cells in the presence and absence of TSA. These experiments served as a first validation of the target genes. Also, they were performed to evaluate the histone deacetylase dependency of the identified genes.

Labeled cRNA from all these samples was hybridized to human GeneFL oligonucleotide arrays to allow simultaneous analysis of about 6,800 human genes. Primary analysis of the resulting data set was performed with Affymetrix Microarray Analysis Suite 4.0. Overall, 4,549 (66.9%) genes showed significant levels of expression in at least one hybridization experiment. Known target genes of the translocation-based fusion proteins were verified by the microarray analyses. For

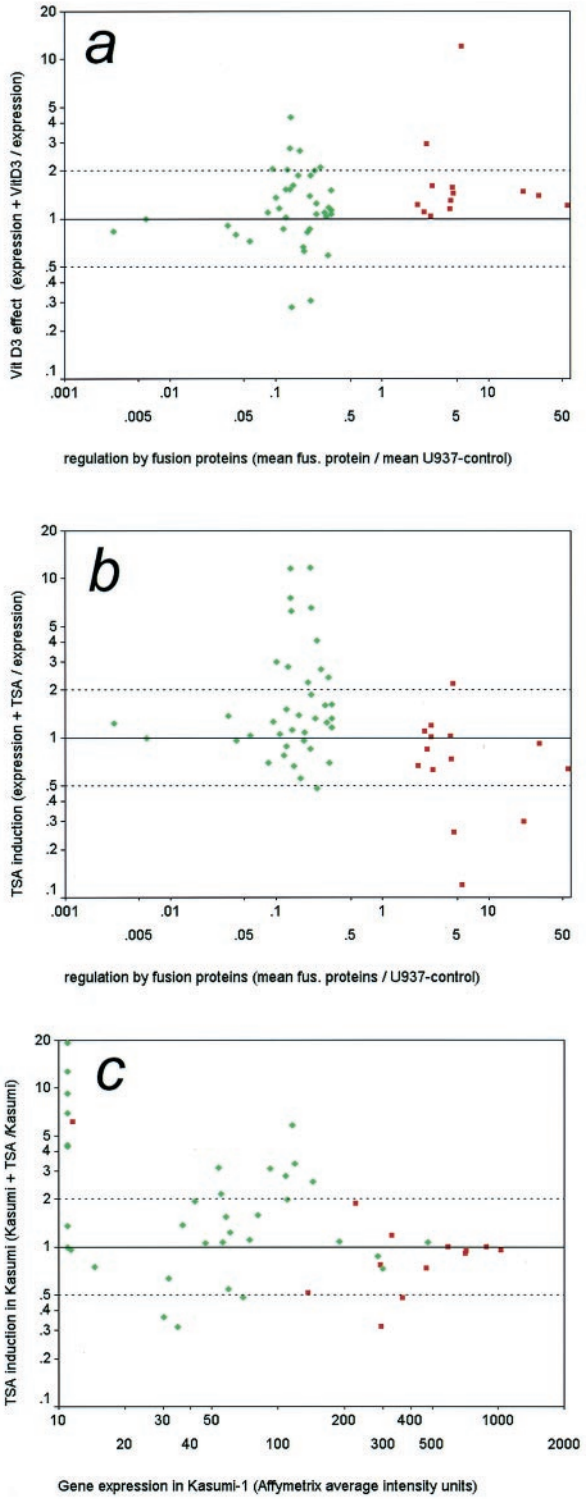

FIG. 2. Regulation of the shared target genes by vitamin D_3, TSA, and expression in Kasumi-1 cells. (a) This scatter plot depicts the association between regulation by the fusion proteins (mean expression levels in fusion protein samples/mean expression levels in control) and gene regulation by vitamin D_3 exposure (mean expression fusion proteins + vitamin D_3/mean expression fusion proteins). Values above 1 indicate induction, and values lower than 1 indicate repression. Dotted lines indicate a twofold induction or repression level. Genes found to be repressed by the fusion proteins are shown in green, and induced genes are indicated as red symbols. (b) Similar analyses were performed for the effect of the histone deacetylase-inhibitor TSA on gene expression in the fusion protein-expressing samples. TSA-induced genes show levels higher than 1 on the *y* axis. (c) Expression levels of the fusion protein-regulated genes in Kasumi-1 cells with the average intensity units as calculated by the Affymetrix software are shown on the *x* axis. Gene regulation by TSA in Kasumi-1 cells is indicated on the *y* axis. Values on the *y* axis higher than 1 indicate induction of the gene by TSA in Kasumi-1 cells. More than 33% of the repressed genes were induced more than twofold by TSA in Kasumi-1 cells. Genes found to be repressed by the fusion proteins are shown in green, and induced genes are indicated as red symbols.

example, we have previously shown that cyclin A1 is induced by PML-RARα and PLZF-RARα but not by AML1-ETO (36). Similar results were obtained with the microarray expression data (data not shown).

To identify target genes shared by all three fusion proteins, we performed a stepwise procedure. First, we used the Significance Analysis of Microarrays (SAM) software to select genes with a significant degree of variation between fusion protein-expressing samples (U937-PML-RARα, U937-AML1-ETO, and PLZF-RARα) and controls (U937-control vector and U937-PML-RARα without zinc). A total of 1,946 genes that were possibly regulated were identified (Fig. 1b).

In a second step, threshold values (see Materials and Methods) were introduced to increase the likelihood of the identified genes being truly regulated and of biological significance.

Altogether, 52 genes were identified as being significantly regulated, with 38 being repressed and 14 being induced by all three fusion proteins (Table 1). Among these, 25% (13 of 52) were surface membrane or secreted proteins. Almost 20% (10 of 52) of the genes are known to function in the nucleus, e.g., in transcriptional regulation and chromatin organization. Four genes that were repressed by the fusion proteins are involved in hematopoietic differentiation. Unexpectedly, several other identified genes act within the cadherin/catenin/Wnt signaling pathway, which was not known to be relevant in the pathogenesis of acute myeloid leukemia.

The 52 genes were analyzed for changes in expression due to vitamin D_3 and TSA exposure (Fig. 2a and b). Interestingly, among the genes repressed by the fusion proteins, 29% (11 of 38) were induced more than twofold by TSA-mediated histone deacetylase inhibition. Almost all of the genes induced by the fusion proteins in U937 cells were also expressed at significant levels in AML1-ETO-positive Kasumi-1 cells (Fig. 2c). Many of the repressed genes were expressed at only very low levels in Kasumi-1 cells. In Kasumi-1 cells, more than one third (34.2%, 13 of 38) of the repressed genes were induced at least twofold by TSA (Fig. 2c).

