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Genome-wide binding patterns and chromatin modifications induced by AML1-ETO and MLL-AF9 fusion proteins in Acute Myeloid Leukemia

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Que Doña **Alba Maiques Díaz** ha realizado el presente trabajo: "**Genome-wide binding patterns and chromatin modifications induced by AML1-ETO and MLL-AF9 fusion proteins in Acute Myeloid Leukemia "** y que a su juicio reúne plenamente todos los requisitos necesarios para optar al grado de Doctor, a cuyos efectos será presentado en la Universidad Autónoma de Madrid. El trabajo ha sido realizado bajo su dirección, autorizando su presentación ante el Tribunal Calificador.

Y para que conste se extiende el presente certificado,

Madrid, Enero de 2014

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Permíteme proponer algunas sugerencias sobre cómo manejar las resistencias naturales que las circunstancias pudieran ofrecer. No asumas que tienes que tener unas condiciones determinadas para hacer tu trabajo mejor. No esperes. No esperes a tener suficiente tiempo o dinero para lograr lo que crees tener en mente. No esperes a tener lo que asumes como ambiente adecuado, libre de estrés. No esperes a tener madurez, conocimiento o sabiduría. No esperes hasta estar seguro de saber lo que estás haciendo. No esperes hasta tener suficiente técnica. Lo que hagas ahora va a determinar la calidad y el alcance de tus actividades futuras.

Anne Bogart (Directora Teatral)

"Resérvate el derecho a pensar: incluso equivocarse es mejor que no pensar nada" *Hypatia*

A mis padres Juan y Loles, y mi hermana Mar.

A mi abuela Lola.

Cambia todo cambia

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RESUMEN

Más del 50% de las leucemias agudas mieloides (LAM) presentan al diagnóstico alteraciones cromosómicas recurrentes. La mayoría producen translocaciones equilibradas que generan proteínas de fusión, las cuales alteran el perfil transcripcional estableciendo el ambiente que permite la transformación leucémica. Entre las translocaciones más frecuentes están la t(8;21)(q22;q22) y la t(9;11)(p22;q23), que producen respectivamente las proteínas de fusión AML1-ETO y MLL-AF9. Mediante estudios de ChIP-chip en progenitores hematopoyéticos que expresan la oncoproteína AML1-ETO hemos identificado nuevos genes diana asociados con modificaciones de la cromatina. La desacetilación de la histona H4 y el aumento de H3K9me3 en los genes diana de AML1-ETO identificados afectan a genes involucrados en vías de señalización esenciales en la diferenciación y auto-renovación de los progenitores hematopoyéticos. La represión inducida por estas marcas de la cromatina se mantiene en muestras primarias t(8;21), y está directa y reversiblemente inducida por la presencia de AML1-ETO. Además, encontramos que más del 50% de los genes diana de AML1-ETO presentan un motivo de unión de Sp1 e identificamos que la proteína Sp1 es crítica para las LAMs dirigidas por AML1-ETO. Estos resultados nos permiten proponer el estudio de terapias dirigidas contra Sp1 en este subtipo leucémico. Por otro lado, observamos que bajas concentraciones del inhibidor de HDACs panobinostat (LBH589) produce toxicidad celular y cambios rápidos en la expresión génica de células leucémicas que expresan la proteína de fusión MLL-AF9. Estudios de ChIP-seq demostraron una hiperacetylacion de histonas consistente con el perfil transcripcional de estas células. Además, más del 50% de los genes diana de MLL-AF9 identificados, presentan H4ac coincidente con la metilación de H3K4 y H3K79 asociada a MLL-AF9. En este contexto, el tratamiento con panobinostat aumenta la presencia de H4ac e induce la activación de genes indirectamente silenciados por MLL-AF9. Sin embargo, mediante arrays de expresión identificamos un aumento de la transcripción de dianas de MLL-AF9, como *HOXA9* y *MEIS1*. Estos resultados dan nuevas pistas sobre el papel de las HDACs en el desarrollo de las LAMs y revelan la sobre-expresión de genes diana de MLL-AF9 como un nuevo mecanismo de acción del panobinostat.

ABSTRACT

Cytogenetic aberrations can be detected in over 50% of newly diagnosed acute myeloid (AML) patients. The majority gives rise to nonrandom chromosomal translocations that often result in fusion proteins, which alter the gene expression profile laying the groundwork for leukemic transformation. Among the most frequently found in AML patients are the t(8;21)(q22;q22) and the t(9;11)(p22;q23), which generates the AML1-ETO and the MLL-AF9 fusion proteins respectively.

By ChIP-chip analysis of human hematopoietic stem/progenitor cells transduced with the AML1-ETO fusion gene we identify new chromatin-modified AML1-ETO target genes. We observed that the presence of either AML1-ETO/HDAC1 complex or increased H3K9me3, on the promoters of AML1-ETO targets, was involved in the repression of hematopoietic differentiation genes and important AML signaling pathways. This silencing is maintained during AML development, as it was also observed on a set of t(8;21) primary samples, and was directly and reversibly induced by AML1-ETO presence. Interestingly, we found a Sp1 binding site in over 50% of AML1-ETO targets and identified that Sp1 protein is critical in myeloid leukemia driven by AML1-ETO, suggesting a role for Sp1 targeted therapy in this leukemia subtype.

Secondly, we investigated the response of AML cells to the pan-HDACi panobinostat (LBH589) and found that low concentrations of panobinostat lead to MLL-AF9 cell toxicity and rapid changes in gene expression. ChIP-seq analysis revealed massive hyperacetylation of histones that was consistent with the transcriptional profile in the presence of MLL-AF9 fusion protein. Furthermore, in over 50% of the identified target genes, H4ac was present along with the known MLL-AF9-induced H3K4me3 and H3K79me2 chromatin marks. In this context, treatment with panobinostat further increases acetylation in H4ac, thus inducing a transcriptional activation of indirectly silenced MLL-AF9 genes. However, by gene expression arrays we also observed increased transcription of active MLL-AF9 target genes such as *HOXA9* and *MEIS1*. Thus, our results provide new insights into the role of HDACs in the leukemogenic process and reveal boosted up-regulation of MLL-AF9 direct targets to be a novel mechanism of action of panobinostat in MLL-rearranged leukemia.

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ABBREVIATIONS

Ac – acetylation

ALL – Acute Lymphoblastic Leukemia

AML **–** Acute Myeloid Leukemia

BPR– break point region.

BRD – Bromodomain

CBF – core binding factor

ChIP – chromatin immunoprecipitation

CR – complete remission

ENL – European LEukemiaNet

FAB – French- American-British

FC – fold change

FDR – false discovery rate

GSEA – Gene Set Enrichment Analysis

H3 – histone H3

H4 – histone H4

HAT – Histone acetyltransferase

HDAC – Histone deacetylase

HDACi – histone deacetylase inhibitor

HSPC – Hematopoietic stem/progenitor cells

ID – Inhibitory domain.

IPA – Ingenuity Pathways Analysis

K – lysine

MDS – Myelodisplastic Syndrome

Me – methylation

Nt – nucleotide

OS – Overal survival

PHD – plant homeodomains

PTM – post-translational modification

qPCR – cuantitative PCR

Seq – sequencing

shRNA – Short Hairpin RNA

TA – transactivation domain.

TAF– transcription activation interaction domain.

tAML - treatment related AML

TFBS – transcription factor binding site

TRM – treatment related mortality

TSS – transcription start site

WBC – white blood count

WHO- Wold Health Organization

Wt – wild type

ZF – Zinc finger

INTRODUCTION

1. Acute myeloid leukemia

Acute myeloid leukemia (AML) is a clonal hematopoietic disorder resulting from genetic alterations in normal hematopoietic stem/progenitor cells. These alterations disrupt normal differentiation and/or cause excessive proliferation of abnormal immature leukemic cells known as blast [1]. As the disease progresses, blast cells accumulate in blood and bone marrow being the presence of over 20% of blast in bone marrow a main criteria for AML diagnosis[2]. Finally, the excessive abnormal hematopoiesis will interfere with the production of normal blood cells, leading to fatigue, infection, bleeding, and organs failure.

AML accounts for approximately 25% of all leukemia diagnosed in adults, with an incidence of 3 to 4 new patients per 100 000 men and women per year. The median age of diagnosis ranges from 66 to 71 years[3]. This myeloid malignancy can arise *de novo* or secondarily either due to the progression of a bone marrow stem cell disorders (such as a myelodisplastic or a myeloproliferative syndrome) or as a consequence of a previous treatment with cytotoxic agents (referred to as therapy-related AML or tAML) [4, 5].

AML is characterized by a complete disruption on normal adult hematopoiesis due to a heterogeneous spectrum of genetic and epigenetic abnormalities. The production of blood is a tightly controlled cell system with remarkable cellular turnover that constantly regenerates from very few hematopoietic stem cells, with self-renewal capacity, through cell divisions and differentiation. The process is driven by early and lineage-specific growth factors and their receptors, which work coordinately with a set of transcription factors and chromatin remodeling genes that regulate the expression of lineage specific genes governing the differentiation decisions. Thus, transcription factors and chromatin remodeling genes are part of a highly regulated network of signaling transduction pathways and inherent cellular programs, which are necessary for the orchestrated regulation of gene expression that defines the phenotype of a given blood cell [6]. Genetic abnormalities on key hematopoietic genes lead to leukemia initiation and progression.

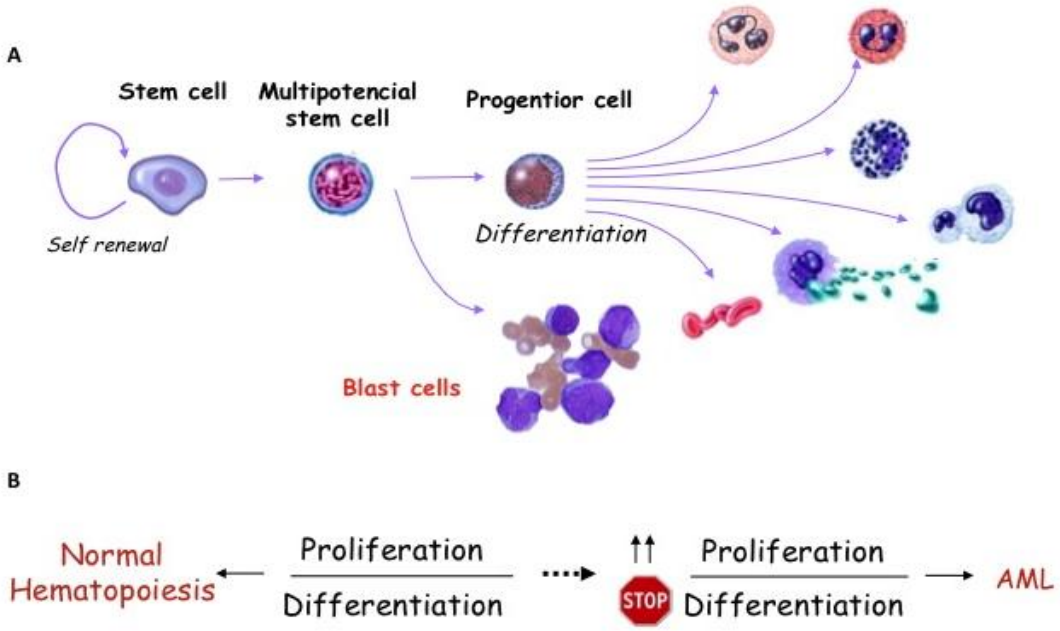


Figure 1. Altered mechanisms on Acute Myeloid Leukemia. (A) The differentiation hierarchy of normal myeloid lineage is shown, starting from left to right. In red the abnormal undifferentiated blast cells are depict. (B) In AML the normal hematopoietic balance between proliferation and differentiation is disrupted, and an increase proliferation with a blockage of differentiation is observed.

2. The importance of genomic characterization on AML

2.2. AML genetics as a prognostic factor

Prognostic factors may be subdivided into those related to patient characteristics and general health condition and those related to characteristics particular to the AML clone. The former subset usually predicts treatment-related mortality (TRM) and becomes more important as patient age increases while the latter predicts resistance to, at least, conventional therapy. AML-related prognostic factors includes white blood count (WBC), existence of prior myelodisplastic syndrome (MDS), previous cytotoxic therapy for another disorder, and cytogenetic and molecular genetic changes in the leukemic cells at diagnosis. High degree of heterogeneity with respect to chromosome abnormalities, gene mutations and changes in expression of multiple genes and microRNAs is observed in AML[2]. Various other factors, such as splenomegaly and elevated serum lactate dehydrogenase (LDH) levels, have been reported to confer some prognostic effect but with variable consistency among studies [7]. The

significance of a prognostic factor is always dependent on the therapy given to a patient.

Cytogenetic aberrations play a central role in the leukemic development: they can be detected in approximately 50% to 60% of newly diagnosed AML patients[8]. The majority of AML cases are associated with nonrandom chromosomal translocations that often result in gene arrangements. The European LeukemiaNet (ELN) proposes a standardized reporting system for genetic abnormalities when presenting data correlating genetic findings with clinical outcome that allows for a better comparison of data among studies (Table 1). This standardized report includes data from cytogenetic analysis and from mutation analyses of the NPM1, CEBPA, and FLT3 genes[7]. Nowadays it is well established that karyotype is an independent predictor, and together with the patient´s age, the most important prognostic factors for predicting complete remission (CR) rate, relapse and overall survival (OS). Indeed the ELN classification divides AML patients into 4 risk groups according to genetic and genomic characterization (Table 1). This classification reflects the high variability among different prognostic groups, ranging from a CR of 80% to 95% in the favorable risk group to only 30% of patients categorized in the adverse-risk group[3].

