Chromatin modifications induced by the AML1-ETO fusion protein reversibly silence its genomic targets through AML1 and Sp1 binding motifs

A Maiques-Diaz[1], FS Chou[2], M Wunderlich[2], G Gómez-López[3], FV Jacinto[4], S Rodriguez-Perales[1], MJ Larrayoz[5], MJ Calasanz[5], JC Mulloy[2], JC Cigudosa[1] and S Alvarez[1]

The AML1-ETO fusion protein, which is present in 10–15% of cases of acute myeloid leukemia, is known to repress myeloid differentiation genes through DNA binding and recruitment of chromatin-modifying proteins and transcription factors in target genes. ChIP-chip analysis of human hematopoietic stem/progenitor cells transduced with the AML1-ETO fusion gene enabled us to identify 1168 AML1-ETO target genes, 103 of which were co-occupied by histone deacetylase 1 (HDAC1) and had lost the hyperacetylation mark at histone H4, and 264 showed a K9 trimethylation at histone H3. Enrichment of genes involved in hematopoietic differentiation and in specific signaling pathways was observed in the presence of these epigenetic modifications associated with an 'inactive' chromatin status. Furthermore, AML1-ETO target genes had a significant correlation between the chromatin marks studied and transcriptional silencing. Interestingly, AML1 binding sites were absent on a large number of selected AML1-ETO promoters and an Sp1 binding site was found in over 50% of them. Reversible silencing induced by the fusion protein in the presence of AML1 and/or Sp1 transcription factor binding site was confirmed. Therefore, this study provides a global analysis of AML1-ETO functional chromatin modifications and identifies the important role of Sp1 in the DNA binding pattern of AML1-ETO, suggesting a role for Sp1-targeted therapy in this leukemia subtype.

Keywords: AML1-ETO; myeloid leukemia ; histone deacetylation; H3K9me3; Sp1

INTRODUCTION

The presence of the AML1-ETO fusion protein indicates acute myeloid leukemia (AML) with t(8;21)(q22;q22), which accounts for ~10–15% of all AML cases.[1,2] This product is the consequence of a reciprocal translocation that fuses the DNA binding domain of the AML1 transcription factor essential for transcriptional activation of genes involved in normal hematopoiesis in frame with nearly the entire ETO protein, a transcriptional repressor molecule expressed in a variety of tissues.[3,4]

In murine models, full-length AML1-ETO alone is not sufficient to induce AML, unless secondary mutations take place.[5,6] Nevertheless, as is the case in murine models, AML1-ETO expression in CD34+ human hematopoietic stem cells promotes self-renewal and interferes with efficient myeloid and erythroid differentiation.[7–9]

AML1-ETO was originally characterized as a transcriptional repressor, recruiting NCoR/SMRT Class I histone deacetylase (HDAC) complexes through its ETO moiety to DNA sequences containing AML1 binding sites and thereby interfering with AML1 function.[4,10–14] However, AML1-ETO has been shown to interact with a number of other transcription factors (for example, E proteins and ETS proteins), hypothesizing to target DNA through other motifs, such as E-Box motifs, as the result of these physical interactions.[2,15–17]

Aberrant gene expression patterns observed in AML with balanced chromosomal translocations are increasingly attributed to the effect of leukemia-specific fusion proteins on the profile of epigenetic modifications of malignant cells.[18] Histone H4 deacetylation (H4Ac) and K9 trimethylation of histone H3 (H3K9me3) are functionally validated chromatin marks already described as direct repressive effects of AML1-ETO on specific locus.[15] H4Ac is catalyzed, among others, by HDAC1 enzyme that reverses lysine acetylation and restores the positive charge of the lysine, stabilizing the local chromatin architecture and shutting down active loci.[19] H3K9me3 is catalyzed by SET domain proteins, as lysine methyltransferase enzyme suppressor of variegation 3–9 homolog 1 (SUV39H1), leading to the formation of high-affinity binding site for heterochromatin protein 1.[20–22] This chromatin-modifying enzyme has been previously shown to interact with AML1[(ref. 22)] and PML/RARA[23] to specific target promoters and to induce H3K9me3 that correlates with transcriptional silencing.

The above observations underscore the significant complexity of the AML1-ETO function as a transcriptional repressor. Although previous studies have shown the genomic DNA binding of AML1-ETO,[14] to date no global analysis of the AML1-ETO functional repressive chromatin modifications have been performed. To achieve this objective, we had used the unique model of

[1]Molecular Cytogenetics Group, Human Cancer Genetics programme, Centro Nacional Investigaciones Oncologicas (CNIO), Centro de Investigaciones de Enfermedades Raras (CIBERER), Madrid, Spain; [2]Division of Experimental Hematology and Cancer Biology, Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA; [3]Bioinformatics Unit, Structural Biology and Biocomputing Programme, Centro Nacional Investigaciones Oncólogicas (CNIO), Madrid, Spain; [4]Chromatin and Disease Group, Cancer Epigenetics and Biology Program (PEBC), Bellvitge Biomedical Research Institute (IDIBELL), Barcelona, Spain and [5]Department of Genetics, University of Navarra, Pamplona, Spain. Correspondence: Dr S Alvarez, Molecular Cytogenetics Group, Human Cancer Genetics Program, Centro Nacional de Investigaciones Oncológicas (CNIO), C/Melchor Fernández Almagro 3, 28029 Madrid, Spain.
E-mail: salvarez@cnio.es

human stem/progenitor hematopoietic cells (HSPCs) retrovirally transduced with the AML1-ETO fusion protein[7] to perform a comprehensive analysis of the AML1-ETO DNA binding map at the promoter regions and its association with the repressive chromatin features of deacetylation of histone H4 and increase of H3K9me3. We observed these epigenetic events as important mechanisms through which AML1-ETO modifies the transcriptional pattern of the HSPCs. Furthermore, our findings were found maintained on primary samples and were validated to be direct effects of AML1-ETO binding that could be reverted upon AML1-ETO knockout. The interaction with the Sp1 transcription factor, previously described to interact with AML1-ETO,[24] was identified as a crucial element driving the AML1-ETO DNA binding pattern and thus supporting the role of Sp1-targeted therapy.