Regulation of a high percentage of the identified target genes was confirmed by quantitative real-time RT-PCR analyses. Several genes were confirmed not only in the cell lines expressing fusion proteins, but also in primary patient samples. For example, the myeloid elf factor (MEF) was found to be repressed by AML1-ETO, PML-RARα, and PLZF-RARα in the cell lines (Fig. 3a). In primary AML bone marrow specimens, AML fusion protein-positive cells expressed significantly

TABLE 1. Target genes shared by AML1-ETO, PML-RARα, and PLZF-RARα

Category and GenBank accession no.	Name	Regulation	Regulation (fold induction)			>2-fold induction by TSA	
			PMLRARα	PLZF-RARα	AML1-ETO	U937-fusion	Kasumi-1
Cadherin/catenin/Wnt signaling							
Z68228	Plakoglobin	Induced	4.20	5.08	4.62	−	−
L76702	Protein phosphatase 2 A B56-delta	Induced	5.54	3.39	4.16	−	−
AB000895	Cadherin FIB1	Repressed	0.14	0.06	0.36	−	+
AB000896	Cadherin FIB2	Repressed	0.26	0.07	0.31	−	−
Cell surface/secreted protein/extracellular matrix							
M16279	MIC2 mRNA, complete cds	Induced	3.72	2.42	2.92	−	−
D83782	KIAA0199 gene	Induced	5.59	3.36	4.30	−	−
AF008445	Phospholipid scramblase	Repressed	0.03	0.03	0.07	−	−
S76942	Dopamine D4 receptor	Repressed	0.04	0.47	0.12	+	−
Y00787	Monocyte-derived neutrophil chemotactic factor	Repressed	0.11	0.11	0.22	−	−
AB098642	Semaphorin E	Repressed	0.13	0.08	0.16	−	−
M92302	Calcium channel, voltage-gated, beta 1 subunit	Repressed	0.16	0.10	0.04	+	−
AB98642	Osteomodulin	Repressed	0.17	0.02	0.09	−	−
L14561	Atpase, Ca^{2+} transporting, plasma membrane 1	Repressed	0.21	0.23	0.19	−	−
D79206	Ryudocan core protein	Repressed	0.22	0.06	0.13	+	−
M32578	MHC class II HLA-DR beta-1 mRNA (DR2.3)	Repressed	0.26	0.17	0.06	−	−
AF014958	Chemokine receptor X	Repressed	0.28	0.07	0.16	−	+
AF000562	Uroplakin II	Repressed	0.45	0.23	0.32	−	+
Myeloid differentiation							
U32645	Myeloid elf-1-like factor	Repressed	0.22	0.07	0.07	−	−
M13207	Granu ocyte-macrophage colony-stimulating factor 1	Repressed	0.29	0.25	0.06	+	+
M97796	Helix-loop-helix protein (Id-2)	Repressed	0.30	0.42	0.17	−	−
M26683	Gamma interferon	Repressed	0.36	0.42	0.23	−	−
Transcription/chromatin/ nuclear proteins							
X73358	hAES-1 mRNA	Induced	61.77	54.95	54.95	−	−
Z36714	Cyclin F	Induced	30.13	20.07	14.67	−	−
M31523	Human transcription factor (E2A)	Induced	5.77	4.16	4.22	−	−
U65093	*msg1*-related gene 1 (*mrg1*)	Induced	2.10	2.63	3.26	−	−
X70683	SOX-4 protein	Induced	2.09	2.01	2.48	−	−
U61145	*Enhancer of zeste* homolog 2 (EZH2)	Repressed	0.24	0.32	0.18	−	−
AB000905	H4 histone	Repressed	0.30	0.29	0.28	−	+
D87673	Heat shock transcription factor 4	Repressed	0.33	0.04	0.05	+	+
X79200	SYT-SSX, synovial sarcoma translocation junction	Repressed	0.34	0.43	0.24	−	+
Z49825	Hepatocyte nuclear factor 4 alpha	Repressed	0.34	0.31	0.29	−	+
Other or unknown function							
D63391	Platelet-activating factor acetylhydrolase IB gamma	Induced	39.56	31.36	20.04	−	−
D31764	KIAA0064 gene	Induced	6.66	6.31	3.91	−	−
M57567	ADP-ribosylation factor (hARF5)	Induced	3.06	2.17	2.31	−	−
L36983	Dynamin (DNM)	Induced	3.42	2.23	3.08	−	−
D16480	Mitochondrial enoyl-coenzyme A hydratase	Induced	2.76	3.42	2.54	−	−
AB000467	RES4-25	Repressed	0.00	0.00	0.01	−	−
AB000466	RES4-24C	Repressed	0.01	0.01	0.01	−	−
U60206	Stress-responsive serine/threonine kinase Krs-1	Repressed	0.03	0.04	0.04	−	−
AB000450	VRK2	Repressed	0.03	0.04	0.10	−	−
U57450	EPC-1 gene	Repressed	0.06	0.33	0.33	+	−
AB000449	VRK1	Repressed	0.08	0.07	0.11	−	−
D49818	mRNA for 6-phosphofructo-2-kinase/ fructose-2,6-bisphosphate	Repressed	0.09	0.21	0.02	−	−
U92015	Defective mariner transposon Hsmar2	Repressed	0.14	0.10	0.14	+	+
D31897	Doc2 (double C2)	Repressed	0.17	0.07	0.14	−	−
U87964	Putative G-protein (GP-1)	Repressed	0.18	0.18	0.05	+	−
AB000115	Human mRNA expressed in osteoblast	Repressed	0.18	0.17	0.19	−	+
AB000462	SH3 binding protein, clone RES4-23A	Repressed	0.20	0.11	0.12	−	−
K03183	Chorionic gonadotropin beta subunit	Repressed	0.28	0.05	0.30	+	−
AB000409	MNK1	Repressed	0.29	0.27	0.38	+	−
AB002314	KIAA0316	Repressed	0.37	0.16	0.17	−	+
M16750	Pim-1 oncogene	Repressed	0.44	0.27	0.09	+	+

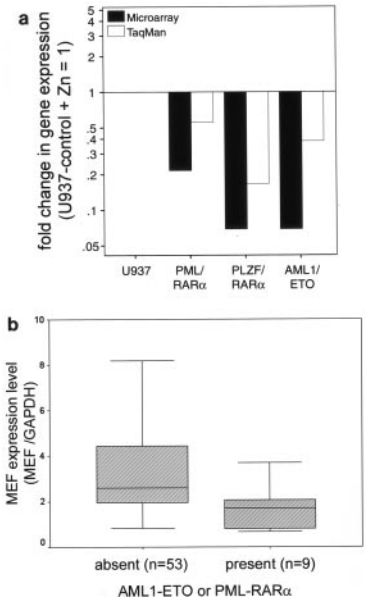

FIG. 3. MEF is repressed in fusion protein-associated AML. (a) Expression analysis of MEF by microarray analysis and real-time RT-PCR confirmation. (b) Levels of mRNA expression in a cohort of 62 AML patients. Bone marrow samples were obtained at the time of the initial diagnosis, and the percentage of blast cells was higher than 70% in all samples. Real-time RT-PCR expression data are shown for patients separated into those expressing and not expressing one of the fusion proteins. The box plots indicate the expression level distribution of the patient samples, with the median being indicated by the horizontal line within the boxes. Differences in expression were statistically significant ($P = 0.001$).

lower levels of MEF than other AML samples ($P < 0.001$, Mann-Whitney U test) (Fig. 3b).

Induction of plakoglobin by AML fusion proteins in vitro and in AML in vivo. Among the 14 induced genes, two genes (PP2A and plakoglobin) regulate events downstream of Wnt signaling. Plakoglobin has recently been shown to have transforming abilities upon overexpression in epithelial cells (22). Real-time RT-PCR verified the induction of plakoglobin mRNA in fusion protein-expressing U937 cells (Fig. 4a). In subsequent Western blotting experiments, plakoglobin was also induced on the protein level. Similar to the findings on the RNA level, AML1-ETO had the strongest effect on plakoglobin induction (Fig. 4b). Also, cells that naturally harbor PML-RARα (NB4) or AML1-ETO (Kasumi-1) expressed plakoglobin (Fig. 4b). Another U937-AML1-ETO cell line, regulated by tetracycline, expressed high levels of plakoglobin as well (Fig. 4c). Among the fusion protein-negative leukemia cell lines, some did express plakoglobin, while others did not. (Fig. 4c).