Table 1. AML classification risk groups according to the European LeukemiaNet (ENL) (modified from Schlenk, 2013).

Genetic group	Subset
Favorable	t(8;21)(q22;q22); RUNX1-RUNX1T1 inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11 Mutated NPM1 without FLT3-ITD (CN-AML) Mutated CEBPA (CN-AML)
Intermediate-I	Mutated NPM1 and FLT3-ITD (CN-AML) Wild-type NPM1 and FLT3-ITD (CN-AML) Wild-type NPM1 without FLT3-ITD (CN-AML)
Intermediate-II	t(9;11)(p22;q23); MLLT3-MLL AML Cytogenetic abnormalities not classified as favorable or adverse
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1 t(6;9)(p23;q34); DEK-NUP214 t(v;11)(v;q23); MLL rearranged AML with maturation -5 or del(5q); -7q; abnl(17p); complex karyotype

Furthermore, during the last years, new whole-genome sequencing studies have offered insights into the pathogenesis of AML relapses by identifying specific mutations along the clonal evolution. In some patients the founding clone in the primary AML have been found to gain mutations and evolve into the relapse clone; in other cases a subclone of the founding one survives initial therapy, gains additional mutation, and expands[9]. In both scenarios, to know the genetic background in each step of the disease will allow to design better the patient´s treatment and an impact in prognostic classifications is expected.

2.2. Fusion proteins as a molecular mechanism of AML development

A total of 1.603 chromosomal aberrations have been catalogued in AML (data from august 2013, available at: http://cgap.nci.nih.gov/Chromosomes/Mitelman [10]). The frequencies of the 4 most common translocations are between 3% and 10%, while for others the prevalence is significantly smaller (Table 2). It was early realized that most AML-typical chromosomal alterations cause fusion genes that involve transcription factors as *AML1* or essential genes for myeloid differentiation as *MLL*. The resulting fusion protein interferes with the normal function of the wt protein. Frequently the oncofusion protein retains the DNA-binding motifs of the wild type transcriptional regulator and gains the capacity to interact with corepressors and/or coactivators through its fusion moiety [11]. These findings have set the paradigm that AML is a disease of transcriptional control in disarray: the aberrant silencing or constitutive activation of target genes essential for myeloid development lays the groundwork for leukemic transformation. Thus, the potential targeting of this interaction has become a major focus for the development of novel therapeutics.

Table 2. Most prevalent AML-associated oncofusion proteins and their specific clinical features (modified form Kumar, 2011; Scandura, 2002)

Translocations	Oncofusion proteins	Frequency of occurrence (% of AML)	Clinical features
t(8;21)	AML1-ETO	10%	Primary AML. Good prognosis when patients receive high-dose cytarabine consolidation therapy, common in older children and younger adults
t(15;17)	PML-RARa	10%	Primary AML. Remission induction with ATRA, sensitive to anthracyclines. Common in older children and younger adults, good risk
inv(16)	CBF-MYH11	5%	Primary AML. Good prognosis when patients receive high-dose cytarabine consolidation therapy. Common in older children and young adults.
der(11q23)	MLL-fusions	4%	Primary or secondary AML, t-AML associated with exposure to Topoisomerase II inhibitors, depending on the fusion. Poor risk
t(9;22)	BCR-ABL1	2%	Secondary AML arising from blastic CML, present with leukocytosis, poor risk.

The present thesis has focused on two of the most frequent chromosomal translocations: t(8;21) and t(9;11) which generates AML1-ETO and MLL-AF9 fusion proteins respectively (Figure 2A and 3A).

AML1-ETO fusion protein results from the chromosomal translocation t(8;21)(q22;q22) appearing in approximately 15-10% of all AML. It is included on the favorable risk group of the ENL, and its phenotype is defined by the French-American-British (FAB) classification as an M2 subtype with granulocytic differentiation [12, 13]. Patients with t(8;21) leukemia have a relative favorable prognosis compared with other types of AML patients (Table 1), with 60% overall survival at 5 years [3, 14].

Genomic rearrangements of the human chromosomal band 11q23 involving *MLL* gene are frequent events in leukemia, appearing in more than 80% of infant acute lymphoblastic leukemia (ALL) and approximately 10% of AML cases [15]. *MLL*-rearranged AML is included on the intermediate-II genetic risk group of the European LeukemiaNet recommendations and is recognized as a distinct disease entity in the WHO classification [12, 13]. The most common *MLL* gene rearrangement, which is seen

in 49% of *MLL*-positive AML patients, involves the translocation t(9;11)(p22;q23), which generates the MLL-AF9 fusion protein (also known as the MLL-MLLT3) [15].

3. Epigenetics and chromatin dynamics

The aberrant recruitment of the epigenetic machinery, as chromatin modifiers, by the oncofusion proteins to the promoters of key differentiation genes plays a central role in triggering AML[16, 17]. This aberrant presence of epigenetic modifiers will either induce the silencing, maintenance and/or increase of gene expression depending on the oncofusion and cellular context. Thus, the aberrant gene expression patterns observed in AML with balanced chromosomal translocations are being increasingly attributed to specific epigenetic modifications induced by the protein complexes recruited by each of the fusion proteins [18-21].

Chromatin is the macromolecular complex of DNA and histone proteins, which provides the scaffold for the packing of the entire genome. The basic functional unit of chromatin is the nucleosome that contains 147 base pairs of DNA wrapped around a histone octamer, formed by histones H2A, H2B, H3, and H4. All chromatin components (DNA and histones) are subjected to covalent modifications, which fundamentally alter its organization and function. At least four different DNA modifications and 16 classes of histone post-translational modifications (histone PTMs) have been described[22]. Such modifications are dynamically laid down and removed by DNA- or chromatin-modifying enzymes in a highly regulated manner. It has been generally accepted that histone PTMs correlate with either positive or negative transcriptional states (Table 3). Two mechanisms for how histone PTMs affect transcription have been proposed, (1) chromatin packing is altered directly (either by change in electrostatic charge or through internucleosomal contacts) to open or close the DNA polymer, thus controlling access of DNA-binding proteins such as transcription factors; (2) the attached chemical moieties alter the nucleosome surface to promote the association of chromatin-binding proteins [16, 23].

Table 3. Examples of chromatin modifications. Well-characterized sites with regard to residues within histones for PTMs in mammals (modified from Berger, 2007)

Histone PTM	Transcriptionally relevant sites*	Transcriptional role
Acetylated lysine (Kac)	H3 (9,14,18,56), H4 (5,8,13,16), H2A, H2B	Activation
Phosphorylated serine/threonine (S/Tph)	H3 (3,10,28), H2A, H2B	Activation
Methylated lysine (Kme)	H3 (4, 36, 79) H3(9,27), H4(20)	Activation Repression
Ubiquitinated lysine (Kub)	H2B (120) H2A (119)	Activation Repression

The great diversity in histone modifications introduces a remarkable complexity that is slowly being elucidated. Typically, in response to cytoplasm signaling, transcription factors recruitment of enzymes leads to establishment of positive-acting chromatin marks across promoters and open reading frames. In turn, negative-acting marks are laid down across genes during repression by DNA-bound repressor recruitment or across heterochromatic regions of the genome [23]. An extension of this idea is that simple combinations of consistently behaving marks correspond to definable and predictable outcomes of transcription. However, these modification patterns are not static entities but a dynamically changing and complex landscape that evolves in a cell context-dependent fashion. Moreover, active and repressive modifications are not always mutually exclusive, as evidenced by "bivalent domains"[22]. There are plenty of examples of how histone modifications are implicated on leukemia development [16]. Among them, it is well establish that AML1-ETO oncoprotein functions as a transcriptional repressor by recruiting NCoR/SMRT/HDAC complexes[14], inducing a histone deacetylation landscape on its target genes; while MLL-AF9 fusion protein alters the histone methylation profile inducing an abnormal transcriptional profile of its targets[15]. Thus, only histone acetylation and methylation have been uncovered in this section.

3.1. Histone acetylation and deacetylation

Acetylation of lysine residues is a major histone modification involved in transcription, chromatin structure and DNA repair. Acetylation neutralizes lysine´s positive charge and may consequently weaken the electrostatic interaction between histones and negatively charged DNA. Thus, it has been generally associated with transcriptional activation, both initiation and elongation, while deacetylation results in relative closed chromatin conformation that often leads to repressed transcription[17]. It is a reversible process, controlled by the equilibrium between histone acetyl transferase (HAT) and histone deacetylase (HDAC) activities. HAT enzymes transfer an acetyl group to the lysine residues of histones, neutralizing the positive charge of the histone and conferring a state associated with an “open” chromatin conformation. HATs are primarily nuclear and can be broadly classified into the GNAT, MYST, and CBP/p300 families [22]. Consistent with this, ChIP-seq analyses have confirmed the distribution of histone acetylation at promoters and enhancers and, in some cases, throughout the transcribed region of active genes[24]. Importantly, lysine acetylation also serves as the nidus for the binding of various proteins with bromodomains (BRD) and tandem plant homeodomains (PHD) fingers, which recognize this modification [25]. It is clear that global histone acetylation patterns are perturbed in cancers, but it is important to mention that several nonhistone proteins, including important oncogenes and tumor suppressors such as MYC, p53, and PTEN, are also dynamically acetylated [22].

In contrasts, the reestablishment of the positive charge in the amino-terminal tails of histones, catalyzed by HDACs, tightens the interactions between histones and DNA, conferring a “close” chromatin state. There are 18 HDACs identified which are subdivided in four classes, depending on sequence homology: class I (HDAC1-3 and HDAC8), class II (HDAC4-7 and HDAC9-10) and class IV (HDAC11) are zinc-dependent amidohydrolases; whereas class III (the so-called Sirtuins) requires NAD+ for the deacetylation reaction [22]. Analogous to the HATs, HDACs target both histone and nonhistone proteins.

Interestingly, genome-wide mapping has revealed that binding of both HATs and HDACs occurs at regulatory regions positively correlated with gene expression. HDACs are thus proposed to have an important role in the transcription regulation of genes modified or “primed” for transcription by the presence of the histone H3 lysine 4 (H3K4) methylation. At these primed regions HDACs may act as fine tuners of transcription, avoiding aberrant transcription to proceed [26].

3.2. Histone methylation and demethylation

Histones are methylated on the side chains of the arginine, lysine and histidine residues, but the best-characterized sites are those that occur on lysine residues. Methyltransferases (HMTs) are much more specific than the acetyltransferases. They usually transfer a methyl group to a single residue, thus leading to transcriptional activation or repression depending on the modified amino acid residue. Moreover, methylation, unlike acetylation, does not alter the overall charge of the molecule, and lysines may be mono-, di-, or tri-methylated. Although many lysine residues on the various histones are methylated, the best studied are histone H3 lysine 4 (H3K4), H3K9, H3K27, H3K36, H3K79 and H4K20. Of these H3K4, H3K36, and H3K79 are often associated with active genes in euchromatin, whereas H3K9, H3K27, and H4K20 are associated with heterochromatic regions in the genome [22]. Furthermore different methylation states on the same residue can also localize differently (e.g. H3K4me2/3 are usually present at TSS of active genes and H3K4me1 is associated with active enhancers); or be associated with different chromatin states (e.g. H3K9me1 is found in active genes, whereas H3K9me3 is associated with gene repression).

Enzymes such as EZH2 and MLL family members that contain a conserved SET domain, with methyltransferase activity, or DOT1L, the only exception that has not SET domain, catalyze lysine methylation [27]. It is now known that histone lysine methylation is not a stable, nondynamic modification as it was believed. Two classes of lysine demethylases have been characterized: the LSD1 (KDM1A) that catalyze the demethylation of mono- and dimethyllysine; and the Jumonji classes, which can demethylate all three methyl lysine states. Moreover, the various states of lysine methylation are read and interpreted by proteins containing different specialized

recognition motifs. Broadly speaking the aromatic cages that engage methyl lysine can be divided into two major families, the Royal Family (Tudor domains, Chromo domains and MBT domains) and PHD fingers [25].

4. Fusion Proteins and Chromatin Modifications

Although the molecular pathogenesis of AML has been studied with the use of cytogenetic analysis for more than three decades, the relationship between a specific fusion proteins and the epigenetic phenotype is not clear. A more complete understanding of the crosstalk between genetics and epigenetics is relevant not only to better understand the pathogenesis of AML but also, to find better approaches to therapy.

4.1 AML1-ETO Fusion Protein

a) Core Binding Factor AML

The Core Binding Factor (CBF) is a heterodimeric transcription factor, composed by a non-DNA-binding CBFβ protein and a DNA-CBFα binding protein. The DNA-CBFα is formed by one of Runt-related transcription factors: *RUNX1* or *AML1; RUNX2* or *AML3;* or *RUNX3* or *AML2.* They have a conserved 128-amino acid Runt domain, so-called because of its homology to the Drosophila *runt* gene, which plays a role in the segmented body pattering. All three Runx proteins bind to a common DNA motif TGT/cGGT and heterodimerizes with the CBFβ, which makes no direct contact with DNA, but rather increases DNA binding to Runx proteins. Both DNA binding and heteromerization with CBFβ are mediated by the Runt domain. *Runx* genes are essential for the development of specific tissue in vertebrates, and *AML1* has a primary role in the development of all hematopoietic cell types (definitive erythroid cells, granulocytes, macrophages and lymphoid cells) [28]. The DNA-binding of AML1 promotes the expression of essential target genes for the development and differentiation of all hematopoietic lineages [29].