MATERIALS AND METHODS

Cell cultures, plasmids and patient samples

HSPCs from human CD34+ umbilical cord blood samples stably transduced with retroviruses expressing the HA-tagged AML1-ETO fusion protein (HSPC-AE) have been studied in depth elsewhere.[7,25] Eight HSPC samples stably transduced with conditional AML1-ETO knockout retroviruses and with control vectors were analyzed after 9 weeks in culture (for construct information, see Supplementary Figure S1). RNA from HSPC-AE cells treated with 150 ng/ml of mithramycin A (M681, Sigma-Aldrich, St Louis, MO, USA) for 24 h was also obtained. RNA from 15 *de-novo* AML t(8;21)(q22;q22) samples was collected from the leftover material after a diagnosis at the cytogenetic laboratories of the Universidad de Navarra (Pamplona, Spain). Informed consent was obtained in accordance with the Declaration of Helsinki (October 2008). The control samples comprised six CD34+ selections from normal umbilical cord blood.

Antibodies

Immunopurified anti-HA (clone H9658 Monoclonal Antibodies Unit, CNIO, Madrid, Spain) was used against HA-AML1-ETO. Commercially available antibodies against HDAC1 (PA1-860; Affinity BioReagents, Rockford, IL, USA), AcetylH4 (06-598; Millipore, Billerica, MA, USA), H3K9me3 (ab8868; Abcam, Cambridge, UK), Sp1 (17-601; Millipore) and SUV39H1 (ab12405; Abcam) were used for ChIP assays.

ChIP-chip

ChIP assays were performed as described elsewhere.[26] qPCR of $p14^{ARF}$ promoter was used to validate the efficiency of each ChIP.[27] Three biological replicates of 10 million cells per ChIP experiment were prepared and hybridized on the same array set. ChIP products were amplified using the GenomePlex Complete Whole Genome Amplification Kit (Sigma, St Louis, MO, USA) and purified using the UltraClean GelSpin DNA Purification Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). Immunoenriched DNA and whole-cell extract DNA were labeled using the Bioprime DNA-Labeling System protocol (Life Technologies, Carlsbad, CA, USA) with random priming and Cy5/Cy3 fluorescent dyes, respectively. One microgram of each labeled DNA was mixed and hybridized in Human Proximal Promoter ChIP-chip 244k arrays (Agilent Technologies, Palo Alto, CA, USA) for up to 40 h at 65 °C at 20 r.p.m. The arrays were then washed and scanned using the Agilent scanner, and the images obtained were processed using the Agilent Feature Extractor software application (v.9.5.3.1). The microarray data have been deposited in GEO (http://www.ncbi.nlm.nih.gov/geo/) and can be retrieved as a Data Series with accession number GSE27663.

Quantitative chromatin immunoprecipitation (qChIP)

ChIP products were obtained for the AML1-ETO protein using the ChampionChIP One-Day kit (SABiosciences Corporation, Frederick, MD, USA) following the manufacturer's protocol. For proteins such as Sp1, HDAC1 and SUV39H1 and the chromatin marks H4Ac and H3K9me3, ChIP was performed as previously described.[26] In all, 5 million of 9 weeks in culture HSPC-AE and HSPC controls were used per ChIP experiment. qPCR of ChIP products was performed on an ABI 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA) with SYBR Green (SABiosciences Corporation). To determine a reliable baseline for enrichment of AML1-ETO binding, we used the media of three negative controls (*ELA2, IRF2* and *DLEU2*) (taken from Gardini *et al.*[14]). Primers and conditions for each promoter were designed to validate regions occupied by AML1-ETO according to the bioinformatics analysis of Agilent ChIP Analytics 3.1 (see Supplementary Methods for oligo references). The percentage of immunoprecipitated DNA relative to input was calculated.

Data analysis

ChIP-chip data were analyzed using Agilent's ChIP Analytics 1.3 software package, incorporating the Whitehead Error Model. The significance threshold for a probe to be considered significant was set at a normalized log ratio >0.7 and $P(X) < 0.001$ (see Supplementary Methods for further information). The transcript or gene ID nearest to each probe was calculated using simple proximity heuristic and the human genome Hg17 (May 2004) as reference. For each ChIP-chip experiment (HA-AML1-ETO, HDAC1, H4Ac or H3K9me3), probes common to HSPC-AE and HSPC control were removed.

Gene lists were functionally annotated using Ingenuity Pathways Analysis software (Ingenuity Systems; http://www.ingenuity.com). Enrichment of genes associated with specific biological functions and canonical pathways was determined relative to the Ingenuity knowledge database, specifying the 244k human promoter platform used as reference. A significance level of *P*-value <0.05 calculated using Benjamin–Hoechberg multiple testing correction (B–H *P*-value) was used as cutoff.

Transcript profiling data from nine clones of 11 weeks in culture HSPC-AE and three HSPC controls samples (GSE8023)[25] were normalized and analyzed for differential expression using Bioconductor's limma package and GEPAS.[28] The significance threshold for differential expression was set at false discovery rate (FDR) >0.01. Gene Set Enrichment Analysis software[29] was used to determine the global effect on gene transcription of AML1-ETO, AML1-ETO/HDAC1 complex and the studied chromatin modifications. The gene list was collapsed to unique gene symbols using the default capabilities (FDR <0.05).

Quantitative real-time RT-PCR

Total RNA obtained with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) was reverse transcribed using random hexamer primers with the TaqMan Gold RT-PCR Kit (Applied Biosystems). The relative mRNA levels of the studied genes were assessed with the 7900HT Fast Real-Time PCR System and software (Applied Biosystems) with specific oligonucleotides and predeveloped TaqMan Assays (Assay-on-Demand; Applied Biosystems) following the manufacturer's protocol. The Calibrated Normalized Relative Quantity, taking into account target- and run-specific amplification efficiencies, was calculated using endogenous GAPDH expression with the Qbase software application.[30] For mithramycin A experiments, KIT (a known Sp1 target) expression was used as positive control.[31]

RESULTS

Identification of AML1-ETO target genes associated with chromatin silencing marks

For this purpose, three HSPC independent clones from human CD34+ umbilical cord blood samples stably expressing the AML1-ETO fusion protein were studied by ChIP-chip. This HSPC-AE model previously characterized[7,25] shows upon 5 weeks in culture a progressive and significant increase of CD34+ cells with an immature myeloid morphology, compared with the control cells which predominantly complete the terminal differentiation.