It has been suggested that plakoglobin overexpression leads to a subsequent increase in β-catenin protein levels by blocking β-catenin degradation (31). Therefore, we performed protein half-life experiments for plakoglobin and β-catenin proteins. We found increased levels of plakoglobin and β-catenin in fusion protein-positive samples. Addition of cycloheximide revealed the higher stability of plakoglobin compared to β-catenin. Since plakoglobin and β-catenin were not detectable in the absence of fusion proteins, no differences in protein half-lives could be analyzed (Fig. 4d). ATRA specifically counteracts the effects of PML-RARα. Therefore, we analyzed whether ATRA influenced plakoglobin expression in NB4 cells. Plakoglobin protein expression was decreased by ATRA in PML-RARα-expressing NB4 leukemia cells (Fig. 4e). To control whether plakoglobin reduction was related to PML-RARα, we performed a similar experiment with NB4-R2 cells, a cell line that expresses a mutant PML-RARα that is not responsive to ATRA (3). In NB4-R2 cells, no decrease in plakoglobin levels was observed after exposure to ATRA (Fig. 4e). These findings indicated that the loss of plakoglobin expression following ATRA treatment was associated with inhibition of the fusion protein.

Next, we analyzed plakoglobin expression in a cohort of patients with acute myeloid leukemia. All patient bone marrow specimens were obtained at the time of diagnosis. A high percentage of blast cells (>70%) was verified morphologically, and blast cells were further enriched by density centrifugation. Among the 62 AML bone marrow aspirate samples obtained from individuals at the time of diagnosis, 9 samples contained either the AML1-ETO ($n = 6$) or the PML-RARα ($n = 3$) fusion protein. None of the samples expressed the rare PLZF-RARα. Overall, plakoglobin mRNA expression was detectable by quantitative real-time RT-PCR analysis in the majority of patients. In general, plakoglobin expression was higher in undifferentiated and myelocytic AML blasts (French-American-British group [FAB] M0, M1, M2, and M3) compared to monocytic or erythroid AML blasts (FAB M4, M5, and M6) ($P = 0.001$). Similar to the findings in the transfected cell lines, fusion protein-positive AML samples expressed significantly higher plakoglobin mRNA levels ($P = 0.04$) than other AML samples (Fig. 4f). These findings verified that plakoglobin induction by the fusion proteins in U937 cells was paralleled by high plakoglobin expression in primary AML leukemic blasts and especially in AML blasts expressing PML-RARα or AML1-ETO.

Activation of the plakoglobin promoter by AML fusion proteins. To analyze the mechanisms of fusion protein-induced plakoglobin expression, we cloned the human plakoglobin promoter by *Pfu* PCR into a luciferase expression construct. AML1-ETO and PML-RARα significantly activated promoter activity following transfection into either HeLa or 32D myeloid cells (Fig. 5a and data not shown). AML1-ETO did not bind directly to the plakoglobin promoter in vivo by chromatin immunoprecipitation analysis (data not shown). Therefore, we analyzed which fragments of the promoter mediated the fusion protein effects. Promoter deletion analyses revealed that at least 197 bp upstream of the transcriptional start site were necessary for AML1-ETO activation, with the region between bp −197 and bp −188 (sequence GGGGCCAATG) being especially important for this effect. C/EBPα, which binds to CCAAT sites, did not regulate the plakoglobin promoter (data not shown). This region corresponds to bp 769 to 778 of the published plakoglobin promoter sequence (AJ276892).

We then analyzed the domains of AML1-ETO that were necessary for plakoglobin promoter activation (Fig. 5b). The expression vectors used for these analyses have been described (25). In these analyses, amino acids 350 to 428, the zinc finger domain, and the hydrophobic heptad repeat domain were necessary for activation of the plakoglobin promoter, Similar

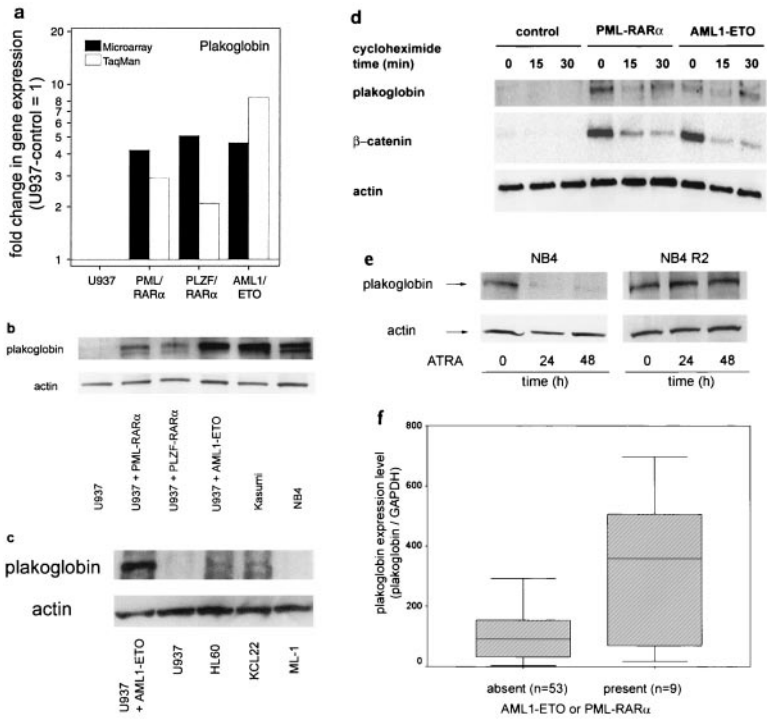

FIG. 4. Induction of plakoglobin by fusion proteins in AML. (a) Comparison of plakoglobin mRNA regulation by fusion proteins in microarray experiments and in real-time RT-PCR analyses in the different U937 cell lines. Expression levels of the U937-vector control cell line were set at 1. (b) Plakoglobin protein expression in fusion protein-expressing U937 cells by Western blot analysis. (c) Western blot analysis of plakoglobin expression in tetracycline-inducible U937-AML1-ETO cells and other myeloid cell lines. (d) Control U937 cells, U937-AML1-ETO, or U937-PML-RARα cells were zinc induced for 24 h before cycloheximide was added to block protein synthesis. Cells were harvested at the indicated time points and analyzed for plakoglobin and β-catenin protein levels. (e) NB4 leukemia cells carry an endogenous t(15;17). These cells but not the resistant NB4-R2 cells differentiate towards granulocytes upon exposure to ATRA. Western blot analysis indicated that plakoglobin protein levels were decreased by ATRA in responsive NB4 cells but not in ATRA-resistant NB4-R2 cells. (f) Expression levels of plakoglobin in bone marrow samples at the time of diagnosis in 62 patients with AML. Plakoglobin expression was significantly higher in fusion protein-positive patients than in others ($P = 0.04$).

AML1-ETO domains were required for promoter activation in HeLa cells and in 32D cells (Fig. 5b and c). These domains recruit corepressors and histone deacetylases. The functionally relevant domains were similar to those that we have previously shown to be important for repression of the $p14^{ARF}$ promoter (25), indicating that AML1-ETO activates the plakoglobin promoter by an indirect mechanism.

Activation of TCF and LEF signaling by AML fusion proteins. Plakoglobin plays an important role in cellular signal transduction and is part of the Wnt signaling pathway. So far, plakoglobin and other components of the Wnt signaling pathway have not been implicated in the pathogenesis of leukemia. We analyzed whether induction of plakoglobin by the fusion proteins activated downstream signaling. Activation of the Wnt signaling pathway leads to stabilization of members of the catenin family that act as transcriptional coactivators for the TCF and LEF transcription factors. Thus, activation of TCF- and LEF-dependent transcription is thought to be an important consequence of Wnt-mediated signaling.