This AML1/CBFβ transcriptional regulatory complex is commonly targeted by translocations, deletions, or point mutations in acute leukemia. The t(8;21)(q22;q22),

t(16;21)(q24;q22), and t(3;21)(q26;q22) fuse the *AML1* gene, which is located on chromosome 21, with *ETO* (a gene located on chromosome 8), *MTG16* (a member of the ETO-gene superfamily, located on chromosome 16), and the *MDS*/ *EVI-1* gene (located on chromosome 3) to create AML1-ETO, AML1-MTG16, or AML1-MDS/EVI-1 fusion proteins, respectively. All of these fusion proteins retain the DNA binding domain (and thus the DNA sequence specificity) of the wild-type AML1 protein [30]. The *CBFβ* gene is rearranged by the inversion 16 abnormality, which juxtaposes *CBFβ* to the myosin heavy chain *MYH11* gene, generating a CBFβ-MYH11 fusion protein [31]. It has been hypothesized that the CBFβ-MYH11 fusion inhibits AML1 function by binding and sequestering AML1 in the cytoplasm, thereby preventing its action in the nucleus. However, CBFβ-MYH11 can also be found in the nucleus and can further stimulate AML1 repressor activity by interacting with corepressor molecules and by the presence of its own repression domain [32]. These activities would further explain its ability to repress the transcription of AML1–dependent genes.

In AML1-ETO fusion, the DNA-binding domain of AML1 is joined to almost full length ETO protein (Figure 2B-C) [33]. Consequently, AML1-ETO functions as a transcriptional repressor by recruiting NCoR/SMRT/HDAC complexes, through its ETO moiety, to DNA sequences containing multiple AML1 transcription factor binding sites (TFBS) [34-38]. The oncofusion exerts a dominant inhibitory effect on AML1 function, thereby interfering with normal hematopoietic differentiation and inducing a preleukemic condition. Such idea was further supported by the results of gene targeted mouse models: mice heterozygous for an AML1-ETO allele show a near identical phenotype to AML1 knockout mice [39, 40]. Moreover, AML-ETO has been described to interact and repress a number of other transcription factors (e.g. E proteins, ETS proteins), hypothesizing to target DNA through other motives, such as E-Box motifs, as the result of these physical interactions [41-43].

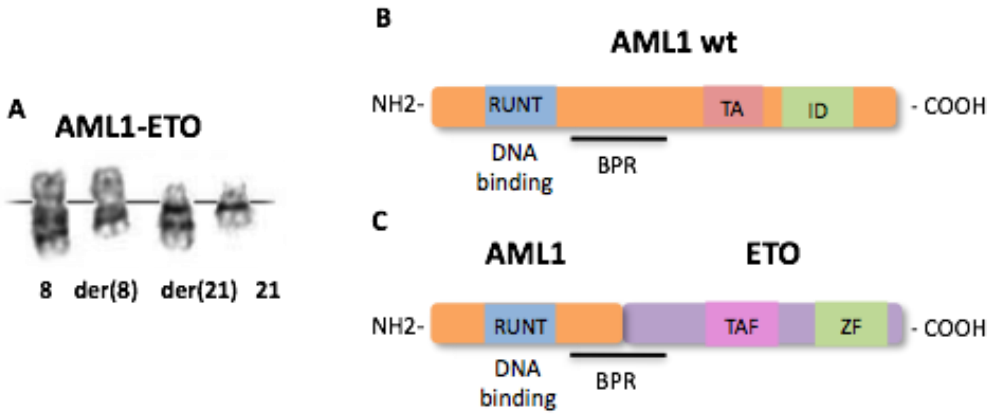


Figure 2. AML1-ETO translocation and correspondent AML1 and ETO regions. (A) Normal and derivative chromosomes of the chromosomal translocation t(8;21)(q22;q22). (B-C) A schematic representation of the protein domains of AML1 wild-type protein (B), and AML1-ETO fusion protein(C). BPR= break point region. TA= transactivation domain. ID= Inhibitory domain. TAF= transcription activation interaction domain. ZF= Zinc finger.

In murine models, full-length AML-ETO alone appears not to be sufficient for leukemogenic transformation, and requires additional genetic events [44-46]. Indeed, other chromosomal aberrations are detected in approximately 70% t(8;21), including del(9q); as well as mutations in *KIT* (up to 40%), in *FLT3* (2-7%) or *RAS* (10%) genes [30]. In line with this, transgenic o conditional expression of AML1-ETO in human hematopoietic stem/progenitor cells (HSPC) did not cause leukemia in immunodeficient mice, but has profound effects: it blocks myeloid cell differentiation at a more immature stage as well as extends their self-renewal potential, thus predisposing to leukemia [44, 47, 48].

b) AML1-ETO & aberrant epigenetic transcriptional silencing.

Although, there are several examples of AML1-ETO upregulation of target genes, the importance of this mechanism is sparse [49, 50] and it is the association of AML1-ETO DNA-binding with chromatin silencing marks the most widely accepted condition related to this fusion protein. On specific loci, histone H4 deacetylation and K9 trimethylation of histone H3 (H3K9me3) have been functionally validated chromatin marks already described as direct repressive effects of AML1-ETO presence [51]. Primary through the NHR2 and NHR4 domains of C-terminal ETO, AML1-ETO recruits NCoR/SMRT/HDAC complexes to AML1 DNA target genes, inducing a repressive

chromatin structure (Figure 3B). The H4 deacetylation is catalyzed, among others, by histone deacetylase 1 (HDAC1) that forms part of the interaction complex. Additionally, the lysine methyltransferase enzyme suppressor of variegation 3-9 homolog 1 (SUV39H1) has been shown to interact with AML1 wt [52]. It has been postulated that this interaction will be maintained on the presence of AML1-ETO at specific target promoters, implicating that SUV39H1 could be the responsible enzyme for the observed H3K9me3. This chromatin mark correlates with transcriptional silencing through the formation of high-affinity binding site for heterochromatin protein 1 (HP1) [53].

Interestingly, the AML1-ETO oncofusion itself is also postranslational modificated by the histone acetyltransferase p300. It has been described that p300 directly interacts through the ETO-NHR1 domain, resulting in AML1-ETO acetylation on lysine 43. This protein acetylation is an essential step required for AML1-ETO leukemogenic effects, as it is necessary for fusion protein stabilization and for bringing p300 to chromatin to acetylate residues [54].

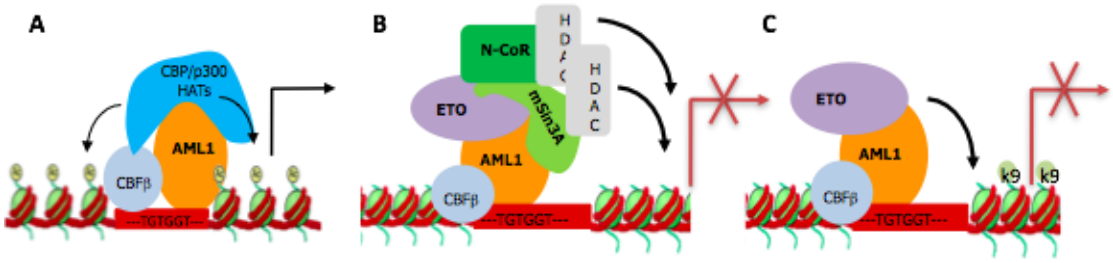


Figure 3. Both AML1 and AML1-ETO proteins binds DNA, but recruit different complexes. (A) The CBF heterodimer formed by AML1/CBFβ binds to specific binding sites and induces histone acetylation by its interaction with coactivators as CBP/p300. (B) AML1/ETO binds to AML1 binding sties but interacts with corepressors generating a histone deacetylation landscape. (C) The presence of AML1-ETO has also been associated with a repressive H3K9me3 chromatin modification.

4.2. MLL-AF9 Fusion Protein

a) MLL-rearranged leukemia

MLL1 gene (also called *MLL, KMT2A* or *ALL1*) is one of the six mixed-lineage leukemia (MLL) family histone methyltransferases in mammals. It is the ortholog of the *Drosophila melanogaster* trithorax (*Trx*) gene, involved in the transcriptional activation of the homeobox (Hox) genes [55]. It is located on the chromosome 11q23 and encodes for a large protein which is proteolitically processed into 2 fragments (MLL^{C} and MLL^{N}) that interact to form the full MLL protein [56]. It contains several domains, including two regions of homology to the TRX protein: the conserved 200 amino acid SET domain and a Plant homeodomain (PHD) zinc fingers, that are involved in protein-protein interactions; and a N-terminal AT-hook and several Zinc finger domains which binds to DNA regions (Figure 4B) [57, 58]. Through the evolutionary conserved SET domain MLL catalyzes mono-, di- and trimethylation of histone H3 on K4 [59]. Both MLL and H3K4me localize across gene promoters, transcription start sites (TSS) and 5' transcribed regions of target genes and facilitate transcription initiation [60]. In addition mouse studies have shown that MLL is essential for fetal and adult hematopoiesis by regulating the expression of Hox genes (e.g. *Hoxa*) and other transcription cofactors promoting hematopoietic stem cell expansion [61-63].

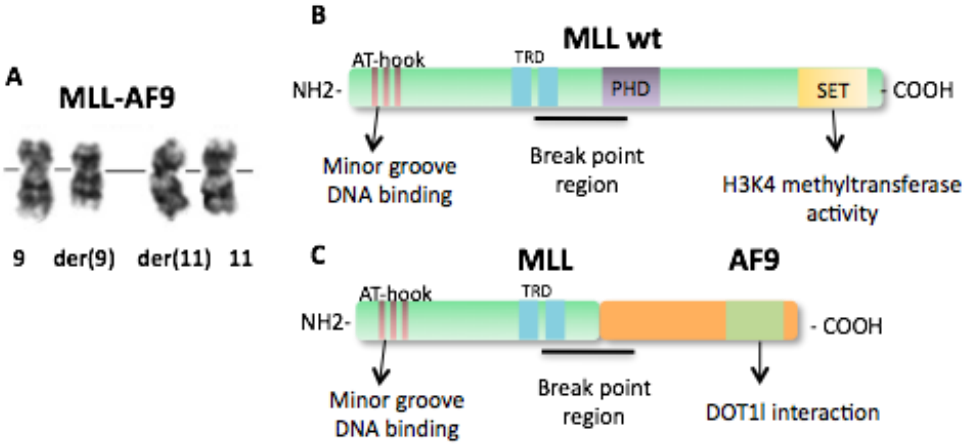


Figure 4. MLL-AF9 translocation and correspondent MLL and AF9 regions. (A) Normal and derivative chromosomes of the chromosomal translocations t(9;11)(p22;q23) are shown. (B-C) A Schematic representation of MLL wild-type protien (B) and MLL-AF9 fusion protein (C).

MLL is known to form part of a large multiprotein complexes composed among others of (1) Menin protein, which makes a bridge between MLL N-terminal domain and the lens epithelium-derived growth factor (LEDGF) chromatin associated protein [64]; (2) histone deacetylases (HDACs) and (3) members of the SWI/SNF chromatin-remodeling complex [57]. Moreover, the regulation of MLL activity is unique since the MLL SET domain has extremely low histone methyltransferase activity, which is dramatically enhanced upon assembly into a core complex with three other proteins: WDR5, ASH2L and RbBP5 [65].

The *MLL* gene was the first trithorax-like gene identified as a proto-oncogene [66]. Deregulation of this gene accounts for 5%-10% of acute myeloid leukemia (AML) in adults and almost 70% of acute lymphoblastic leukemia (ALL) in infants [67]. The most common rearrangements are balanced *MLL* translocations, in which one *MLL* allele is truncated and fused in frame with over 60 partners to produce oncogenic fusion proteins. The aberrant protein therefore retains the MLL N-terminal domains but looses it's C-terminal part, where the methyltransferase SET domain is located (Figure 4C). Interestingly, the wild type MLL is also required for the MLL fusion protein-mediated leukemogenesis *in vivo*: knocking out *MLL* allele leads to loss of the leukemic transformation capacity of MLL-AF9 cells [68]. This indicates a necessity of the H3K4 methylase function for leukemia pathogenesis. Moreover, the MLL-PHD3-Bromo cassette (also called PHD domain) has the ability to recruit HDACs implicating that HDACs may be recruited to MLL-bound promoters resulting in gene expression silencing [69]. In MLL-fusion proteins the PHD the fusion partner replaces domain, and its loss has been confirmed to be required for the transforming activity [70].

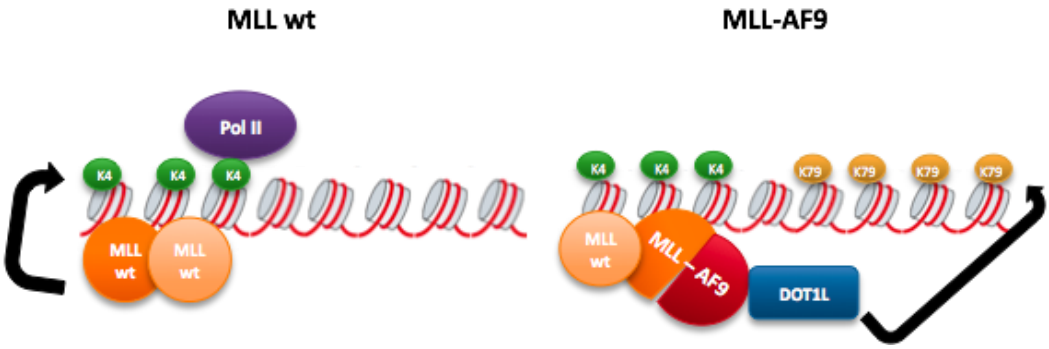
b) MLL-AF9 & aberrant epigenetic signature

Whereas MLL translocations can be found in either ALL or AML, particular translocations show lineage specificity. For example, the t(4;11) is found most often in ALL while the t(9;11) in AML [15]. Mechanistic studies showed that some of the most common MLL fusion partners are able to interact with the transcription machinery by recruiting a variety of chromatin modifiers to the target genes. The MLL-AF9 oncoprotein is one of those fusion partners (others are AF4, ENL and ELL), which are

known to recruit elongation factors in conjunction with epigenetic cofactors, resulting in an aberrant transcriptional elongation [71]. These protein complexes directly stimulate the transcription elongation by recruiting cofactor complexes such as the polymerase associated factor complex (PAFc) [72]; the positive transcription elongation factor b (pTEFb), that regulates and phosphorylates RNA polymerase II [20, 73] and the histone methyltransferase DOT1L, which catalyzes the histone H3 on lysine 79 methylation (H3K79me2) [74, 75]. MLL-AF9 leukemia is largely the result of epigenetic and transcription elongation deregulation which may be sufficient for initiation and progression of leukemogenesis (Figure 5).