To identify those AML1-ETO target genes where histone deacetylation occurs and to determine the role of HDAC1 in that process, ChIP-chip experiments with antibodies against the HA-tagged fusion protein, HDAC1 and the chromatin mark H4Ac were performed. ChIP-chip data analysis enabled the identification of 1168 unique genes (Supplementary Table S1) and 6 miRNAs

Table 1. Summary of the results obtained in each ChIP-chip experiment

Samples studied[a] *(array sets analyzed)*[b]	*3 HSPC-AE (1)* *3 HSPC-CB (1)*	*3 HSPC-AE (1)* *3 HSPC-CB (1)*	*3 HSPC-AE (1)* *3 HSPC-CB (1)*	*3 HSPC-AE (1)* *3 HSPC-CB (1)*
Antibody against	HA tag	HDAC1	Histone 4 lysine 5, 8, 12 and 16 acetylation	Histone 3 trimethylated lysine 9
Aim	Analysis of AML1-ETO binding in human promoters	Correlation of AML1-ETO and HDAC1 binding in human promoters	Correlation between AML1-ETO-HDAC1 and the loss of the H4Ac active chromatin mark	Correlation between AML1-ETO and the presence of the H3K9me3 inactive chromatin mark
Significant peaks[c] specific to HSPC-AE	1525	3990	1468	3376
Unique genes identified	1168	1826	943	1202
Unique miRNAs identified	6	10	7	22
Unknown peaks	16	39	18	61

Abbreviations: AML, acute myeloid leukemia; ChIP, chromatin immunoprecipitation; HDAC1, histone deacetylase 1; HSPC, human stem progenitor cells; H3K9me3, K9 trimethylation of histone H3; H4Ac, histone H4 deacetylation.
[a]HSPCs transduced with the control vector (HSPC control) and with the retrovirus expressing the HA-tagged AML1-ETO were studied (HSPC-AE).
[b]ChIP samples were hybridized on an Agilent 244k Human Promoter Array set.
[c]See Data analysis in Materials and methods.

Table 2. List of the miRNAs identified in each independent ChIP-chip experiment

ChIP-chip	*miRNAs*	*Total number*
AML1-ETO	hsa-mir-124a-3, hsa-mir-193a, hsa-mir-203, hsa-mir-424, hsa-mir-607, hsa-mir-345	6
HDAC1	hsa-let-7i, hsa-mir-137, hsa-mir-15b, hsa-mir-17, hsa-mir-193b, hsa-mir-23a, hsa-mir-301, hsa-mir-594, hsa-mir-7-1, hsa-mir-7-2	10
H4Ac	hsa-mir-497, hsa-mir-659, hsa-mir-142, hsa-mir-193a, hsa-mir-594, hsa-mir-642, hsa-mir-99b	7
H3K9me3	hsa-mir-124a-3, hsa-mir-124a-2; hsa-mir-137, hsa-mir-148a, hsa-mir-149, hsa-mir-212, hsa-mir-335, hsa-mir-339, hsa-mir-340, hsa-mir-424, hsa-mir-498, hsa-mir-512-1, hsa-mir-512-2, hsa-mir-515-1, hsa-mir-517a, hsa-mir-519d, hsa-mir-520a, hsa-mir-520e, hsa-mir-526b, hsa-mir-594, hsa-mir-615, hsa-mir-638	22

Abbreviations: AML, acute myeloid leukemia; ChIP, chromatin immunoprecipitation; HDAC1, histone deacetylase 1; H3K9me3, K9 trimethylation of histone H3; H4Ac, histone H4 deacetylation.

bound by AML1-ETO, and 1826 genes and 10 miRNAs by HDAC1 protein ($P(X) < 0.001$; Normalized Log Ratio > 0.7) (Tables 1 and 2). Interestingly, only 10% of the 1168 identified AML1-ETO target showed a significant increase on H4Ac compared with the HSPC control cells, indicating the presence of a deacetylation process at the majority of the AML1-ETO binding sites (Figure 1a). However, only 103 AML1-ETO deacetylated target genes were co-occupied by HDAC1 (Figure 1b). Among the 103 genes co-occupied by both AML1-ETO and HDAC1, 75% showed a distance of < 1200 bp between AML1-ETO and HDAC1 probes. Thus, these 103 genes could be considered as AML1-ETO/HDAC1 complex functional target genes (Supplementary Table S3). As the majority of the deacetylation events at AML1-ETO binding sites did not involve HDAC1 in our model, further studies are needed to determine the combinatory effect of other HDAC class 1 enzymes on the generation of this repressive chromatin landscape.

To explore the histone H3 lysine9 methylation, another previously known AML1-ETO-related silencing chromatin mark,[32] ChIP-chip analysis of HSPC-AE samples was performed. An enrichment of the chromatin mark H3K9me3 on 1202 promoter regions compared with the HSPC control (Table 1) was observed. The simultaneous presence of H3K9me3 and AML1-ETO occupancy was observed for 2 miRNAs and 264 unique genes, with 91.7% of the probes located closer than 1200 bp (Figure 1c; Table 2; Supplementary Table S5). Only 22 of these 264 AML1-ETO-H3K9me3 target genes were coincident with the 103 AML1-ETO/HDAC1 functional target genes, suggesting that the simultaneous presence of H3K9me3 and deacetylated histone H4 is an exclusive repressive mechanism.

Enrichment of specific biological functions and pathways among the AML1-ETO epigenetically repressed target genes

The functional classification of the AML1-ETO/HDAC1 and the AML1-ETO-H3K9me3 identified target genes revealed an enrichment of categories like tumorigenesis, differentiation, gene expression, and hematological system function and development (B–H *P*-value < 0.05) according to Ingenuity Systems Analysis (Ingenuity Systems; http://www.ingenuity.com) (Figure 1d; Supplementary Tables S2a, S4a and S6a). Furthermore, among the 103 targets of the AML1-ETO/HDAC1 complex the TGF-β pathway (*MAP3K7, MAPK1, RUNX3* and *SMAD7*) and ERK/MAPK pathway (*ELK3, PPP1R12A* and *YWHANG*) were found enriched (Figure 1d; Supplementary Table S4b); while the AML1-ETO-H3K9me3 associate with genes within the canonical Wnt/β-catenin pathway (*AXIN2, GSK3A, PPARD, SOX18, SOX7* and *WNT9A*) and canonical stem cell pluripotency pathway (*BMP2, FGFR1 GNAS, SMAD7* and *WNT2B*) (Figure 1d; Supplementary Table S6b). In light of these data, we conclude that the presence of the H3K9me3 or deacetylated histone H4 at the AML1-ETO target genes is enriched on different biological pathways but targeting the same functional categories.