TCF- and LEF-associated promoter activation depends on the physical binding of β-catenin or plakoglobin to either the LEF or TCF transcription factor. To analyze the effects of the fusion proteins on the amount of LEF-associated catenins, U937 cells were transfected with either AML1-ETO, PML-RARα, or the control vector in combination with an HA-LEF-1 expression construct. Two days after transfection, protein lysates were immunoprecipitated with an anti-HA affinity matrix and analyzed for the amount of plakoglobin-bound LEF-1 (Fig. 6a). Interestingly, expression of the fusion proteins led to the formation of plakoglobin/LEF-1 complexes which were not detected in U937 cells transfected with the control vector. HA-LEF-1/plakoglobin complexes were also detected in PML-RARα-positive NB4 cells. ATRA treatment of the HA-LEF-1-transfected NB4 cells strongly reduced the amount of plakoglobin bound to HA-LEF-1 (data not shown).

Since β-catenin expression was also increased by AML fusion proteins, we analyzed whether LEF-1-bound β-catenin was increased by the fusion proteins as well (Fig. 6b). In these experiments, we observed a dose-dependent increase in LEF-1-bound β-catenin after expression of PML-RARα or AML1-ETO. Chromatin immunoprecipitation was used to analyze plakoglobin localization to TCF and LEF target genes in vivo. We analyzed the c-*myc* promoter, because this promoter is preferentially activated by plakoglobin (22). In U937 cells, plakoglobin specifically precipitated with the c-*myc* promoter when AML1-ETO was expressed (Fig. 6c). Taken together, these experiments showed evidence that the fusion proteins increased the amounts of plakoglobin and β-catenin that could

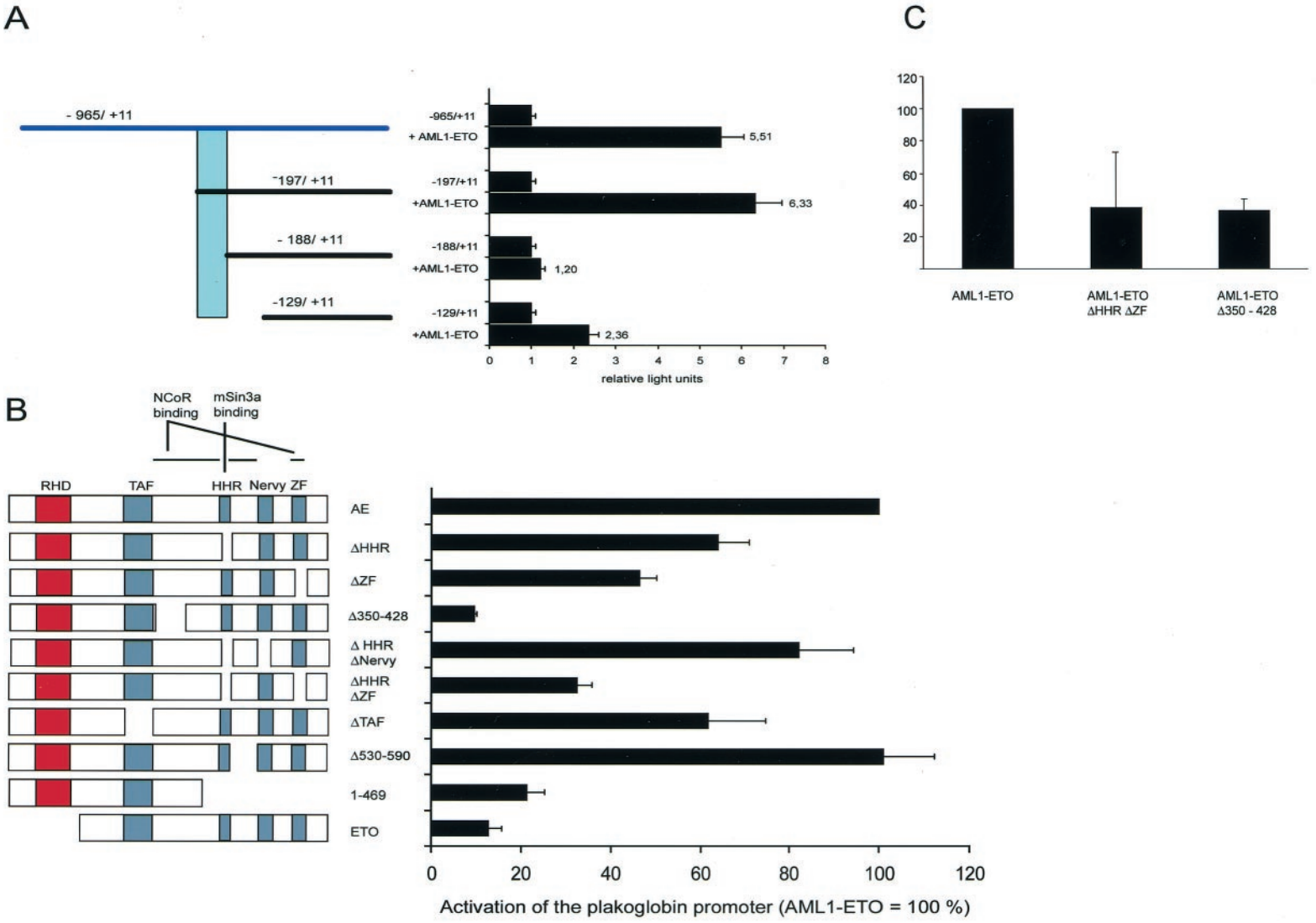

FIG. 5. AML1-ETO activates the human plakoglobin promoter. (a) The plakoglobin promoter was cloned by *Pfu* PCR into pGL3-Basic, and the full-length promoter fragment as well as several deletion mutants generated by restriction digestion were analyzed for activation by AML1-ETO. Promoter fragment numbering is according to the published plakoglobin promoter sequence (44). (b) Several deletion mutants of AML1-ETO were tested for activation of the plakoglobin promoter in HeLa cells, which were used for ease of transfection. The plakoglobin promoter activity induced by the full-length AML1-ETO expression construct was set at 1. (c) AML1-ETO and the relevant deletion mutants identified in b were analyzed in 32D hematopoietic progenitor cells in luciferase assays with the plakoglobin promoter. Again, constructs lacking the corepressor binding domains failed to induce the plakoglobin promoter.

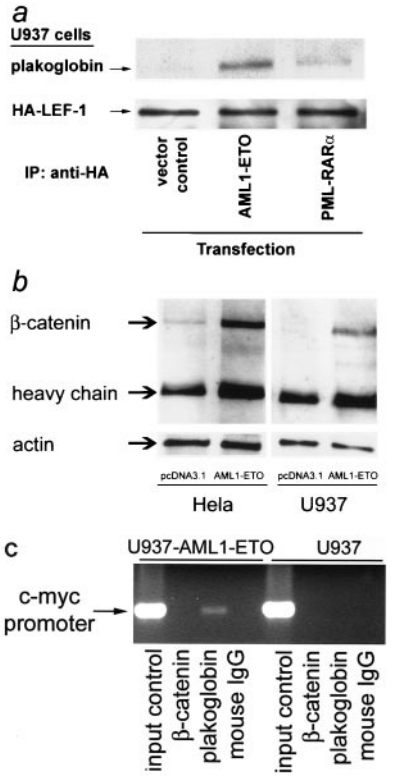

FIG. 6. Activation of TCF and LEF transcriptional activity by AML fusion proteins. (a) Analysis of LEF-1-bound catenin levels in the presence of AML fusion proteins. HA-LEF-1 was transfected along with the indicated fusion protein or a control vector into U937 cells. Equal protein concentrations of the transfected cell populations were used to immunoprecipitate HA-LEF-1 by an anti-HA affinity matrix. Significantly increased levels of LEF-1-bound plakoglobin were detected in Western blot analyses of the immunoprecipitates. Subsequent Western blot analysis of HA-LEF-1 served as control for transfection and equal loading. (b) Following transfection of HA-LEF1, PML-RARα, and AML1-ETO, a dose-dependent increase in LEF-1-bound plakoglobin and β-catenin was demonstrated by anti-HA immunoprecipitation and Western blotting. The immunoglobulin heavy chain and actin served as loading controls. (c) Chromatin immunoprecipitation analysis of the c-*myc* promoter. U937 cells with or without AML1-ETO induction were formaldehyde cross-linked, and DNA-protein complexes were precipitated with either antiplakoglobin or anti-β-catenin antibodies or a control antibody. Samples were washed and cross-links were reversed before promoter sequences were amplified by PCR.