Moreover, the polycomb gene CBX8 has been shown to mediate the interaction between MLL-AF9 and the histone acetyltransferease TIP60. This member of the MYST family is associated with transcription elongation, and is able to catalyze, among others, H4K5, K8, K12 and K16 residues at promoters and gene bodies of active genes. The presence of Tip60 on MLL-AF9 cells positively contributes to the leukemic transformation, and has been implicated at least in *HOXA9* gene transcription regulation [76]. However, the presence and function of histone acetylation associated to MLL-AF9 binding remains elusive.

Hematopoietic stem and progenitor cells (HSPCs) retroviral expressing MLL-AF9 have been able to model the initiation and progression of AML on both xenograft and pure murine genetic models [77-79]. However, as knock-in mice models containing *MLL-AF9* fusion gene under control of the *MLL* promoter spontaneously develop AML with a latency of 4 months to > 1 year, the requirement for a second genetic event during leukemogenesis has been postulated. Identification of mutations in FLT3 and RAS in MLL-rearranged leukemia further supports this idea [80].



Figue 5. MLL and MLL-AF9 induced chromatin landscape. (A) The MLL wt protein, formed by MLL^{c}/MLL^{N}, binds DNA and induces H3K4me3 at promoter regions, allowing Pol II presence. This is associated with transcription initiation (B) MLL-AF9 presence is able to interact with DOT1L, which induces H3K79me2 along the gene bodies. The presence of MLL wt is also necessary, establishing a H3K4me3 mark on promoters. In this situation a chromatin landscape associated with transcription elongation is established.

5. Treatment and outcomes in AML

Most adults with AML die from their disease. The standard treatment paradigm for AML is remission induction chemotherapy with an anthracycline/cytarabine combination, followed by either consolidation chemotherapy or allogeneic stem cell transplantation, depending on the patient's ability to tolerate intensive treatment and the likelihood of cure with chemotherapy alone. A complete remission (CR) can be achieved in 65% to 75% of younger adult patients (<60 years) and in approximately 40 to 60% of older patients (>60 years) [3]. Several strategies have been employed to intensify the induction regimen in the hope of improving the remission rate; however, none have provided a clear advantage to induction therapy with cytarabine and anthracycline [81-83]. Recently the combination of bortezomib with daunorubicin and cytarabine in induction resulted in an encouraging remission frequency of 65% in older patients. A similar remission frequency was previously observed in patients with relapsed AML using this combined regimen [84] .

Though, even with the most recent approaches the chemotherapy-based therapy has changed little in the last three decades. Because of toxicity and intrinsic disease resistance, cytotoxic chemotherapy has reached its limit in the treatment of AML patients: we need new therapeutic strategies. It is why there is a need for increased understanding of AML pathogenesis and better molecular genomic technologies. Big

efforts are being done in those directions, leading to novel drug targets and the development of personalized, risk-adapted treatment strategies.

5.2. New therapeutic strategies for t(8;21) and t(9;11) AMLs

The simplest therapeutic strategy will be targeting the fusion protein itself, as well as its aberrant interactions with cofactors. This has been clearly demonstrated in patients with acute promyelocytic leukemia [5]. Regarding AML1-ETO, a small molecule that allosterically inhibits RUNT/CBFβ binding has already been developed [85], but there are reports indicating that the transforming activity of AML1-ETO is independent of CBFβ interaction and such therapies might not be sufficient.

On the other hand, big efforts are being done to target the MLL or the MLL-rearranged leukemia complex. Several compounds that inhibit most of its components are under development. For example, targeting the P-TEFb core component CDK9 is a possibility using flavonoids such as flavopiridiol [86]. Furthermore, the exposure of leukemic cells to the selective DOT1L inhibitor EPZ004777 results in selective killing of those cells bearing the MLL gene translocation, with little effect on non-MLL-translocate cells [87]. Recently, inhibition of MLL methyltransferase activity using a newly developed MM-401 inhibitor that blocks MLL-WDR5 interaction has also shown promising results *in vitro* [88]. However, yet there is not a good molecular candidate with good toxicity-effectiveness ratio.

5.2. Histone deacetylase inhibitors (HDACi)

Another promising possibility is the pharmacological targeting of chromatin alterations. Epigenetic inhibitors of DNA methylation, histone deacetylation and histone methylation may have a better chance to reset the deregulated transcription in AML, and there are been widely study in several AML subtypes including t(8;21) and t(9;11) AMLs [80].

Histone deacetylase inhibitors (HDACi) are a novel class of anticancer agents that blocks HDAC activity, with a significant activity against different tumors. Seven classes of HDACi have been characterized to date, and currently, the US Food and Drug Administration (FDA) has approved two, vorinostat and romidepsin, for the treatment

of cutaneous T-cell lymphoma[89, 90]. Others, as panobinostat (or LBH589) are showing promising results in Phase I/II clinical studies in patients with advanced hematologic malignancies [91]. Panobinostat is a potent pan-HDACi with greater inhibitory activity of HDACs than current approved agents [92]. This new class of drugs is promising but there is an urgent need to better understand their mechanisms of action and to find biomarkers that may predict the clinical response.

Finally, an important consideration that must be taken into account is the genetic heterogeneity of AML: it is very unlikely that the already established or new agents (including combination therapies) will be equally effective in all genetic subgroups. Therefore, the identification of the genetic determinants of response to treatment, including achievement of a CR and survival end points, is of high importance. Thus a better characterization of the genome wide effects of oncofusion proteins as AML1-ETO and MLL-AF9 is highly needed.

AIMS

Nonrandom chromosomal translocations resulting in gene rearrangements are primary genetic events in AML that determine leukemic cells phenotype and clinical outcome [8]. These rearrangements lead to the production of new fusion genes that involve essential genes for myeloid development, modifying their capacity to interact with corepressors and/or coactivators through its fusion moiety [11]. The aberrant silencing or constitutive activation of fusion protein target genes lays the groundwork for leukemic transformation. This aberrant gene expression is largely attributed to the epigenetic modifications induced by each specific fusion protein on the malignant cells [16]. Integrated analysis of expression signatures, global DNA fusion protein binding pattern and induced chromatin modifications will provide a framework for improvement of new AML therapies.

PROJECT I: AML1-ETO fusion protein: DNA Binding Map and Chromatin modifications

1. To map the DNA binding pattern of AML1-ETO/HDAC1 complex characterizing and quantifying the associated chromatin modifications.
2. To identify the DNA binding motives of the AML1-ETO target regions.
3. To correlate the aberrant epigenetic modifications and AML1-ETO expression signatures.
4. To determine the reversibility of AML1-ETO induced chromatin modifications.

PROJECT II: Targeting Acetylation on MLL-Leukemia

1. To evaluate the *in vitro* sensibility of AML cell lines to the HDAC inhibitor panobinostat.
2. To map the MLL-AF9 DNA binding and induced chromatin modifications.
3. To correlate the aberrant epigenetic modifications with MLL-AF9 expression signature.
4. To determine the correlation between chromatin acetylation and transcriptional modifications after panobinostat exposure on MLL-AF9 leukemia models.

MATERIALS AND METHODS

1. Cell cultures, plasmids and patient samples

Umbilical cord blood CD34+ cells selected using immunomagnetic beads (Miltenyi Biotech), stable transduced with retroviruses expressing empty vectors (HSPC control), the MLL-AF9 (HSPC-MA9) or the AML1-ETO (HSPC-AE) fusion protein cDNAs. For AML-ETO knockout two loxP sites were located on both sides of the AML1-ETO construct, and the Cre recombinase cDNA was subcloned on the same MIGR1 retroviral expression. All cellular models were kindly provided by Dr. Mulloy (Cincinnati Childrens Hospital Medical Center, OH, USA). Previous studies describe and characterize these models in depth have been reported elsewhere [93]. In both models the MIGR1 vector, in which the murine stem cell virus long terminal repeat, known to be optimally expressed in human CD34+, drives the expression of the cDNA, was used (Figure 6). The HSPC cultures were maintained in IMDM, 20% with fetal Bovine Serum (FBS) for HSPC-MA9 or with the serum substitute BIT for HSPC-AE, β-mercaptoetanol, and the following cytokines: TPO, SCF, IL3, IL6 and FLT3-L (10ng/ml of each). The THP-1, NOMO-1 and K562 cell lines were maintained in RPMI with 10% FBS; while 20% FBS was used for SKNO1. All cell lines were obtained from the DSMZ repository.

The leftover material of 15 de novo AML t(8;21)(q22;q22) samples after diagnosis at the cytogenetic laboratories of the Universidad de Navarra (Pamplona, Spain) were used for RNA extraction. Informed consent was obtained according to the Declaration of Helsinki (October 2008**).**

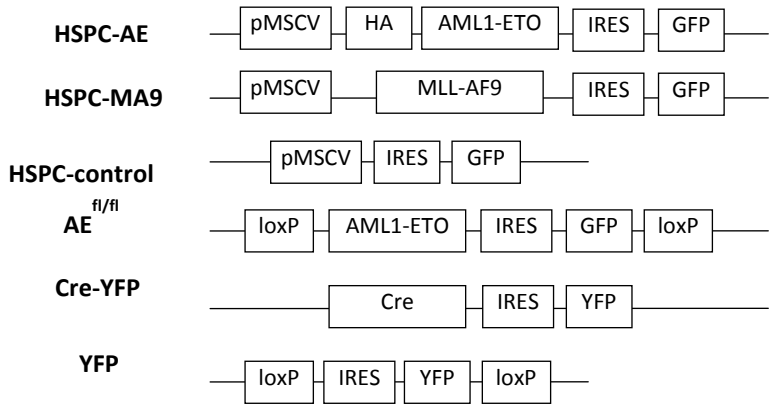


Figure 6. Schematic representation of all the constructs used.

2. *In vitro* studies

2.1. Antibodies

The antibodies used for immunoblotting and/or chromatin immunoprecipitation assays were the following: anti-HA (clone H9658) and GAPDH (monoclonal antibodies, CNIO); HDAC1 (PA1-860) from Affinity BioReagents; H4ac (06-598), H3k79me2 (04-835); H3K4me3 (17-614); and Sp1 (17-601) from Millipore; H3K9me3 (ab8868), SUV39H1 (ab12405), RNA polII (ab5408) from Abcam; MLL N-terminus (A300-086A) and AF9 C-terminus (A300-597A) from Bethyl; and CDKN1A (sc-397) from Santa Cruz.

2.2. Immuoblotting

Whole cell lysates were loaded onto SDS-PAGE gels. After transfer, PVDF membranes (Millipore) were incubated with primary antibodies for 1h or overnight, followed by either DyLight conjugated secondary antibodies (Thermo Scientific) and *LICOR* platform analysis (Biosciences) or Chemiluminiscence HRP substrate (Luminata-Western, Millipore).

2.3. Quantiative RT-PCR

RNA was isolated using the RNeasy Mini Kit (Qiagen), then reverse-transcribed using TaqMan® Gold RT-PCR Kit (Applied Biosystems). cDNA was analyzed using quantitative PCR with SYBR Green thechnology (Applied Biosystems) with the 7900HT Fast Real-Time PCR System (Applied Biosystems). Pre-developed TaqMan Assays (Applied Biosystems) and in house designed primers purchased from Sigma-Aldrich were used. All the analysises were normalized to endogenous PGK or GAPDH gene expression. The relative or absolute mRNA levels using ΔCt method or quantity referred to the standard curve was calculated. Standard curves were performed to assess the good quality of the primers. When using SybrGREEN dissociation curves were performed for each set of primers. All primer sequences used are shown in tables 1 and 2.

Table 4. Pre-developed Taqman Assays to study AML1-ETO target genes.

Assay ID	Gene Symbol	Target Exons	Context Sequence	Dye
Hs00231079_m1	AML1	5	GTCGACTCTCAACGGCACCCGACCT	FAM
Hs00923894_m1	CDKN2A	2	GGTCCCTCAGACATCCCCGATTGAA	FAM
Hs00736972_m1	YES1	1	TCCTGCTGGTTTAACAGGTGGTGTT	FAM
Hs00180312_m1	MLLT3	9	ACCAACAACAACCAGATTCTTGAAG	FAM
Hs00902008_m1	CTCF	11	ACAGAACCAGCCAACAGCTATCATT	FAM
Hs01046830_m1	MAPK1	6	GCTGACTCCAAAGCTCTGGACTTAT	FAM
Hs01009006_m1	SIRT1	8	AGATTAGTAGGCGGCTTGATGGTAA	FAM
Hs00357218_g1	RPS19	1	CGGAGGCCGCACGATGCCTGGAGTT	FAM

Table 5. qRT-PCR primers used to study MLL-AF9 target genes.