AML1-ETO induced inactive chromatin state involves key target genes

Hematopoietic differentiation blockage was the main feature observed on the functional characterization of the HSPC-AE model[7] and concordantly, this function was enriched among the AML1-ETO epigenetically repressed genes (Figure 1d). Thus, seven selected genes identified as AML1-ETO/HDAC1 functional targets and involved in cell differentiation (*CTCF, MAPK1, SIRT1, YES1, AML1, MLLT3* and *RPS19*) were analyzed using qChIP. In all the genes studied, the HSPC-AE samples were found significantly enriched for the co-occupancy of AML1-ETO and HDAC1 with a loss of H4 hyperacetylation compared with the HSPC controls

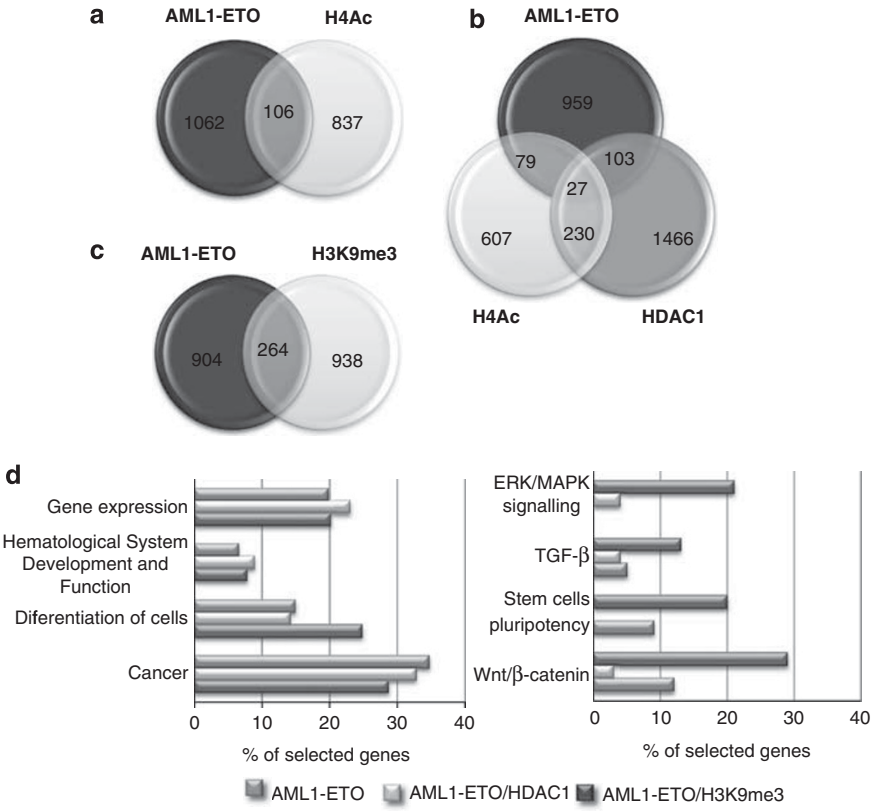

Figure 1. Identification and functional characterization of AML1-ETO target genes in the presence of silencing chromatin marks. (**a**, **c**) Venn diagrams of the overlap of target genes identified by ChIP-chip analysis using anti-AML1-ETO fusion protein (tagged with HA), anti-HDAC1, anti-histone H4 acetylation (H4Ac) and anti-H3K9me3 chromatin mark antibodies. (**b**) Significant biological functions and (**d**) canonical pathways represented by the AML1-ETO target genes, AML1-ETO targets in the presence of HDAC1 and H4 deacetylation, and AML1-ETO in the presence of the H3K9me3 chromatin mark.

(Supplementary Figure S2). These results further confirm the simultaneous presence of both proteins at the same genomic location, even for those genes where the ChIP-chip analysis revealed a distance between the fusion protein and the HDAC1 >800 bp (Supplementary Table S3). Interestingly, no differences on the occupancy of HDAC1 at *MLLT3* promoter were observed between HSPC-AE and HSPC controls, suggesting an independency between HDAC1 and AML1-ETO binding at this target gene.

The qChIP analysis at the promoter of five selected genes (*MLLT3*, *RPS19*, *GSK3*, *SOX18* and *OCLN*) among the list of AML1-ETO-H3K9me3 targets confirmed the significant enrichment of H3K9me3 in HSPC-AE cells compared with HSPC control, but no significant differences were observed for the co-occupancy by SUV39H1 in the identified AML1-ETO-H3K9me3 target genes (Supplementary Figure S3). Therefore, our results identified an enrichment of the H3K9me3 in the presence of the fusion protein at target loci, but no conclusion about the methyltransferase activity linked to AML1-ETO could be made. Further studies are needed to identify the effect and proteins involved in the H3K9 trimethyl transferase activity associated with AML1-ETO on leukemia cells.

DNA binding sequence analysis of functional AML1-ETO targets

The AML1-ETO DNA bounded regions were found equally distributed along the 5.5 kb upstream and the 2.5 kb downstream of the transcription start site. AML1-ETO binds DNA through the AML1 RUNT domain, mainly at AML1 transcription factor binding sites (TFBSs), although association with other motifs (for example, E-box motifs) has been previously described.[16,14] In our study, an AML1 TFBS was identified at $>30\%$ of AML1-ETO functional targets (Supplementary Figure S4; Supplementary Table S1), suggesting that the AML1-ETO DNA association may rely on additional transcriptional complexes with different sequence specificities.