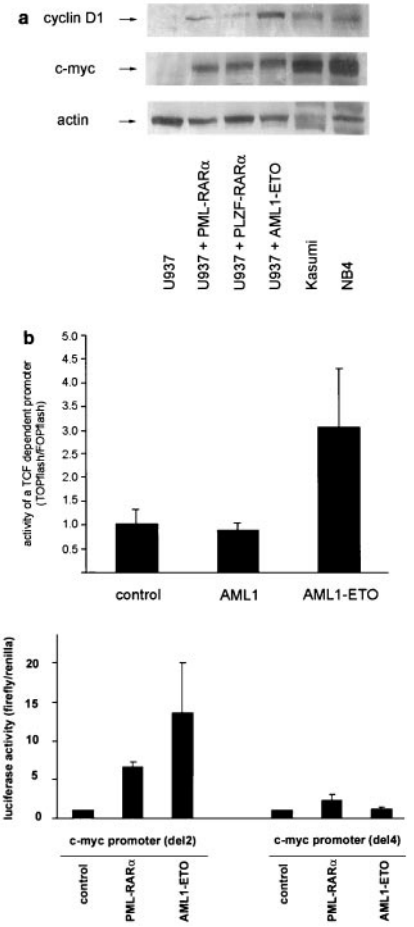

FIG. 7. Induction of TCF and LEF target genes and signaling by AML fusion proteins. (a) Western blot analysis of the TCF and LEF target genes cyclin D1 and c-Myc protein in fusion protein-expressing U937 cells, in AML1-ETO-positive Kasumi-1 cells, and in PML-RARα-positive NB4 cells. All cells expressing one of the fusion proteins expressed high levels of plakoglobin. (b) The TCF- and LEF-dependent Topflash/Fopflash promoter system was used to analyze the effects of the fusion proteins on plakoglobin downstream signaling. The vector control, AML1, or AML1-ETO expression vector was transfected along with the reporter constructs and a *Renilla* luciferase reporter used for normalization purposes. AML1-ETO but not AML1 induced TCF and LEF transcriptional activity. The mean ± standard error of three independent experiments is shown. (c) The effects of AML1-ETO and PML-RARα on the c-*myc* promoter were analyzed in primary hematopoietic $CD34^+$ progenitor cells. AML1-ETO and PML-RARα induced the TCF binding element containing the del-2 promoter. No effect of the fusion proteins on the activity of the TCF binding element-lacking del-4 construct was observed. The results of four independent experiments (mean and standard error) are shown.

bind to the TCF and LEF transcription factors and that plakoglobin was found at a target gene promoter in vivo.

Next, we looked for the induction of TCF and LEF target genes by AML fusion proteins. Two functionally important targets of TCF and LEF transcription factors are c-myc and cyclin D1 (16, 53). In U937 cells, induction of each of the leukemogenic fusion proteins for 12 h was associated with an increase in c-Myc and cyclin D1 protein expression (Fig. 7a). In addition, PML-RARα-positive NB4 cells and AML1-ETO-positive Kasumi-1 cells also expressed significant levels of c-Myc and cyclin D1. The transcriptional activity of TCF-catenin complexes was tested by using luciferase reporter constructs with TCF and LEF binding sites (Topflash). A reporter construct with mutated TCF and LEF sites (Fopflash) was used to correct for non-TCF- and non-LEF-dependent effects. We co-transfected these reporter plasmids with either AML1b or AML1-ETO (Fig. 7b). AML1b did not show any activity, whereas AML1-ETO consistently increased luciferase activity more than threefold. While the increase in Topflash activity by AML1-ETO was significant, it was lower than the effects of mutant β-catenin transfection. One possible explanation is that the effects of AML1-ETO occurred indirectly via plakoglobin induction and thus might be weaker in in vitro experiments. Furthermore, it has been reported that plakoglobin shows relatively weak activity in the Topflash/Fopflash system. Also, it is

conceivable that cell type-specific differences might be important.

Since c-*myc* was induced by the fusion proteins in leukemic cells (Fig. 7a) and plakoglobin was bound to the c-*myc* promoter in vivo (Fig. 6c), we also used c-*myc* promoter reporter constructs. To analyze whether AML1-ETO and PML-RARα could lead to activation of fragments of the c-*myc* promoter that contained TCF binding elements, we transfected primary human $CD34^+$ cells, which were enriched to high purity (>95%) by magnetic bead isolation procedures. AML1-ETO or PML-RARα expression vectors were electroporated into $CD34^+$ cells along with either a construct that contained two TCF binding elements (del-2), or a shorter fragment lacking TCF binding elements (del-4) (16). Both promoter fragments showed significant promoter activity. These constructs were used previously to verify plakoglobin transactivation (22). PML-RARα and AML1-ETO induced the TCF- and LEF-dependent c-*myc* promoter fragment between 6- and 12-fold (Fig. 7c). No activation of the promoter construct lacking these sites was found (Fig. 7c). In combination with our chromatin immunoprecipitation results, these findings provide evidence that AML1-ETO and PML-RARα induce c-*myc* promoter activity through plakoglobin-TCF and -LEF activity in hematopoietic cells.

These experiments established that AML fusion proteins enhanced the formation of plakoglobin-LEF complexes, led to TCF- and LEF-dependent promoter transactivation, and induced TCF and LEF target gene expression.

Plakoglobin increased 32D cell clonogenic growth and enhanced leukemogenesis in vivo. Based on these findings, we were interested whether plakoglobin and TCF- and LEF-dependent transcription could contribute to the malignant phenotype in fusion protein-expressing leukemias. We analyzed the biological consequences of TCF-dependent transcription in leukemic and nonleukemic hematopoietic progenitor cells. First, we established 32D hematopoietic progenitor cell lines that stably overexpressed plakoglobin. Plakoglobin expression was verified by real-time RT-PCR (data not shown) and Western blotting (Fig. 8a). Plakoglobin levels in transfected 32D cells were increased about threefold compared to the levels in control vector-transfected cells. The protein levels of plakoglobin were similar to those observed in NB4 and U937-AML1-ETO cells but lower than the levels in AML1-ETO-positive Kasumi-1 cells. Two independent control cell lines and two plakoglobin-expressing cell lines were tested for cellular proliferation and clonal growth. Cellular proliferation, as assessed by growth curves and [^{3}H]thymidine incorporation, was significantly increased by plakoglobin (Fig. 8b and c). Furthermore, plakoglobin enhanced colony growth by 60% (Fig. 8d).