Gene symbol	Forward	Reverse
PGK	GGGAAAAGATGCTTCTGGGAA	TTGGAAAGTGAAGCTCGGAAA
MEIS1	GCATGAATATGGGCATGGA	CATACTCCCCTGGCATACTTTG
HOXA9	AAAACAATGCTGAGAATGAGAGC	TATAGGGGCACCGCTTTTT
PBX3	CAATCACAGGTGGATACCCTC	GGAGAAGTCACAGAAGATGGAG
JMJDC1	AATGGCATTCTCTCAAAAGCAG	CTTGTCAACATCTTTCCCAGC
ARID2	TATAGAGCAAGTCCAAACCCAG	GAGCATTTAGCCACTGACAAG
SATB1	CTTTAAAACACTCGGGCCATC	CCTTTCTCACCAGCACAAATTC
GATA2	TTCAATCACCTCGACTCGC	GCTGTGCAACAAGTGTGG
ALDH1A1	AGCAGGAGTGTTTACCAAAGA	CCCAGTTCTCTTCCATTTCCAG
DNMT3B	CCCATTCGAGTCCTGTCATTG	TTGATATTCCCCTCGTGCTTC

2.4. Flow cytometry

Cells were analyzed on FACSCanto II (BD Biosciences, San Jose, California, USA). Anti-human CD11b and CD14 were from BD Biosciences. DAPI was used to exclude nonviable cells.

2.5. Cell cycle and analysis of apoptosis

Apoptosis was analyzed using DAPI staining, FITC annexin V and annexin V binding buffer (BD Biosciences). The cell cycle was analyzed using ethanol fixation and treatment with 100 ug/ml ribonuclease and 50 ug/ml propidium iodine (Sigma-Aldrich, St. Louis, Missouri, USA).

2.6.Chromatin immunoprecipitation (qChIP)

Cells were harvested and subjected to chromatin immunoprecipitation (ChIP), as described previously [94]. Antibodies against specific histone modifications, AML1-ETO or MLL-AF9 were used to precipitate DNA fragments associated with those modified histones or proteins respectively. The immunoprecipitation was done using Protein A/G Plus-Agarose beads (Santa Cruz) and DNA was purified with phenol/clorophorm extraction followed by ethanol precipitation. Eluted DNA fragments were analyzed by quantitative PCR using promoter specific primers. Quantitative PCR (qPCR) of ChIP products was performed on an ABI 7900HT sequence detection system with Power-SYBR Green master mix (Applied Byosistems). The percentage of immunoprecitipated DNA relative to input was calculated for each experiment. Primers and cover promoters used as positive controls for the different antibodies are included in table 3.

Table 6. Primers used as positive control for each antibody in the qChIP expermiments. The transcript reference or the oligomer sequences of each is shown.

Antibody	Promoter cover by the primer	reference / sequence
H3K9me and SUV39H1	SATa (heterochromatic region)	NC_000001.10 (+)1A SABiosciences
H3K4me3, H3K79me2 , H4ac and RNA pol II	GAPDH	Forward:TACTAGCGGTTTTACGGGCG Revese: TCGAACAGGAGGAGCAGAGAG
MLL-AF9	HOXA11	NM_005523.5 (+)01Kb SABiosciences
AML1-ETO	CDKN2A	NC_000009.10 (–)01A SABiosciences

ChampionChIP One-Day kit (SABiosciences Corporation) was used for HA-AML1-ETO ChIP, according to manufacturer's protocol. In order to determine a reliable baseline for enrichment of AML1-ETO binding, we used the media of three negative

controls (ELA2, IRF2, and DLEU2) (ref. taken from Gardini et al [41]). The primer sequences used are shown in Table 4 and 5.

Table 7. Primers used for qChIP validation of AML1-ETO target genes Further information about the primers can be found at SAbiosciences website.

Gene symbol	Chromosome RefSeq	Primer reference	Assay position
MAPK1	NC_000022.9	GPH022094(+)01A	681
CTCF	NC_000016.8	GPH005067(+)01A	–18
MLLT3	NC_000009.10	GPH026139(+)01A	836
RPS19	NC_000019.8	GPH006636(+)01A	–399
AML1	NC_000021.7	GPH021919(–)01A	–743
SIRT1	NC_000010.9	GPH001660(+)01A	–393
YES1	NC_000018.8	GPH019642(+)01A	–387
CDKN2A	NC_000009.10	GPH026158(–)01A	–446
ELA2	NC_000019.8	GPH006142(+)01A	601
DLEU2	NC_000013.9	GPH017330(–)1A	–801
IRF2	NC_000004.10	GPH023603(–)1A	–515
SATa	NC_000001.10	GPH110005C(+)1A	-508
GSK3	NC_000019.8	GPH020330(-)1A	-420
OCLN	NC_000005.8	GPH010205(-)1A	-348
SOX18	NC_000020.10	GPH1022261(-)1A	-568

Table 8. Primers used for qChIP validation of MLL-AF9 target genes.

Gene symbol	Forward	Reverse
MEIS1	TCACCACGTTGACAACCTCG	CAGCTGGAGTGGCAGAAAGC
MEIS1 (at TSS)	GGCGCAAAGGGTACGTATTA	CGGCTTTAGGAGCCTCATTT
HOXA9	GCATTAAACCTGAACCGCTG	GGAGAACCACAAGCATAGTCAG
PBX3	GGATGGACGATCAATCCAGGATGC	TGGTCATGATCTGGTGGAGGAT
SATB1	TGGAGACCTTGTCCGCAGTGATTT	TGAGCTGTACACTGTGATGGTG
BMI1	AAACGGGACCCATAGATGTGGTGA	TGTATGCACTGCACTCCAGCAAT
DLEU1	CAACGCTGCGAGAGCAAATGACAA	AGGTTGTAGCGGGCTCTTAGGA
SOX4	AGAGCGCTCGTGAACTGGAATCAA	TGCTGGTTGCTGAAGCTTCCAA

JMJDC1	TACTTTCCCGCGGCCCTCTTCATTT	AACCACCGCAGACGGAAAGTA
ARID2	AGCAGCACCATGTTGAATCGCGT	AATCGGAGCGAAACTAGAAGG
GATA2	AGACATGCCACACACTGCTACAGA	TGGAAATGGTTGTGCGTCCCTT

2.7. Dug studies

Cells were plated in triplicate with titrating doses of panobinostat (purchased from Selleck) (range, 0 µM to 1 µM) for 72 hours. After adding WST1 cell proliferation reagent (Roche, Basel, Switzerland) plates were read at 450 nm to measure optical density. Three independent HSPC-MA9 clones were used for gene expression profiling after culture with 30 nM panobinostat for 6 and 24 hours, RNA extraction, and quality assessment with the Agilent 2100 Bioanalyzer.

3. High-throughput studies

3.1. Gene expression analysis

Sureprint G3 Human Gene Expression 8x60v2 microarrays were hybridized (Agilent Technologies) in NIMgenetics (Madrid, Spain). Arrays were examined using the DNA Microarray Scanner C (Agilent Instruments) and images were extracted with Agilent Feature Extraction software (v10.5). The transcript profiling data from HSPC-MA9 (n=9), HSPC-AE (n=5) and HSPC control samples (n=3) (GSE8023)[77, 95], MLL-rearranged primary samples (n=11) and control cells (peripheral blood and bone marrow aspirates from normal donors) (n=8) (GSE1159)[96] were downloaded from Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo). Differentially expressed genes were obtained using Bioconductor's limma package [97] (Bioconductor project). The estimated significance level (p value) was adjusted using the Benjamini & Hochberg False Discovery Rate (FDR) correction. Those genes with a FDR <0.05 were selected as differentially expressed between conditions.

3.2. Characterization of AML1-ETO/HDAC1 DNA binding and induced Chromatin modifications

3.2.1. Chromatin immunoprecipitation with microarray technology (ChIP-chip)

Three biological replicates of 10 million 9-weeks-cultured cells per ChIP experiment were prepared and hybridized on the same array set. The products were amplified using the GenomePlex® Complete Whole Genome Amplification Kit (Sigma) and labeled using the Bioprime® DNA-Labeling System protocol (Life Technologies) with random priming and Cy5/Cy3 fluorescent dyes. One microgram of each labeled DNA was mixed and hybridized in Human Proximal Promoter ChIP-chip 244k arrays (Agilent Technologies) for up to 40 hours at 65°C at 20 rpm. Agilent scanner was used and images processed with Agilent Feature Extractor software application (v.9.5.3.1).

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. 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 (see Results). The relationship between each probe and the nearby gene or transcript identified is defined (assuming a 10kb window) as: “promoter” (when the probe is upstream the TSS), “inside” (when the probe is inside the gene), or as “divergent promoter” (when the probe is upstream of two genes that are transcribed in opposite directions). The HSPC-AE filtered gene lists derived from each experiment were crossed to find the overlapping genes.

3.2.2. Sequence analysis of AML1-ETO-binding regions

Gene data sets identified in the ChIP-chip approach were analyzed through the oPOSSUM motif search system [98] to identify AML1 transcription factor binding sites (TFBS). An area of –5 kb to +5 kb around the TSS was scanned using the vertebrate-specific PSSMs in the JASPAR database [99]. A predicted binding site was considered as PSSM score of <85% and a minimum human-mouse conservation of 70% was observed. To identify other TFBS targeted by complexes that include the AML1-ETO fusion protein and other DNA-binding factors, two unsupervised prediction methods were used, namely, the STAMP [100] and PSCAN [101] algorithms. For the multiple alignment motif study with the STAMP algorithm, the nucleotide sequences were extracted using

the University of California Santa Cruz genome browser (UCSC, human database release Hg18, March 2006) using the standard settings. Each input motif was compared with JASPAR and TRANSFAC using the STAMP algorithm (Sandelin and Wasserman test) to recognize potential similarity with other known TFBS. The obtained list was crossed with the list of known AML1 or ETO interacting proteins downloaded from the STRING database and BioGRID database to select the putative AML1-ETO-complexed transcription factors .The TFBS information was also extracted using the PSCAN web tool, analyzing an area of 1kb upstream the TSS of each gene. Two lists of known TFBS were obtained, one using the JASPAR database and the other using TRANSFAC. Both tables are on Annex I.

3.3. Characterization of Mll-AF9 DNA binding and induced Chromatin modifications

3.3.1. Chromatin immunoprecipitation sequencing (ChIP-seq) protocol

We performed ChIP-seq assay to study the MLL-AF9 oncoprotein DNA binding and induced chromatin modifications. The ChIP precipitated DNA of ~10 million cells with the different antibodies were independently processed into sequencing libraries with the ChIP-Seq sample preparation kit (Illumina), in accordance with the manufacturer´s instructions (Illumina). Libraries were prepared from ~200bp DNA fractions. Input samples from HSPC-MA9 and HSPC controls were pooled in a single library. Libraries were sequenced in a Genome Analyzer IIx (GA2, Illumina) single 36-base read run. Raw sequences were defined as reads passing purity filter before genome alignment. Alignment was performed with BWA versus the human sequence assembly GRCh37/hg19 (Feb 2009) under default settings permitting alignments with 1 mismatch in 36 base reads.

3.3.2. ChIP-seq Analysis

Findpeaks 3.1 [102] was used to identified ChIP-seq MLL and AF9 binding regions. Calibration was performed with the control input, and peaks with a confidence less than 99.9 % were rejected. Subpeaks (0.7) and trim (0.95) were enabled. A custom program was run to search Ensembl database and to find the first gene that lays in sense and antisense direction taking as reference point the defined peaks. Thus, a

window of 5000 nt upstream and 200 nt downstream of the origin of transcription for each gene was defined and the closer gene to the short reads peak was taken. The process was done on sense an antisense strand of the chromosome. The maximum number of genes associated to a peak was two (one in sense and the other in antisense). Software SICER 1.1 [103] was used to analyze the ChIP-enriched signals for the histone modifications H3K4me3, H3K79me2 and H4ac on HSPC-MA9 and HSPC cells. It was used following developer's technical recommendations for histone modifications (200-bp window/ FDR≤ 0.01). The software pipeline for finding differential peaks in pair-wise comparisons (HSPC-MA9 vs HSPC) was applied and significant ChIP-seq peaks were filtered at FC<1.5 and FDR ≤ 0.001.

The generated bed files were uploaded to the USCS genome browser (http://genome.ucsc.edu/) to visualize the enrichment peaks obtained with both Findpeaks and Sicer analyses. Venn diagrams used for gene set comparisons were performed at http://bioinfogp.cnb.csic.es/tools/venny/index.html

4. In silico data analysis

Gene lists were functionally annotated using Ingenuity Pathways Analysis software (Ingenuity® Systems, 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 (Agilent) used as reference for ChIP-chip gene sets. The significance level cut off used was p <0.05 after Benjamin-Hoechberg multiple testing correction (B-H p-value). Gene Set Enrichment Analysis (GSEA) software [104] was used to determine the global effect of gene transcription. Gene sets were considered to be significantly associated with expression when a FDR less 0.25 was present; using a ttest permutation (1000 permutations) of gene sets comparison. The enrichment score from the gene set after it has been normalized across analyzed gene set (the so called NES) was also shown.

5. Accession numbers

All the high-troughput data generated have been deposited in GEO (http://www.ncbi.nlm.nih.gov/geo/) and can be retrieved as GSE27663

RESULTS

PROJECT I. AML1-ETO fusion protein: DNA Binding Map and Chromatin modifications

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

1.1. Analysis of ChIP-chip arrays

Three HSPCs 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, shows after 5-weeks, in culture a progressive differentiation blockage with a significant increase of CD34+ cells with an immature myeloid morphology, compared to the control cells which predominantly complete the terminal differentiation [44]

In order 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 (Table S1) and 6 miRNAs bound by AML1-ETO, and 1826 genes and 10 miRNAs by HDAC1 protein ($p(X)<0.001$; Normalized Log Ratio>0.7) (Tables 9 and 10). Interestingly, only 10% of the 1168 identified AML1-ETO targets showed a significant increase on H4Ac compared to the HSPC control cells, indicating the presence of a global deacetylation process. However, only 103 AML1-ETO target genes were identified associated with HDAC1 binding. This result suggests that other HDAC class I enzymes may be playing a role inducing the observed deacetylation signature, confirming previous studies that proposed a combinatory effect of HDAC class I enzymes on the generation of a repressive chromatin landscape.