Detailed sequence analyses using unsupervised methods (Supplementary Tables S7 and S8) identify an enrichment of Sp1 TFBS among AML1-ETO target genes. The supervised analysis of Sp1 TFBS, in a region covering $+2$ kb to -2 kb of the transcription start site using the oPPOSUM motif search system revealed that $>50\%$ of the AML1-ETO target genes present this binding motive (Fisher's score $=3.59E{-}13$). This finding is of particular interest, since Sp1 is known to interact with AML1-ETO through the RUNT domain and is an essential transcription factor involved in hematopoietic differentiation.[24,33]

The frequency of AML1, Sp1 or combined AML1 $+$ Sp1 TFBS was analyzed in the AML1-ETO, AML1-ETO/HDAC1/H4Ac-neg, AML1-ETO/H3K9me3, AML1-ETO/H4Ac-Neg and AML1-ETO/AcH4 gene sets. This analysis revealed a discrete enrichment of 1.5 times of AML1 TFBS in the AML1-ETO/HDAC1 gene set (Supplementary Table S9), supporting previous studies that indicate an enrichment of AML1 TFBS in the promoters of downregulated genes.[34] Furthermore, three times less Sp1 TFBS was found in the AML1-ETO/AcH4 gene set (Supplementary Table S9), suggesting an inverse association between this chromatin mark and Sp1 consensus motif. This fact points to an involvement of Sp1 transcription factor only in the repressive role of AML1/ETO.

The functional classification of the 625 AML1-ETO target genes in which an Sp1 motif has been identify revealed an enrichment of categories like development and gene expression (B–H *P*-value

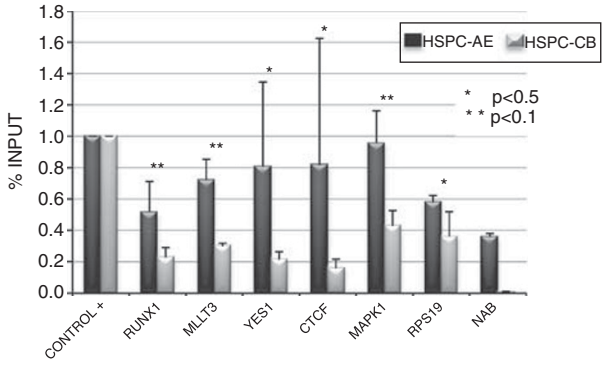

Figure 2. Sp1 binding is enriched at AML1-ETO target genes on the HSPC-AE cells. qChIP was performed using an anti-Sp1 transcription factor antibody on two HSPC-AE and two HSPC control cells. The *DHFR* gene was used as a positive control. Standard deviation is represented by the error bars.

Table 3. Statistical significance of the gene set enrichment analysis

Gene set list	*No. of genes*	*FDR*
AML1-ETO target genes	1168	0.001
AML1-ETO/HDAC1/H4Ac-neg	103	0.0001
AML1-ETO/H4Ac-neg	1063	0.0001
AML1-ETO/H3K9me3	264	0.01
AML1-ETO/H4Ac-neg/H3K9me3	222	0.005

Abbreviations: AML, acute myeloid leukemia; FDR, false discovery rate; H3K9me3, K9 trimethylation of histone H3; H4Ac, histone H4 deacetylation.

<0.05) according to Ingenuity Systems Analysis (Ingenuity Systems; http://www.ingenuity.com). Furthermore, the canonical Wnt/β-catenin pathway (*AXIN2, CCND1, CDH3* and *FZD7)* and canonical stem cell pluripotency pathway (BMP2, FGFR1 and WNT2B) were found enriched (Supplementary Tables S10a and b).

A significant enrichment of Sp1 presence in all the promoters studied was confirmed in the HSPC-AE samples compared with HSPC controls (Figure 2). The fold increase assessed in this assay was independent of the presence of an AML1 or a non-AML1 TFBS on the studied target. This fact further supports previous data indicating that AML1-ETO and Sp1 are recruited to promoters as a complex.[31] Furthermore, our study indicates that this DNA binding mechanism occurs in a genome-wide manner.

AML1-ETO occupied targets with inactive chromatin marks are transcriptionally repressed

To confirm the transcriptional repression conferred by the studied inactive chromatin marks, a Gene Set Enrichment Analysis[29] was performed. The HSPC-AE expression signature was cross-compared with the AML1-ETO targets with associated chromatin modifications. As expected, a significant enrichment of downregulated genes was confirmed among the 1068 AML1-ETO target genes (FDR <0.001). Interestingly, those deacetylated genes, with and without HDAC1 binding, showed the most significant correlation with repressed genes (FDR <0.0001) pointing to H4 deacetylation as the most significant epigenetic mark induced by AML1-ETO binding (Table 3; Supplementary Figure S5).

Individual analysis confirmed a significant downregulation ($P<0.0001$) of six out of seven selected candidate genes previously studied for AML1-ETO, HDAC1 and Sp1 occupancy in HSPC-AE samples (Figure 3a). No differences were observed with regard to the presence of an AML1 or a non-AML1 TFBS. Consistent with our qChIP result, no differences were found in *MLLT3* mRNA levels, as a similar occupancy level of HDAC1 protein was observed in HSCP-AE and control cells (Figure 3a; Supplementary Figure S2).

Finally, we analyzed the expression levels of AML1-ETO targets relative to the studied chromatin marks and the presence of an Sp1 or AML1 TFBS. No significant differences were found associated with the presence of the different TFBS or the studied chromatin marks. However, we identify a significant increase on the median expression levels of those AML1-ETO targets with an H4Ac chromatin mark compared with the AML1-ETO total targets (Supplementary Figure S7). This supports previous studies showing that AML1-ETO could also mediate the upregulation of some of its targets.[35]

AML1-ETO transcriptional repression is restored upon AML1-ETO knockout and Sp1 inhibition

To further characterize the importance of AML1-ETO direct epigenetic repressive effect on the survival and proliferation of the HSPC-AE cells, a Cre-loxP AML1-ETO system was generated. HSPC cells were transduced with AML1-ETO constructs flanked by two loxP sites and a Cre-recombinase expression vector (for construct information, see Supplementary Figure S1). AML1-ETO mRNA levels were found absent after 48 h (Supplementary Figure S1), but no effect on HSPC proliferation was observed until day 10 (Chou *et al*., manuscript under review). Thus, we examine the expression levels of the AML1-ETO target genes at days 4, 6 and 8. AML1-ETO knockout significantly restored the expression of the *SIRT1, AML1, CTCF, RPS19, YES1*, GSK3A and *MAPK1* genes at day 8, but no effect was observed in the *MLLT3* gene (Figure 4). The absence of *MLLT3* reactivation further confirms the previous observation of an independency of HDAC1 and AML1-ETO DNA binding at that promoter on the HSPC-AE model (Supplementary Figure S2). The transcriptional activation observed on the genes studied further support the direct and reversible repressive epigenetic effect mediated by AML1-ETO DNA binding, on the presence of an AML1 or non-AML1 TFBS.