To analyze the effects of plakoglobin in vivo, syngeneic mice were injected with 32D cells transfected either with plakoglobin or with the empty expression vector. After a latency period of 50 days, eight of nine plakoglobin-transfected mice developed a rapidly fatal disease that on pathological examination resembled acute leukemia (Fig. 9a). On macroscopic examination, the mice had heavily enlarged spleens and livers. Histologic examination revealed multifocal infiltration by a largely immature, blast-like population of hematopoietic cells of the liver, the spleen, the bone marrow, and the lung (Fig. 9c). The expression of plakoglobin in the organs infiltrated by 32D

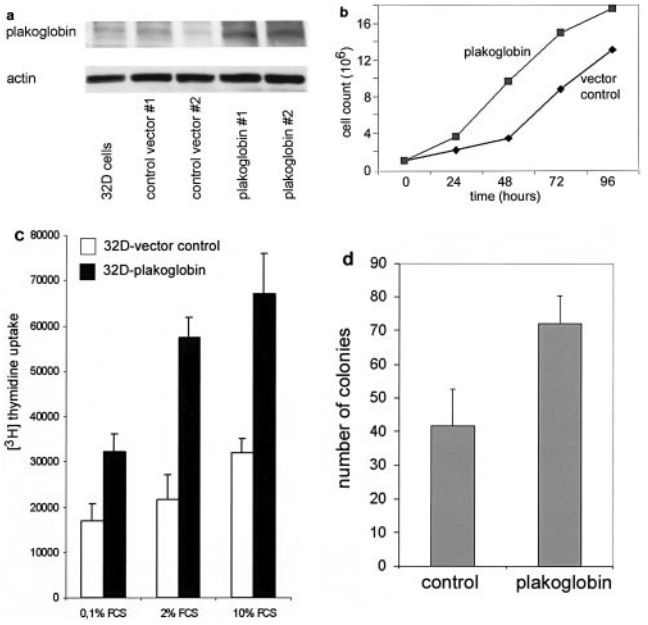

FIG. 8. Plakoglobin enhances growth of the 32D hematopoietic progenitor cells. (a) The murine 32D cell line was stably transfected with a plakoglobin expression vector (pCGN-plakoglobin). Single clones were expanded and tested by Western blotting for plakoglobin expression. Levels of plakoglobin protein expression were comparable to those induced by AML1-ETO in U937 cells. (b) Plakoglobin-expressing 32D cells grew faster than their control vector-transfected counterparts. Shown are the means of three experiments. In each experiment, two independent cell lines each of the control- and the plakoglobin-transfected cells were analyzed. (c) Expression of plakoglobin in 32D cells was associated with increased proliferation, as shown by a [^{3}H]thymidine incorporation assay. The plakoglobin effect was present at low as well as at high serum concentrations. The assay was independently performed three times. For each experiment, all conditions were tested in quadruplicate.(d) To analyze colony-forming abilities, control vector- or plakoglobin-expressing 32D cells were plated in colony assays. The number of colonies formed by plakoglobin-expressing cells was significantly higher than the number of colonies formed by control-transfected cells. Indicated are the mean and standard error of four different experiments performed with two independent control and plakoglobin cell lines each.

plakoglobin cells was verified in the spleen and liver by quantitative RT-PCR (Fig. 9b). In the control group, a few of the mice died after a much longer latency period. Their livers and spleens were smaller than those detected in the 32D-plakoglobin-carrying mice. Histological examination of their livers revealed infiltrating nodules consisting of more mature hematopoietic cells (data not shown). Kaplan-Meier survival curves were drawn and established the significant difference in survival between the two groups ($P = 0.0016$, log-rank test) (Fig. 9a).

TCF and LEF signaling is important for growth of AML1-ETO-positive leukemia cells. These findings showed evidence that plakoglobin could enhance the leukemogenic growth of a nontransformed hematopoietic progenitor cell line. We also wondered whether TCF and LEF signaling was important for the growth and survival of leukemic cells that expressed one of the fusion proteins. Therefore, we studied the consequences of TCF and LEF inhibition on clonal growth of the AML1-ETO-positive Kasumi-1 leukemia cell line. Kasumi-1 cells or U937

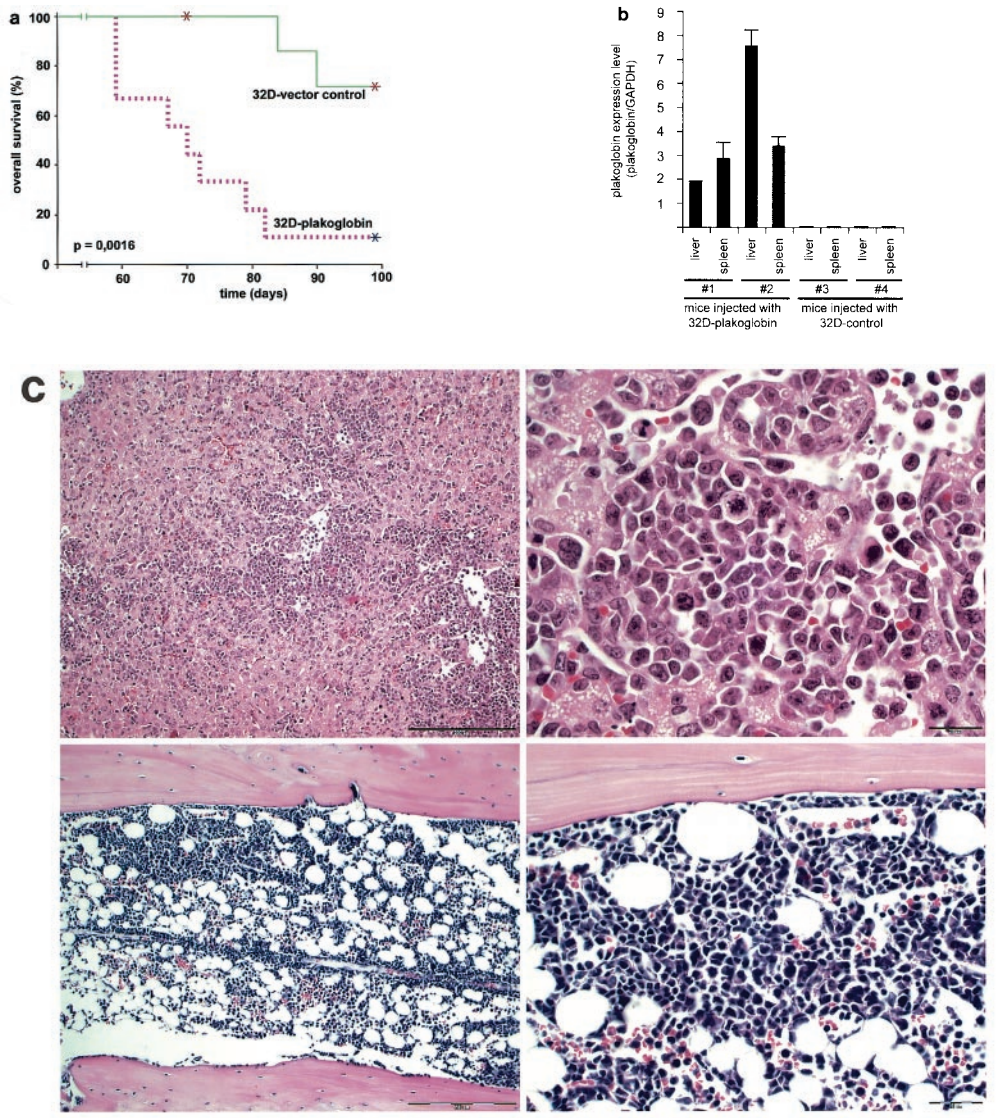

FIG. 9. Plakoglobin accelerates leukemogenic growth in syngeneic mice. (a) A total of 2×10^5 32D cells transfected with either a plakoglobin expression vector or a control vector were injected into syngeneic mice. The mice were monitored over time, and moribund mice were sacrificed. The observed differences in the Kaplan-Meier plot are statistically highly significant ($P = 0.0016$). The censored control mouse (day 70) was sacrificed for comparison of the organs with those of a moribund plakoglobin mouse. (b) Plakoglobin expression levels were analyzed in the organs of two mice injected with either 32D-plakoglobin or 32D-control cells. The relative expression levels and standard deviation of two quantitative real-time RT-PCR analyses are shown. (c) These photos depict the histological findings in 32D-plakoglobin-transfected mice. The upper two pictures present liver histology, and the lower ones show bone marrow sections.

cells were electroporated with either a dominant negative TCF (dnTCF-4E, N-terminally deleted) expression vector or an empty control vector. Following transfection, the cells were seeded in colony assays in the presence of neomycin for selection (Fig. 10a). The dominant negative TCF4 expression vector inhibited colony growth more than fourfold, indicating the importance of TCF and LEF signaling for AML1-ETO-positive Kasumi-1 cells. On the other hand, only a minor, nonsignificant decrease in colony growth was observed in naive U937 cells. This indicated that TCF and LEF signaling is important for AML1-ETO-positive leukemia cells but not necessarily for all leukemia cell lines.