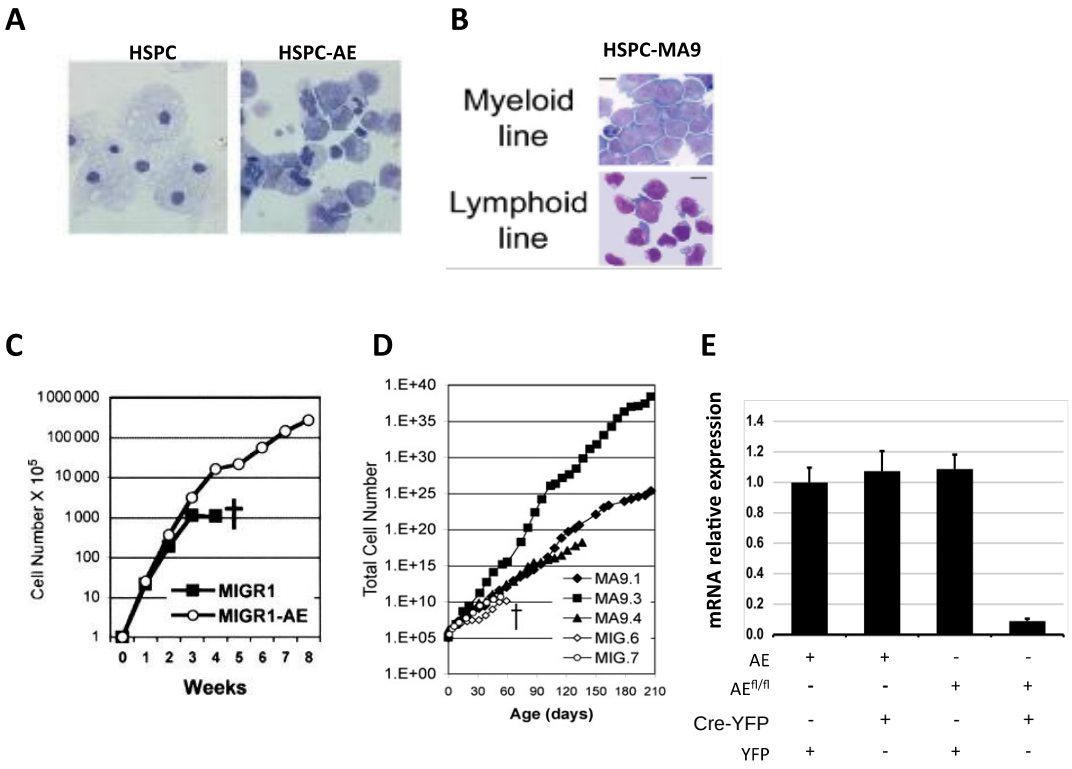


Figure 7. Characterization of the cellular models used (B-E are modified from Mulloy, 2002 & Wei, 2008). (A-B) Whrigh-Giemsa staining of nonadherent cells after 5 weeks in culture showing macrophague morphology in the HSPC cultures and an immature myeloid phenotype in the HSPC-AE (A), or in cytospins of long term cultured HSPC-MA9 cells under myeloid and lymphoid conditions (B). Magnification is 1000x. (C-D) Growth curve charts the differences in proliferative capacity between HSPC (MIGR1) and HSPC-AE (MIGR1-AE) (C) or HSPC (MIG) and HSPC-MA9 (MA9) cells (D). (E) qRT-PCR of AML1-ETO expression levels at 48 hours upon removal of AML1-ETO in the HSPC-floxed system.

Table 9. Summary of the results obtained in each ChIP-chip experiment.

Samples studied (Array sets analyzed)	3 HSPC-AE (1) 3 HSPC (1)	3 HSPC-AE (1) 3 HSPC (1)	3 HSPC-AE (1) 3 HSPC (1)	3 HSPC-AE (1) 3 HSPC (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 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

Among the 103 genes co-occupied by both AML1-ETO and HDAC1, 75% showed a distance of less than 1200bp between AML1-ETO and HDAC1 probes. Thus, these 103 genes could be considered AML1-ETO/HDAC1 complex functional target genes (Figure8B; Table S3). To explore the histone H3 lysine 9 trimethylation (H3K9me3), another previously known AML1-ETO related silencing chromatin mark [51], ChIP-chip analysis of HSPC-AE samples was performed. An enrichment of the chromatin mark H3K9me3 on 1102 promoter regions compared to the HSPC control was observed (Table 9).

Table 10. 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

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 1200bp (Figure 8C; Tables 10 and 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 presence of H3K9me3 and deacetylated histone H4 are exclusive repressive mechanism.

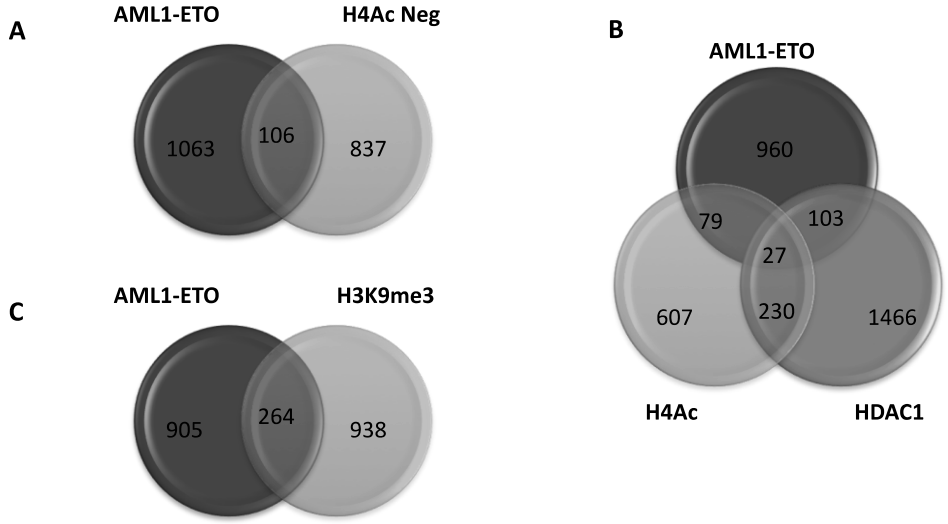


Figure 8. Number of overlapping target genes identified by ChIP-chip analysis. Each Venn diagram represents comparisons between the AML1-ETO, HDAC1, H4ac or H3K9me3 ChIP-chip results. H4ac-neg stands for those regions that showed significant H4ac presence in HSPC controls but not on HSPC-AE.

1.2. Analysis of biological functions and pathways directly altered by AML1-ETO

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) (Table S2-S6) according to IPA (Ingenuity® Systems, www.ingenuity.com). Furthermore, among the 103 targets of the AML1-ETO/HDAC1 complex the TGF-β pathway (i.e. *MAP3K7, MAPK1, RUNX3, SMAD7*) and ERK/MAPK pathway (i.e. *ELK3, PPP1R12A, YWHANG*) were found enriched (Figure 9 and Tables S4a-b); while the AML1-ETO-H3K9me3 associate with genes within the canonical Wnt/β-catenin pathway (i.e. *AXIN2, GSK3A, PPARD, SOX18, SOX7, WNT9A*) and canonical stem cell pluripotency pathway (i.e. *BMP2, FGFR1 GNAS, SMAD7, WNT2B*) (Figures 8B and 9; Tables S6a-b). In light of these data, we conclude that the presence of the H3K9me3 or deacetylated histone H4 at the AML1-ETO target genes are enriched on different biological pathways but targeting the same functional categories.

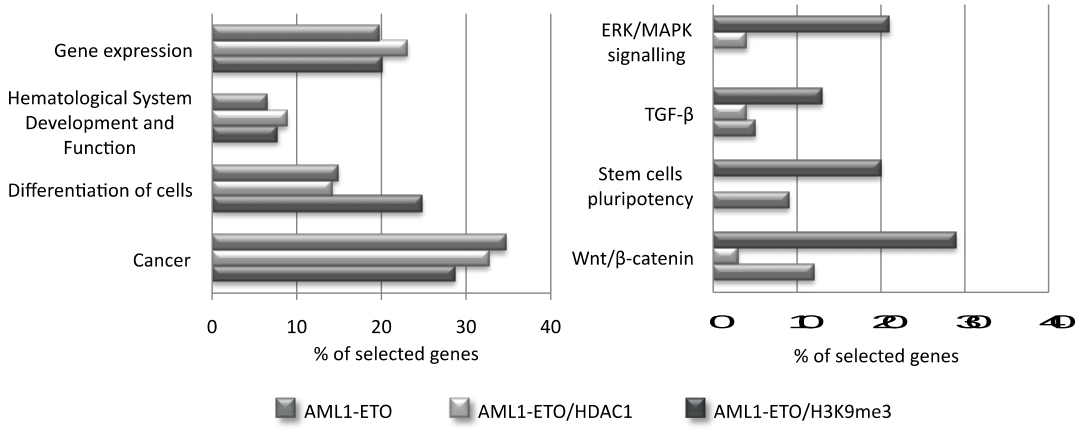


Figure 9. Functional characterization of AML1-ETO target genes in the presence of silencing chromatin marks. The number of genes significantly associated on biological functions (left) and canonical pathways (right) of each gene sets are shown

1.3. qChIP validation of AMl1-ETO functional targets

Hematopoietic differentiation blockage was the main feature observed on the functional characterization of the HSPC-AE cellular model [44] and one of the specifically enriched functions among the AML1-ETO functional targets. Thus, for validation purposes 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 hyper-acetylation compared to the HSPC controls (Figure 10A-C). 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 over 800bp (Table S3). Interestingly, no differences on the occupancy of HDAC1 at MLLT3 promoter was 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 5 selected genes (*MLLT3, RPS19, GSK3, SOX18, OCLN*) among the list of AML1-ETO-H3K9me3 targets confirmed the significant enrichment of H3K9me3 in HSPC-AE cells compared to HSPC controls (Figure 10D), but no significant differences were observed for the co-occupancy by SUV39H1 in the identified AML1-ETO-H3K9me3 target genes (data not shown). 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 methyltransferase activity associated with AML1-ETO.

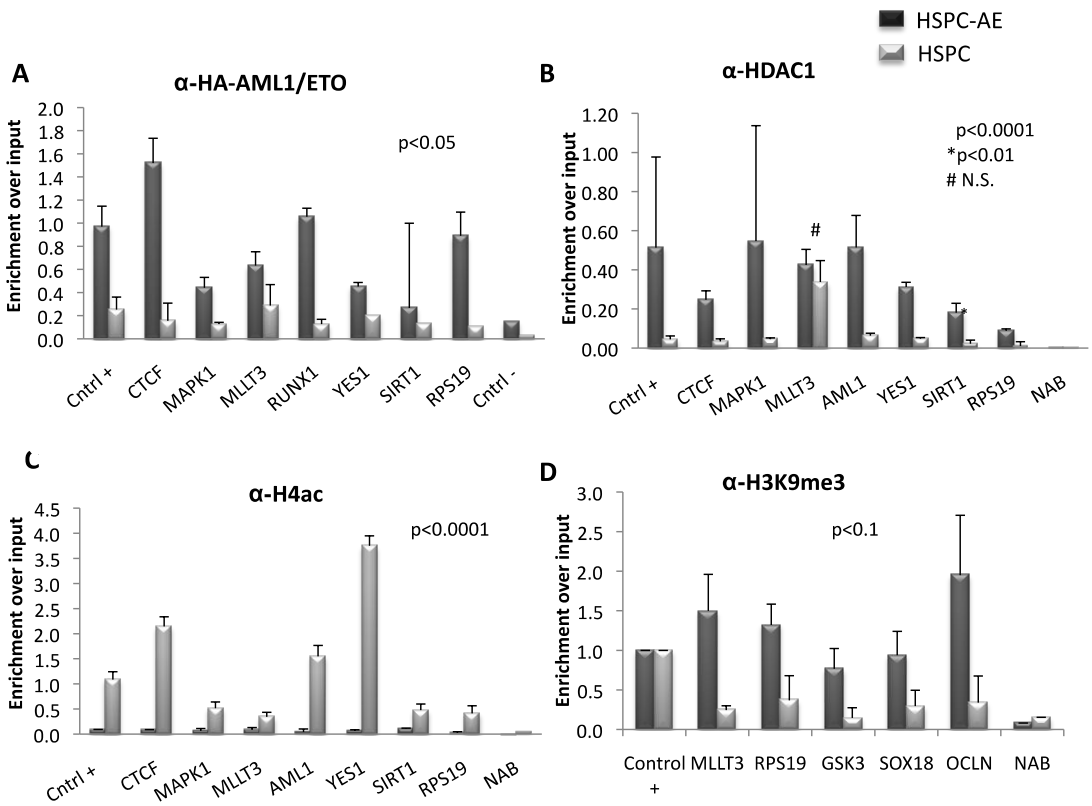


Figure 10. qChIP analysis of AML1-ETO target genes in HSPC-AE and HSPC cells. qChIP was performed on the promoter of selected AML1-ETO identified target genes using antibodies against (A) HA-tagged AML1-ETO, (B) HDAC1, (C) H4Ac, and (D) H3K9me3. The negative control for the enrichment of HA-AML1-ETO binding was calculated using the media of three negative controls (ELA2, IRF2, and DLEU2) taken form Gardini et al. The $p14^{ARF}$ promoter was used as positive control for figures A-C; heterochromatic SATa region was used as postive control for figure D. Each experiment was replicated at least twice. Bars represent standard error.