The role of Sp1 in AML1-ETO target gene repression was further elucidated by treating HSPC-AE clones with mithramycin A, a previously reported Sp1 inhibitor.[31] Mithramycin A exposure led to a decrease in mRNA levels only on those target genes with Sp1 and non-AML1 TFBS at their promoters (that is, *CTCF* and *SIRT1*) but showed no effect on those genes with Sp1 and AML1 TFBS (that is, *AML1* and *YES1*; Figure 5), supporting our hypothesis of Sp1 driving AML1-ETO binding to genes where Sp1 but not AML1 TFBS is present.

AML1-ETO direct transcriptional repression in AML patients

The expression status of these selected genes was analyzed in a set of 15 AML primary samples expressing the AML1-ETO oncoprotein. All the studied genes were found downregulated on the primary samples compared with the controls (Figure 3c), supporting the relevance of AML1-ETO epigenetic silencing of key genes on the leukemic process. However, different levels of significance were observed between the studied genes.

All the genes containing an AML1 binding site (that is, *AML1, YES1* and *MLLT3*) were found as the most significantly downregulated ($P<0.005$) and a large variability between patients was observed for the identified target genes without an AML1 TFBS (Figures 3b and 4). For genes containing an Sp1 TFBS (*CTCF* and *SIRT1*), only a moderate decrease on the mRNA levels was observed. We confirmed that this difference between the HSPC model and AML patients was not related to differences in Sp1 abundance (Supplementary Figure S6). Further studies are needed to better characterize if the variability on transcriptional repression induced by this fusion protein is relative to different mechanisms of AML1-ETO DNA binding, the levels of AML1-ETO transcripts or the presence of secondary mutations.

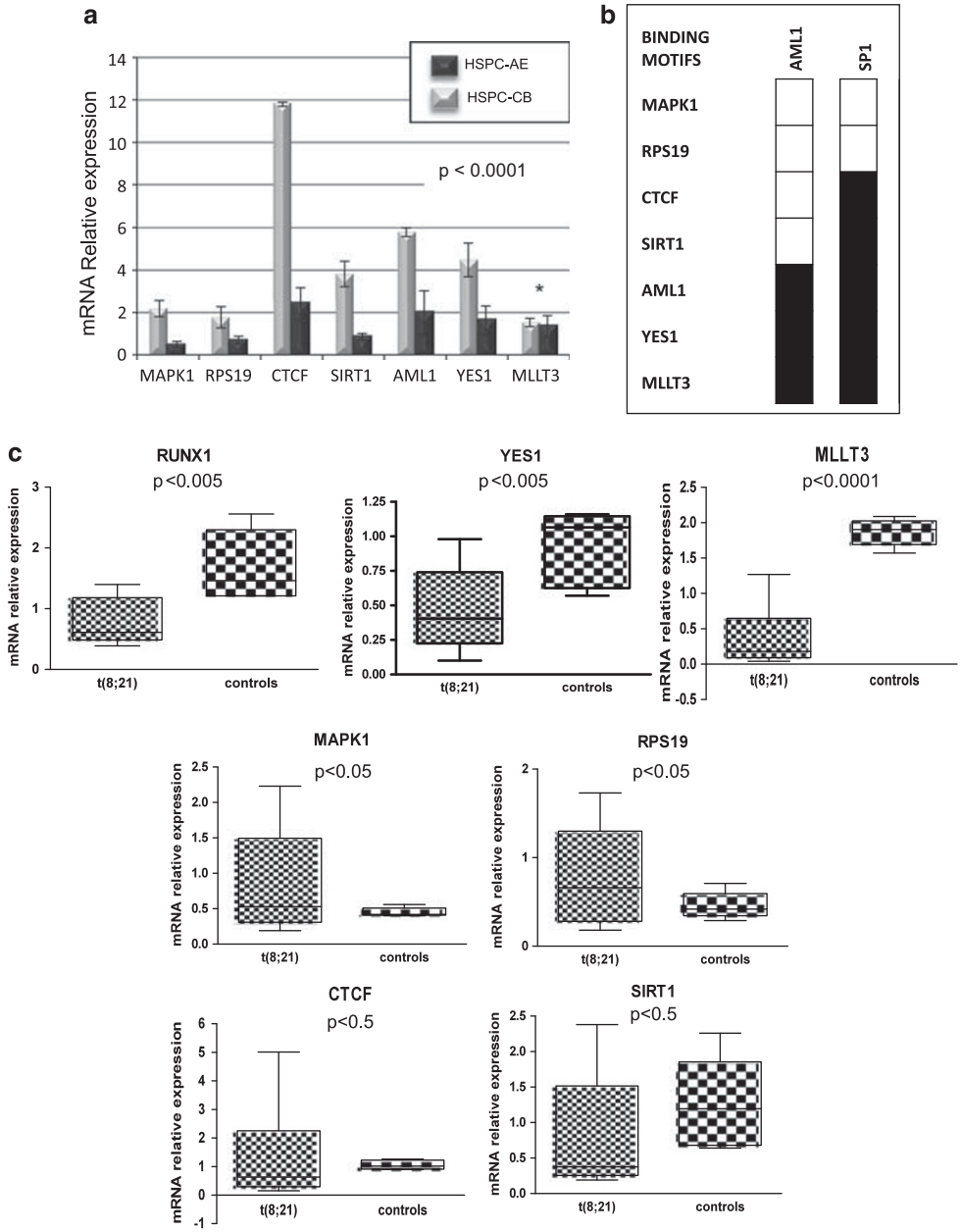

Figure 3. *In-vivo* transcriptional analysis of AML1-ETO targets. (**a**) Analysis of the expression level of seven AML1-ETO/HDAC1 target genes on HSPC samples (*P = n.s.). (**b**) Schematic representation of the AML1 and/or Sp1 TFBS present in the seven selected genes. (**c**) Box plot representing the expression levels of the selected genes in 15 t(8;21) AML primary samples and CD34+ cells (controls).