Plakoglobin-expressing hematopoietic progenitors retain an immature phenotype. Wnt signaling plays an important role in self-renewal and cell fate determination. Recently, AML1-ETO has been shown to enhance self-renewal in hematopoietic progenitors (39, 54). Activation of plakoglobin-induced TCF and LEF signaling could be a mechanistic explanation for AML fusion protein-induced self-renewal. Therefore, we analyzed the effects of plakoglobin on hematopoietic progenitors. ScaI-positive, lineage-negative murine hematopoietic progenitor bone marrow cells were purified by using immunomagnetic beads. Subsequently, progenitor cells were infected with the retroviral vector PINCO as a control or PINCO-plakoglobin (14). Cells were grown in growth factor-supplemented medium and plated in colony assays on day 5. After 15 days, green fluorescent protein-positive colonies were analyzed for surface marker expression by flow cytometry with antibodies for Sca1

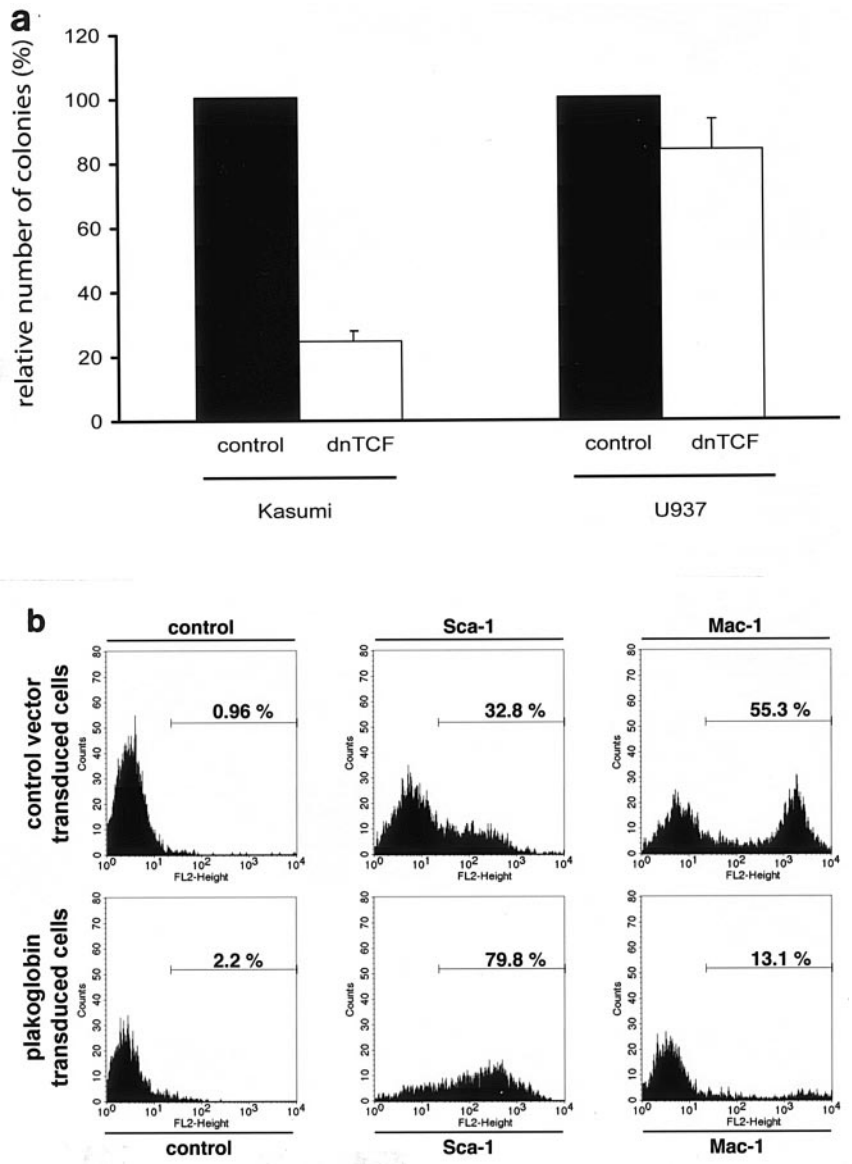

FIG. 10. Dominant negative TCF4 inhibits leukemic cell growth and plakoglobin affects hematopoietic progenitors. (a) AML1-ETO-positive Kasumi-1 cells were transfected with a dominant negative TCF4 expression vector. In subsequent triplicate colony assays with neomycin selection, dnTCF4-containing Kasumi-1 cells showed reduced clonal growth. The mean and standard error of two independent experiments are shown. (b) $ScaI^+$ lin^- mouse bone marrow cells were transduced with the indicated retroviral vectors; 15 days after seeding in methylcellulose, the expression of the stem cell antigen ScaI or the differentiation marker Mac1 of the green fluorescent protein-positive cells was determined by flow cytometry. The results of one representative experiment out of three are shown.

and Mac1 expression (Fig. 10b). These analyses demonstrated that the majority of the cells in the control transfections underwent differentiation, as indicated by loss of the stem cell antigen ScaI and induction of the differentiation antigen Mac1. In contrast, cells expressing plakoglobin retained the immature, stem cell-like phenotype ($ScaI^+$ $Mac1^-$) of the initial cell population (Fig. 10b).

DISCUSSION

The high incidence of balanced translocations in leukemia is a nonrandom event. Different types of leukemia are associated with specific balanced translocations (26). In acute myeloid leukemias, the two most frequent balanced translocations are t(8;21) and t(15;17). About 20% of newly diagnosed acute myeloid leukemia patients show evidence of the presence of one of these translocations. In the current study, we provide strong evidence that AML1-ETO, PML-RARα. and the related PLZF-RARα share important target genes. Importantly, the findings in the cell lines translated directly to differential gene expression in primary AML patient samples at the time of diagnosis. Our further analyses deciphered TCF and LEF signaling as an oncogenic pathway that was specifically induced by several AML fusion proteins.

Identification of relevant target genes in the microarray data

set was performed with a two-step procedure. In a first step, we identified 1,945 genes as potential targets for the fusion proteins. The large number of genes demanded further selection. Therefore, only genes which showed consistent regulation by all three fusion proteins were considered significant. This algorithm limited the number of identified genes to 52. Currently, no established single method that allows easy identification of all truly regulated genes from microarray analyses is available (6). The combination of different algorithms as used in our study is very likely to increase the percentage of biologically relevant genes.

The number of repressed genes ($n = 38$) in our experiments was more than twice the number of induced genes ($n = 14$). Since AML1-ETO, PML-RARα, and PLZF-RARα act primarily as transcriptional repressors on most of their target gene promoters, the larger number of repressed genes could be expected.