2. DNA binding sequence analysis of functional AML1-ETO targets

2.1. DNA Sequence Analysis

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 (TSS). AML1-ETO binds DNA through the AML1 RUNT domain, mainly at AML1 transcription factor binding sites (TFBS), although association with other motifs (e.g. E-box motifs) has been previously described [34]). In our study, an AML1 TFBS was identified at over 30% of AML1-ETO functional targets (Figure 11B-C; Table S1), suggesting that the AML1-ETO

DNA association may rely on additional transcriptional complexes with different sequence specificity.

Detailed sequence analyses using unsupervised methods (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 TSS using the oPPOSUM motif search system, revealed that 50% of the AML1-ETO target genes present this binding motive (Fisher 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 [105].

2.2. Analysis and validation of the association between Sp1 at AML1-ETO fusion protein

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 <0.05) according to Ingenuity Systems Analysis. Furthermore, the canonical Wnt/β-catenin pathway (i.e. *AXIN2, CCND1, CDH3, FZD7*) and canonical stem cell pluripotency pathway (i.e. *BMP2, FGFR1, WNT2B*) were found enriched. A significant enrichment of Sp1 presence in all the promoters studied was confirmed in the HSPC-AE samples compared to HSPC controls (Figure 11C). 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 finding further supports the previous data of AML1-ETO and Sp1 protein interaction, and indicates that AML1-ETO and Sp1 bind DNA as a complex[106]. Furthermore, our study indicates that this DNA binding mechanism occurs in a genome wide manner.

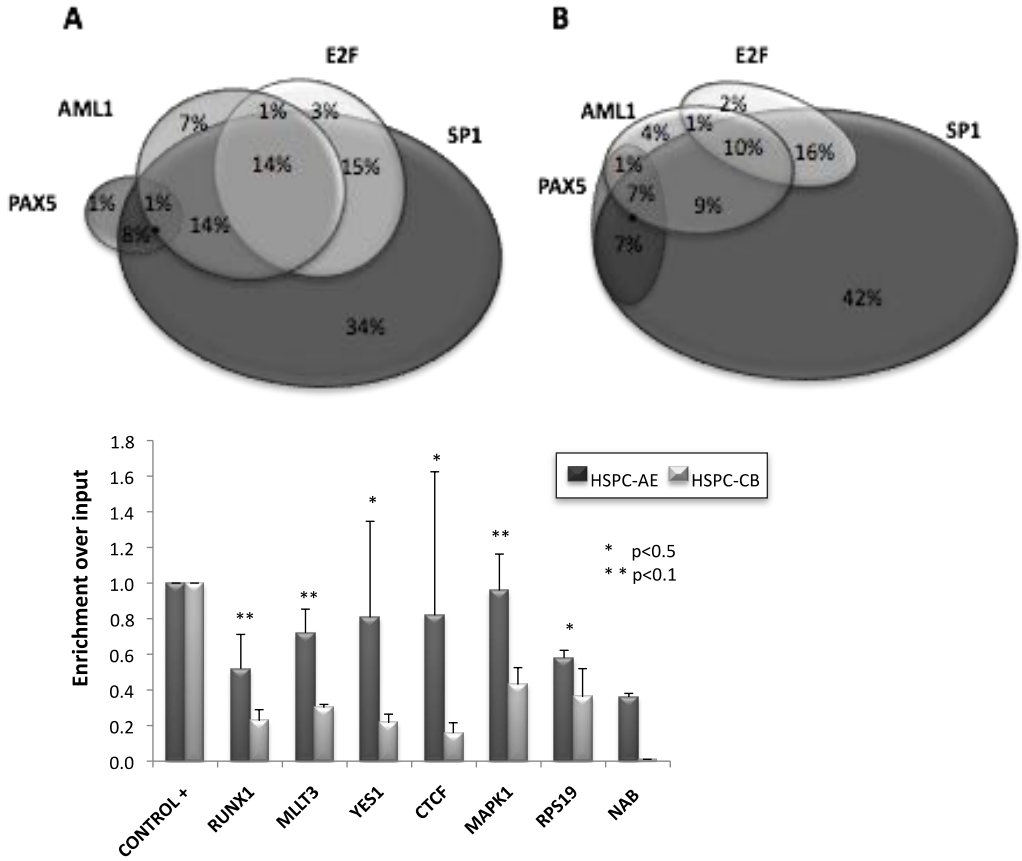


Figure 11. Transcription factor binding sites analysis identified Sp1 binding presence in AML1-ETO target genes. (A) Distribution of the identified TFBS among the 103 AML1-ETO/HDAC1 target genes with deacetylated histone H4. A known motif was found in 68% (71/103) of the genes. (B) 264 AML1-ETO target genes with the H3K9me3 chromatin mark. A known motif was found in 58% (153/264) of the genes. (C) qChIP was performed using an anti-Sp1 transcription factor antibody on 2 HSPC-AE and 2 HSPC-control cells. The DHFR gene was used as a positive control. Error bars represent standard errors.

2.3. Role of Sp1 transcription factor in AML1-ETO expressing cells

The biological importance of Sp1 on t(8;21) AML was further studied by lentiviral shRNA knockdown system. Interestingly, the HSPC-AE cells were highly sensible to Sp1 knockdown. The stably suppression of Sp1 leads to a complete abrogation of self-renewal of two independent HSPC-AE clones, and no viable cells were obtained after 2 weeks upon Sp1 knockdown (Figure 12A). To further study this effect the AML1-ETO derived cell line SKNO-1 and the MLL-AF9 cell line THP-1 were analyzed. A significant inhibition on Sp1 expression was obtained with lentiviral transduction on both cell lines with 2 selected shRNA sequences (Figure 12B). In SKNO-1 this silencing resulted in a significant growth decline and increased apoptosis, while no effect were observed on THP-1 (Figure 12C-E). Moreover, HSPC-AE and SKNO-1 cells showed high sensitivity to titrating concentrations of Mithramycin a, a previously known DNA binding inhibitor of

Sp1 protein [106], while THP-1 cells were fairly affected (Figure 13). Interestingly, we identified that Sp1 protein levels were augmented on AML1-ETO expressing cells compared to normal HSPC (Figure 12E-D), while no mRNA upregulation was found (data not shown). These results indicate that Sp1 protein is critical in myeloid leukemia driven by AML1-ETO. Further studies are needed to evaluate the mechanisms and the efficiency of Sp1 protein targeting drugs (as proteasome inhibitors like Bortezomib) in AML patients.

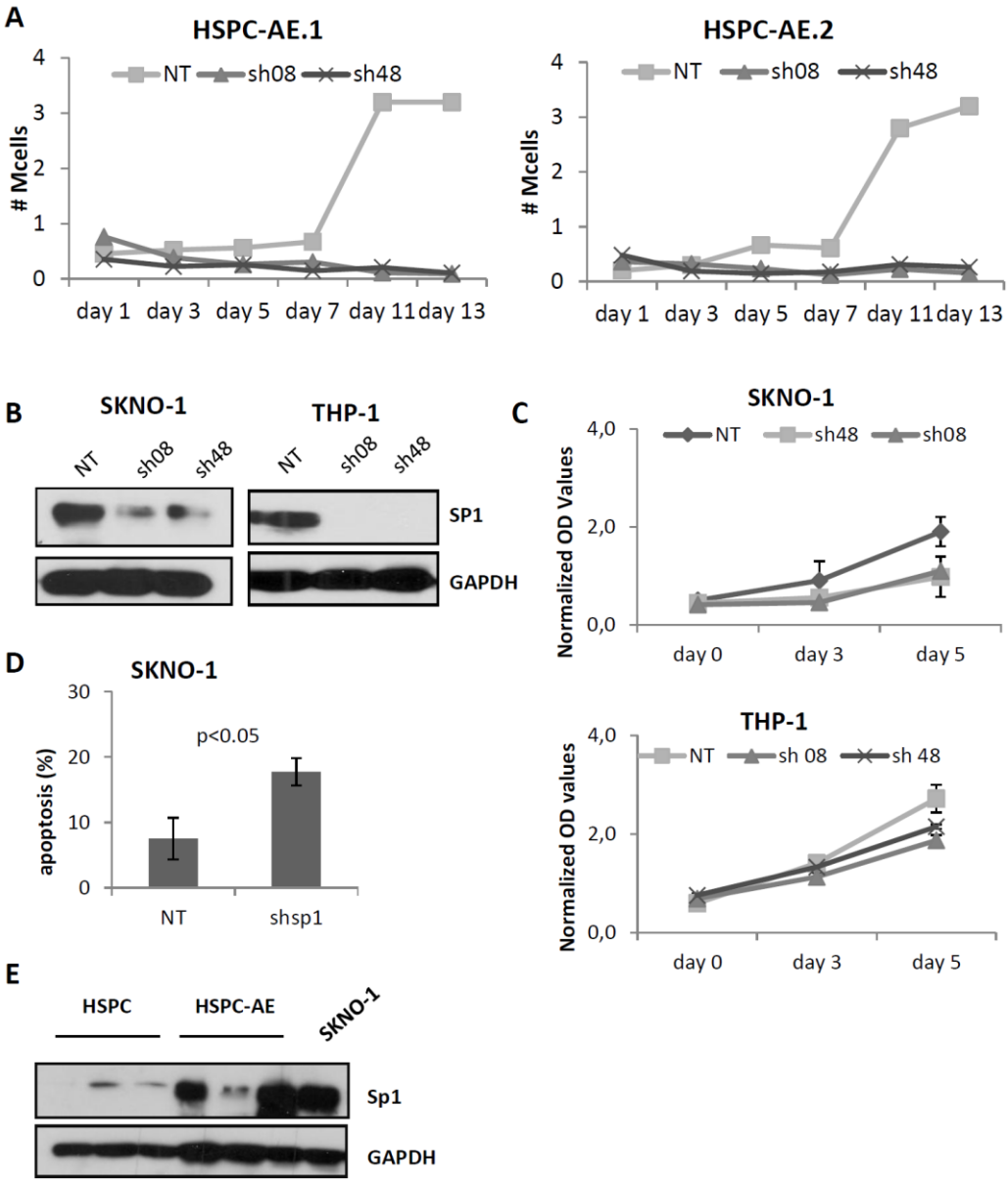


Figure 12. Sp1 knockdown impairs AML1-ETO cell survival and proliferation. (A) Lentiviral Sp1 knockdown impairs proliferation on two independent HSPC-AE clones after puromycin selection.(B) Sp1 immunoblot demonstrated the efficiency of Sp1 knockdown (sh08

& sh48) on SKNO-1 (left) and THP-1 cells (right). (C) Cells were selected with puromycin and then platted and grown in normal media; at the indicated times WST-1 mix was added. Averaged normalized OD values to day 0 are shown. (D) Two independent Sp1-knockdown and NT SKNO-1 cells were stained with annexin V were analyzed by flow cytometry. The averaged fold changes of 3 experiments are shown.(E)Immunoblot analysis of Sp1 protein on three independent HSPC-AE and HSPC clones. NT= non-targeting sequence.

3. Integrative analysis of AML1-ETO functional targets and transcriptional modifications

3.1. GSEA Analysis

To confirm the transcriptional repression conferred by the studied inactive chromatin marks a Gene Set Enrichment Analysis (GSEA) 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<0.0001). Interestingly, those deacetylated genes, with and without HDAC1 binding, showed the most significant correlation with repressed genes (FDR<0.0001 and FDR<0.001, respectively) pointing to H4 deacetylation as the most significant epigenetic mark induced by AML1-ETO binding (Table 11).

Table 11. 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

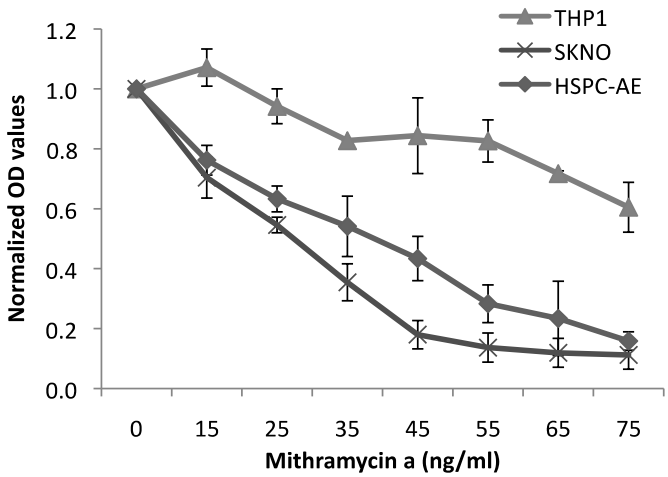


Figure 13. **Sp1 pharmacological inhibtition.** HSPC-AE, SKNO-1 and THP-1 cells were exposed 72 h to serial dilutions of Mithramycin a followed by WST-1 addition. Averaged normalized OD values to vehicle are shown.

3.2. Validation studies at selected genes on the HSPC model and primary samples

Individual analysis confirmed a significant downregulation ($p<0.0001$) of 6 out of 7 selected candidate genes previously studied for AML1-ETO, HDAC1, and Sp1 occupancy in HSPC-AE samples (Figure 14A). 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 10B). The expression status of these selected genes was analyzed in a set of 15 AML primary samples expressing the AML1-ETO oncoprotein. All the genes studied were found downregulated on the primary samples compared to the controls (Figure 14C), supporting the relevance of AML1-ETO epigenetic silencing of key genes on the leukemic process. However, different levels of significance were observed between the genes studied.