DISCUSSION

Gene expression profiling studies have revealed the presence of unique gene expression signatures associated with balanced AML chromosomal translocations.[25] Regulation of gene expression involves multilayered mechanisms, in which epigenetic modifications such as DNA methylation and histone tail modifications have a major role.[36] We recently showed that the AML1-ETO fusion protein was not sufficient to induce the specific DNA methylation pattern observed in primary patient samples.[37] The AML1-ETO fusion protein is known to repress transcription by recruiting chromatin modifiers such as the NCoR/SMRT/HDAC complex,[10] thus leading to histone H4 deacetylation and recruitment of the SUV39H1 protein, a histone H3 lysine 9 methyltransferase at DNA binding sites.[38]

Using ChIP-chip, we identified 1168 AML1-ETO target genes involved in functions such as hematopoietic differentiation and self-renewal, the main features reported in the HSPC-AE.[7] Furthermore, we observed that H4 deacetylation is the main consequence of AML1-ETO binding and identified a set of 103 genes functional targets of the AML1-ETO/HDAC1 complex. The functional importance of this chromatin mark was revealed by the involvement of the modified targets on the TGF-β pathway, which were previously reported to be inhibited by AML1-ETO fusion protein,[39] or of ERK/MAPK, a key pathway in the self-renewal process of hematopoietic stem cells.[40] Among the identified genes, we found novel AML1-ETO target genes previously described to be involved on the hematopoietic development, which repression could be a relevant step in the AML

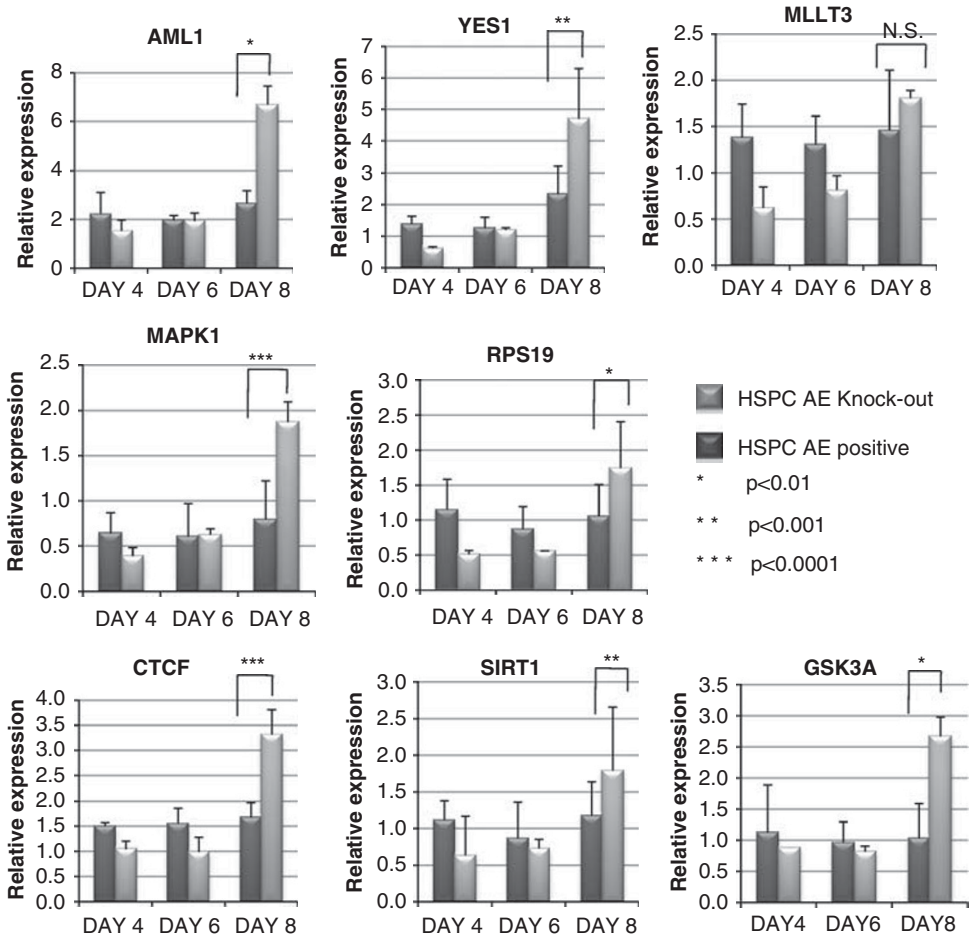

Figure 4. Knockout of AML1-ETO restores the expression of its target genes. qRT-PCR analysis shows increased expression levels in six of the seven genes studied upon AML1-ETO knockout. Measurements of two independent AML1-ETO-ko clones were taken at three different time points (4, 6 and 8 days in culture). The constructs used as HSPC-AE-positive and HSPC-AE-knockout are further described in Supplementary Figure S1.

development induced by t(8;21). Such as *MLLT3,* a positive regulator of the erythroid and megakaryocytic cell fate decision;[41] *RPS19*, a ribosomal protein which silencing decrease the proliferative capacity of hematopoietic progenitors and leads to a defect on erythroid development;[42] *SIRT1*, downregulated during neutrophil differentiation of acute promyelocytic leukemia cells[43] and *CTCF* which knockdown inhibited differentiation into erythroid lineage of the K562 cell line.[44]

The 264 genes identified as AML1-ETO targets with an increased H3K9me3 were found to be significantly involved in the Wnt/β-catenin and stem cell pluripotency pathways. Interestingly, we and others have previously identified aberrant DNA methylation of Wnt antagonists as being responsible for the activation of the Wnt/β-catenin pathway required for the development of leukemic stem cells and for the maintenance of self-renewal on AML1-ETO patients.[37] This study reveals another level of complexity identifying the H3K9me3 modification as an initial event targeting this pathway (*TCF3, SOX18* or *FZD7*) in the HSPC-AE model. SUV39H1 is the only member of the mammalian H3K9 histone methyltransferases known to interact with AML1 and DNA methyltransferase 1.[45] However, the interaction of SUV39H1 and AML1-ETO remains unclear and we had not found an enrichment of SUV39H1 binding on the AML1-ETO studied targets. Therefore, further studies are needed to determine the mechanism underlying the observed increase of H3K9me3 and its relation with the unique DNA aberrant methylation profile in primary AML1-ETO samples.