So far, most studies have focused on identification of target genes of a single aberration. For example, AML1-ETO specifically represses the $p14^{ARF}$ tumor suppressor (25). Therefore, it is not unexpected that most of the genes identified in our study have not previously been regarded as target genes for AML fusion proteins.

Among the genes that were repressed by the fusion proteins, 29% were induced at least twofold by TSA in the fusion protein-expressing U937 cell lines and more than 30% could be induced by TSA in AML1-ETO-containing Kasumi leukemia cells. Some of these genes act as differentiation-associated transcription factors (e.g., Id2 and MEF) (7, 18, 20, 33). The functional relevance of these genes needs to be studied further.

Several of the induced genes regulate effects downstream of Wnt signaling (Table 1). Regulation of the protein levels of catenins is central to the activation of this pathway. While β-catenin levels are predominantly regulated by the protein degradation machinery, this mechanism is less effective for plakoglobin (γ-catenin) (49). This is in accordance with our data that plakoglobin was induced by all three fusion proteins on the mRNA as well as on the protein level. Importantly, plakoglobin was also expressed at significantly higher levels in fusion protein-positive AML patient samples compared to AML patient samples that did not express a fusion protein. Thus, our a priori hypothesis, derived from microarray experiments in a cell culture system, was independently confirmed with primary AML patient samples in vivo.

The protein level of β-catenin was also increased in cells expressing one of the fusion proteins. However, β-catenin was not regulated on the mRNA level (data not shown). While we cannot exclude that the fusion proteins have direct effects on β-catenin stability, the increased levels of β-catenin protein may well be a direct consequence of the transcriptional regulation of plakoglobin. Other groups have shown several mechanisms that mediate induction of β-catenin by plakoglobin. Overexpression of plakoglobin results in the accumulation of endogenous β-catenin in the nucleus (31, 51). This localization within the nucleus could reduce the accessibility of plakoglobin to the cytosolic degradation machinery. In addition, plakoglobin interacts with the APC protein and thereby possibly elevates the level of β-catenin by interfering with its degradation (31).

Taken together, our data indicate that, in addition to the direct influence of plakoglobin on TCF- and LEF-dependent promoters, plakoglobin-induced stabilization of the β-catenin protein may contribute to the observed induction of Wnt target genes in hematopoietic cells.

Further analyses deciphered the mechanisms leading to plakoglobin induction. AML fusion proteins activate the plakoglobin promoter through an indirect mechanism which is dependent on the corepressor and histone deacetylase recruiting mechanisms. The direct effector protein is not known yet. Site-directed mutagenesis of a CCAAT site in the plakoglobin promoter did not alter promoter activation by AML1-ETO (data not shown).

Plakoglobin is a structural protein in the organization of cell-cell contacts, but it also plays a role in the Wnt signaling pathway (60). In this context, plakoglobin functions as a coactivator of the TCF and LEF transcription factors. The Wnt signaling pathway plays a crucial role in cell fate and differentiation decisions in a variety of organs (8, 23). In addition, downstream signaling through the TCF and LEF transcription factors induces proliferation and cellular transformation (43). Wnt signaling pathway alterations are a major factor in the pathogenesis of several types of cancer, with colon cancer being the best-studied example.

The importance of this pathway in the pathogenesis of leukemia was largely unknown. Wnt proteins are able to determine the cell fate of hematopoietic progenitors, and expression of several members of the Wnt family led to an expansion of multilineage progenitor cells (2, 4). While this article was under consideration, it was reported that Wnt3A plays an important role in self-renewal of hematopoietic stem cells (46). This is an intriguing observation, given that AML fusion proteins are presumed to function partially by enhancing self-renewal (39). It is possible that the self-renewal capacity conferred by AML fusion proteins is mediated by Wnt signaling pathway activation.

This is the first report to demonstrate that plakoglobin and TCF and LEF factors have a role in leukemia. Interestingly, several additional recent findings provide hints that hematopoiesis and leukemia might be more closely associated with the Wnt signaling pathway than was previously anticipated. For example, Wnt signaling prevents adipocyte differentiation by inhibiting C/EBPα (47). C/EBPα is a major inducer of granulopoiesis and is frequently mutated in the FAB M2 subtype of AML (42, 45, 52, 59). Also, ATRA was shown to inhibit β-catenin-TCF and β-catenin-LEF signaling, and β-catenin interacted with PML (9, 50).

In the current study, we provide evidence that AML1-ETO and PML-RARα induce TCF and LEF transcription factor-dependent activity. In Western blots, plakoglobin appeared as a double band, possibly due to posttranslational modification. Plakoglobin induction was associated with the formation of plakoglobin/LEF-1 and β-catenin/LEF-1 complexes in vivo. Subsequently, we demonstrated plakoglobin binding to the c-*myc* promoter, c-*myc* promoter activity, and increased c-*myc* expression. Plakoglobin preferentially induces c-*myc* compared to other Wnt target genes (22). For the first time, we demonstrated plakoglobin induction by AML fusion proteins in hematopoietic cells. Plakoglobin in turn activates Wnt signaling and leads to expression of functionally relevant target genes. While expression of c-*myc* and cyclin D1 was clearly induced by

the fusion proteins, these genes were not identified in the microarray analyses, which is likely to be based on the strict selection criteria in the microarray analyses. This strict regulation decreased the number of false-positives but also implied that several genuine target genes were not detected. With regard to c-*myc*, the mechanisms that lead to its induction by AML fusion proteins are indirect. This provides a possible explanation for why Myc was not found to be induced in the microarray analyses. The c-Myc oncoprotein appears to be regulated by AML1-ETO in several ways. In addition to our findings, it has been described that c-*myc* is induced by AML1-ETO via C/EBPα inhibition (19).

The functional relevance of Wnt signaling induced by AML fusion proteins via plakoglobin induction was verified in vitro and in vivo. In primary hematopoietic cells, retroviral transduction of plakoglobin retained the immature phenotype of the progenitor cells. The 32D cell line showed increased colony formation potential and proliferative activity upon plakoglobin transfection. Interestingly, plakoglobin enhanced the leukemogenic potential of 32D cells in syngeneic mice. Injection of plakoglobin-expressing 32D cells caused rapid death in almost all mice, and their organs were diffusely infiltrated by blast-like hematopoietic cells. The latency period of leukemia development in the 32D-plakoglobin-injected mice was comparable to the time it took for transforming FLT3 internal tandem duplication mutations in our previous experiments to lead to leukemia-related death (34). The bone marrow-infiltrating cells grew in culture like to the initial 32D-plakoglobin cells and still depended on interleukin-3 (data not shown). Thus, no secondary mutations appeared to be necessary for leukemia-like growth of 32D-plakoglobin cells in vivo. The leukemias induced by AML1-ETO in a mouse model also grew factor dependently in vitro (17). Thus, plakoglobin effects in 32D cells are in accordance with AML1-ETO effects in the mouse model. While some of the mice injected with the 32D control cells also died, their deaths occurred much later and the organs were less heavily infiltrated.

Taken together, our analyses identified shared targets of fusion proteins derived from the most frequent translocations in AML. PML-RARα, PLZF-RARα, and AML1-ETO induced plakoglobin expression in cell lines as well as in primary patient samples. Induction of plakoglobin expression resulted in transcriptional activation of the TCF and LEF transcription factors. The TCF and LEF target genes, cyclin D1 and c-*myc*, which are well known oncogenes, are induced by the fusion proteins, and typical functional consequences of oncogenic Wnt signaling were observed. These data provide evidence that AML-associated fusion proteins contribute to leukemogenesis by specifically targeting the Wnt signaling pathway.

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