Although the presence of both the chromatin modifications studied has been reported at the c-FMS locus associated with AML1-ETO binding,[15] we found that the simultaneous presence of H3K9me3 and histone H4 deacetylation is not a genome-wide event, indicating that these two events are mainly independent. Furthermore, cross-comparison between ChIP-chip data and expression signature confirmed a significant correlation between gene silencing and the histone modifications in AML1-ETO target genes.

Sp1 transcription factor, which is essential in hematopoietic differentiation,[24,33] was identified as an important driver in the DNA binding map of the AML1-ETO oncoprotein. In this study, detailed sequence analysis revealed a significant enrichment of the Sp1 TFBS present in $>50\%$ of the target genes independently of the associated silencing chromatin mark. These data support previous studies and suggest that a large portion of AML1-ETO functional targets do not present an AML1 TFBS. Significant and reversible silencing of most AML1-ETO target genes was confirmed in the AML1-ETO knockout model and primary samples, independently of the presence of an AML1 (for example, *YES1* and *AML1*) and/or an Sp1 TFBS (for example, *CTCF* and *SIRT1*). On the contrary, Sp1 inhibition lead only to the activation of those genes with an Sp1 binding site at their promoter (*CTCF and SIRT1*) but no effect was seen on those presenting an AML1 TFBS (*AML1* and *YES1*). Furthermore, on the primary samples those genes containing an AML1 consensus sequence showed a higher significant silencing pattern, suggesting that more effective transcriptional

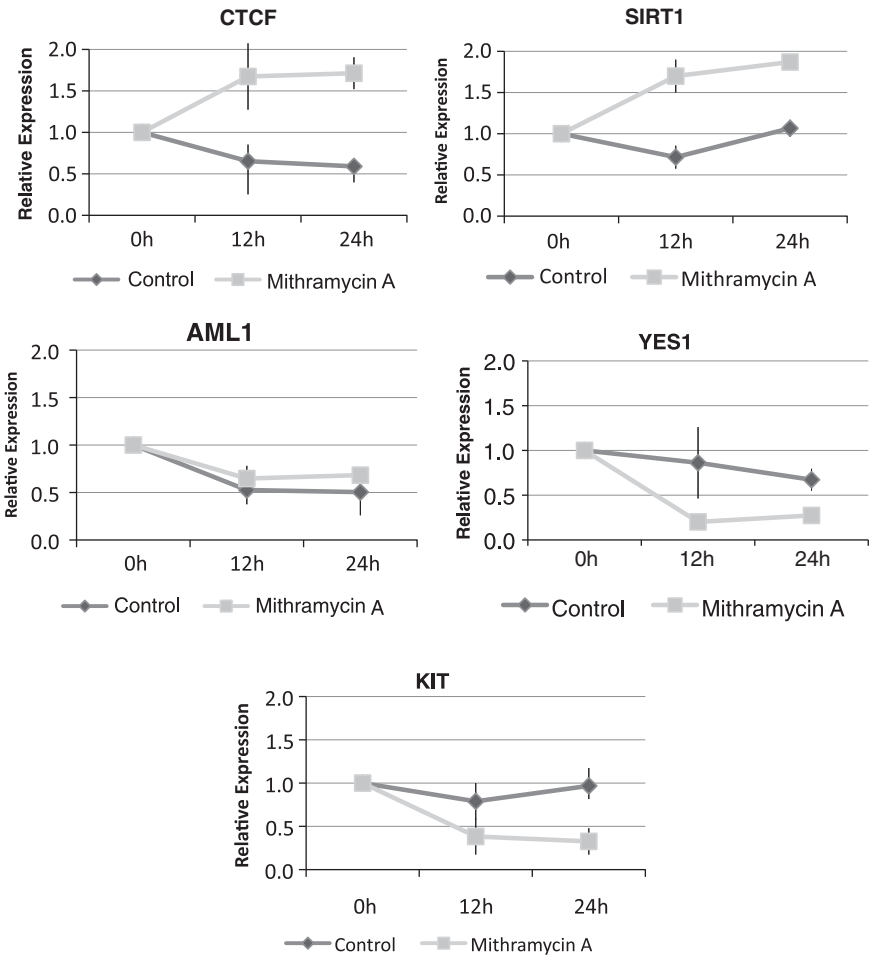

Figure 5. Mithramycin A restores the expression of AML1-ETO target genes with an Sp1 TFBS. qRT-PCR analysis shows increased expression levels in *CTCF* and *SIRT1* genes, no changes were observed on *AML1* or *YES1* expression studied upon Mithramycin A treatment (150 ng/ml). *KIT* mRNA levels were used as a control. Measurements of two independent AML1-ETO clones were taken at three different time points (0, 12 and 24 h in culture).

repression takes place when the fusion protein directly binds DNA through an AML1 TFBS. All together these data support the interaction between AML1-ETO and Sp1, which would lead to Sp1 transactivity inhibition,[24] and identify this event as a genome-wide mechanism involved in t(8;21) leukemia. These results highlight the importance of performing integrated analysis using genome-wide binding profiles, to obtain a comprehensive view of combinatorial interactions between fusion proteins and key regulators as Sp1. Interestingly, previous reports using ChIP-seq analysis of AML1 binding sites also identified DNA binding that was not associated with canonical AML1 response elements.[46]

In conclusion, our investigation provides a genome-wide functional DNA binding map of the AML1-ETO fusion protein characterizing the associated key silencing chromatin modifications, which are important initial steps toward transformation of t(8;21) leukemia. This global approach also enabled us to identify a critical role for Sp1 in the DNA binding of the AML1-ETO fusion protein. As recent studies have shown an *in-vitro* effect of bortezomib, which targets Sp1 complexes in leukemia with t(8;21), our results offer additional evidence for the use of this drug in clinical trials.[31,47]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

AMD and SA designed and performed the experiments, analyzed data and prepared the manuscript. FSC, MW and JCM developed all the used HSPC models and provided the expression data. GGL performed bioinformatics analyses. FVJ, SRP, JCC and SA participated on the project design. MJL and MJC provided the primary samples.

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