All the genes containing an AML1 binding site (i.e. *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 (Figure 14B). For genes containing an Sp1 TFBS (i.e. *CTCF* and *SIRT1*) only a moderate decrease on the mRNA levels was observed (Figure 14C). 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 or Sp1 proteins, or the presence of secondary events.

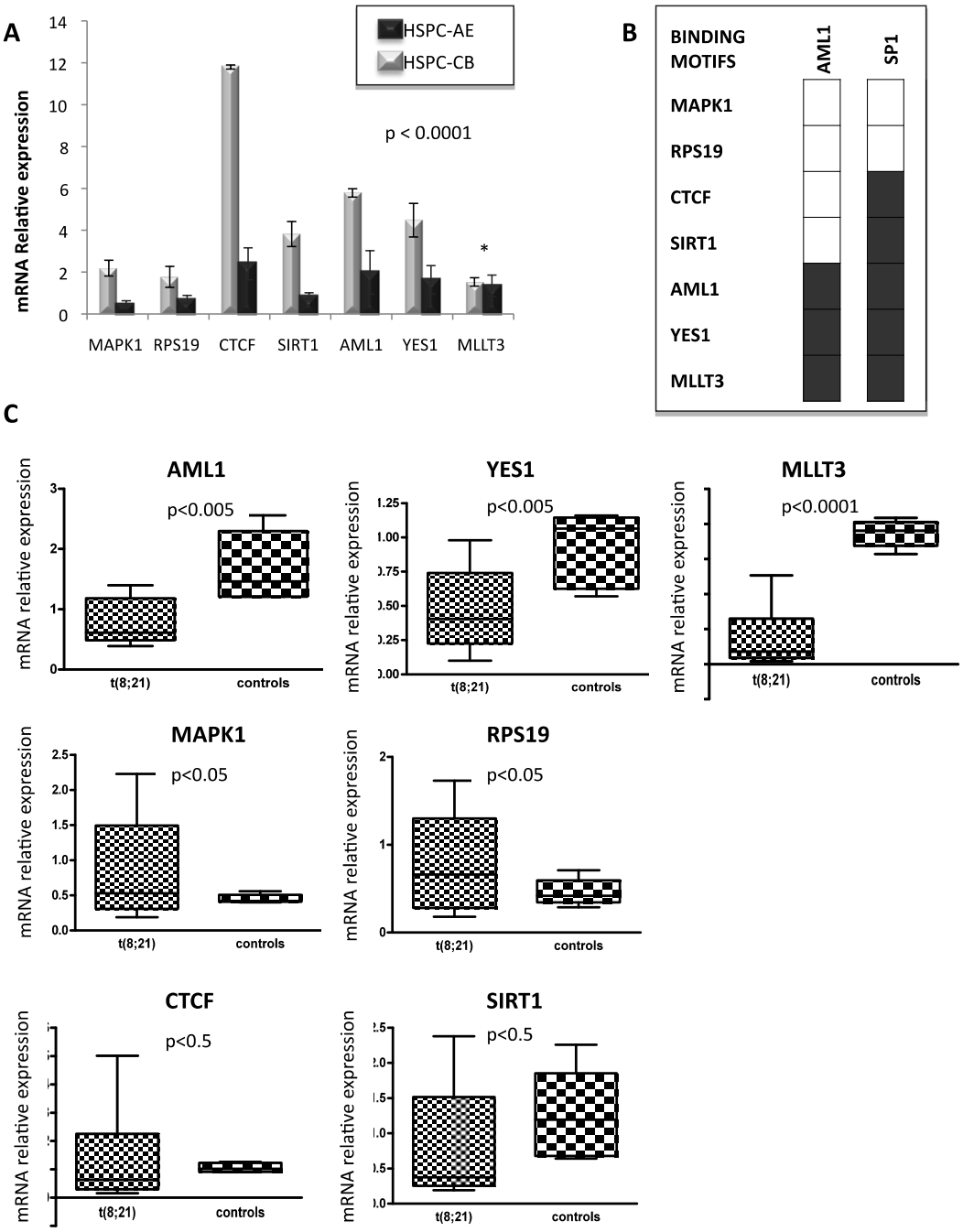


Figure 14. In vivo transcriptional analysis of AML1-ETO targets. (A) mRNA expression of 7 AML1-ETO/HDAC1 target genes on HSPC samples (*p=N.S.). (B) Schematic representation of the AML1 and/or Sp1 TFBS present in the 7 selected genes. (C) Box-plot representing the expression levels of the selected genes in 15 t(8;21) AML primary samples and CD34+ cells (controls). Error bars represent standard error.

4. Analysis of the reversibility of AML1-ETO induced transcriptional repression

4.1. AML1-ETO knockout HSPC model

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. AML1-ETO mRNA levels was found absent after 48 hours but no effect on HSPC proliferation was observed until day 10 (J.C. Mulloy, personal communication). 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 15). 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 (Figure 10B). 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.

4.2. Pharmacological inhibition of Sp1 DNA binding

The role of Sp1 in AML1-ETO target gene reversible repression was further elucidated by treating HSPC-AE clones with Mithramycin a. This exposure led to a decrease in RNA transcription only on those genes with an Sp1 TFBS at their promoter (*CTCF* and *SIRT1*) but showed no effect on those genes with also an AML1 TFBS (*AML1* and *YES1*) (Figure 16), supporting our hypothesis of Sp1 driving a reversible AML1-ETO binding and silencing to genes where Sp1 but not AML1 TFBS is present.

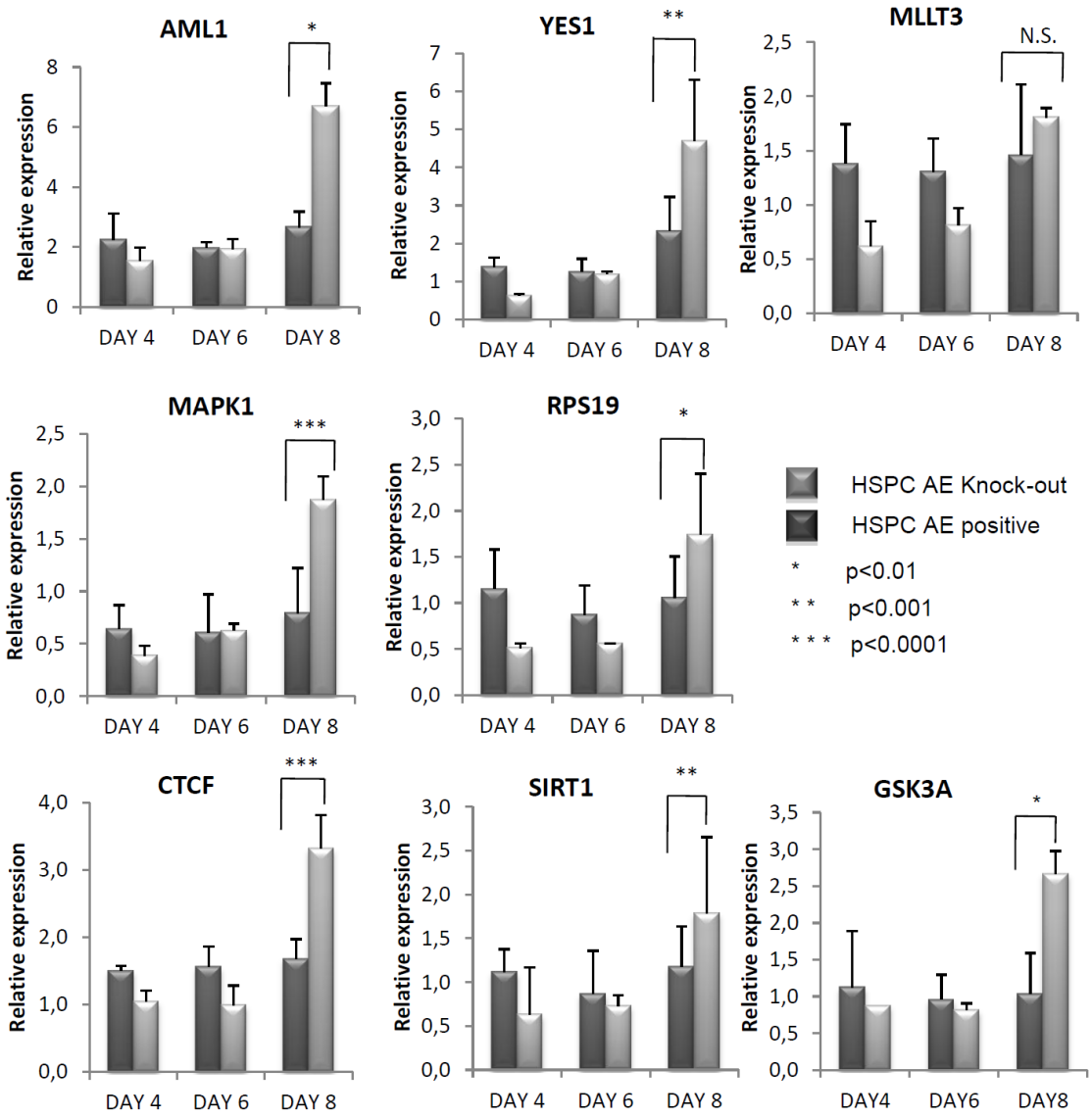


Figure 15. Knockout of AML1-ETO restores the expression of its target genes. qRT-PCR analysis shows increased expression levels in 6 of the 7 genes studied upon AML1-ETO knockout. Measurements of two independent AML1-ETO-ko clones were taken at indicated time points. Error bars represent standard error.

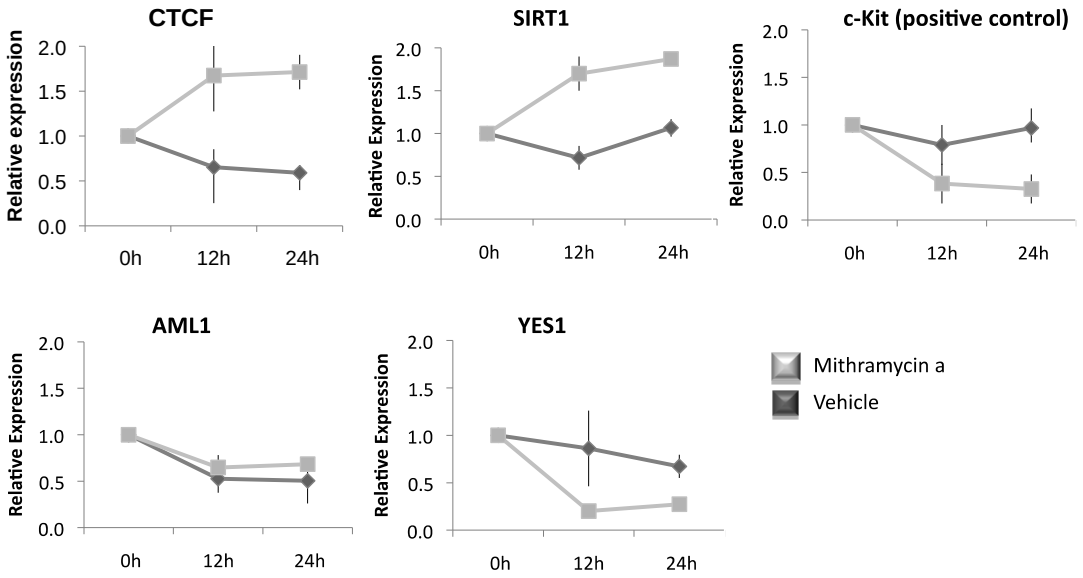


Figure 16. Mithramycin a restores the expression of AML1-ETO target genes with an Sp1 TFBS. qRT-PCR analysis upon Mytramicin A treatment (150 ng/ml). KIT mRNA levels were used as a control. of two independent AML1-ETO clones were taken at indicated time points.

PROJECT II. Targeting Histone Acetylation In MLL-AF9 Leukemia

1. *In vitro* analysis of efficacy of the HDAC inhibitor LBH589 on AML HSPC models and cell lines

1.1. Functional analysis of cell proliferation, apoptosis and cell cycle

Recent promising studies using HDAC inhibitors (HDACi) in several AML models [107] prompted us to evaluate the sensitivity to the pan-HDACi panobinostat of the HSPC expressing either MLL-AF9 (HSPC-MA9) or AML1-ETO (HSPC-AE). Unexpectedly, we observed that HSPC-MA9 clones showed a higher sensitivity ($IC50_{HSPC\text{-}MA9}$≈30 nM) than HSPC-AE ($IC50_{HSPC\text{-}AE}$≈200 nM) (Figure 17A). This finding was confirmed in the MLL-AF9-derived cell lines THP-1 and NOMO-1 (IC50≈5 nM) (Figure 17A). Additionally, cell viability in THP-1 and NOMO-1 was compared with the effects on the resistant BCR-ABL–derived cell line K562. No differences were observed at 6 hours. However, at 24 hours, massive induction of apoptosis was observed in MLL-AF9 cell lines, while no effects were found in K562 cells (Figure 17B). In parallel, with low doses (10 nM) and high doses (30 nM) of panobinostat, no differences in cell cycle were observed after 6 hours of treatment. However, after 24 hours at low doses, a G1 cell cycle blockage was observed in the MLL-AF9 cell lines, with no significant changes in the K562 cells (Figure 17C). Consistent with this G1-blockage effect, CDKN1A protein levels were induced in HSPC-MA9 cells after 24 hours of exposure to 30 nM of panobinostat (Figure 17D). When the cell lines were treated with high doses, the proportion of sub-G0 cells increased in THP-1 and NOMO-1, and a G1 cell cycle blockage was then observed in the K562 cell line (Figure 17C).

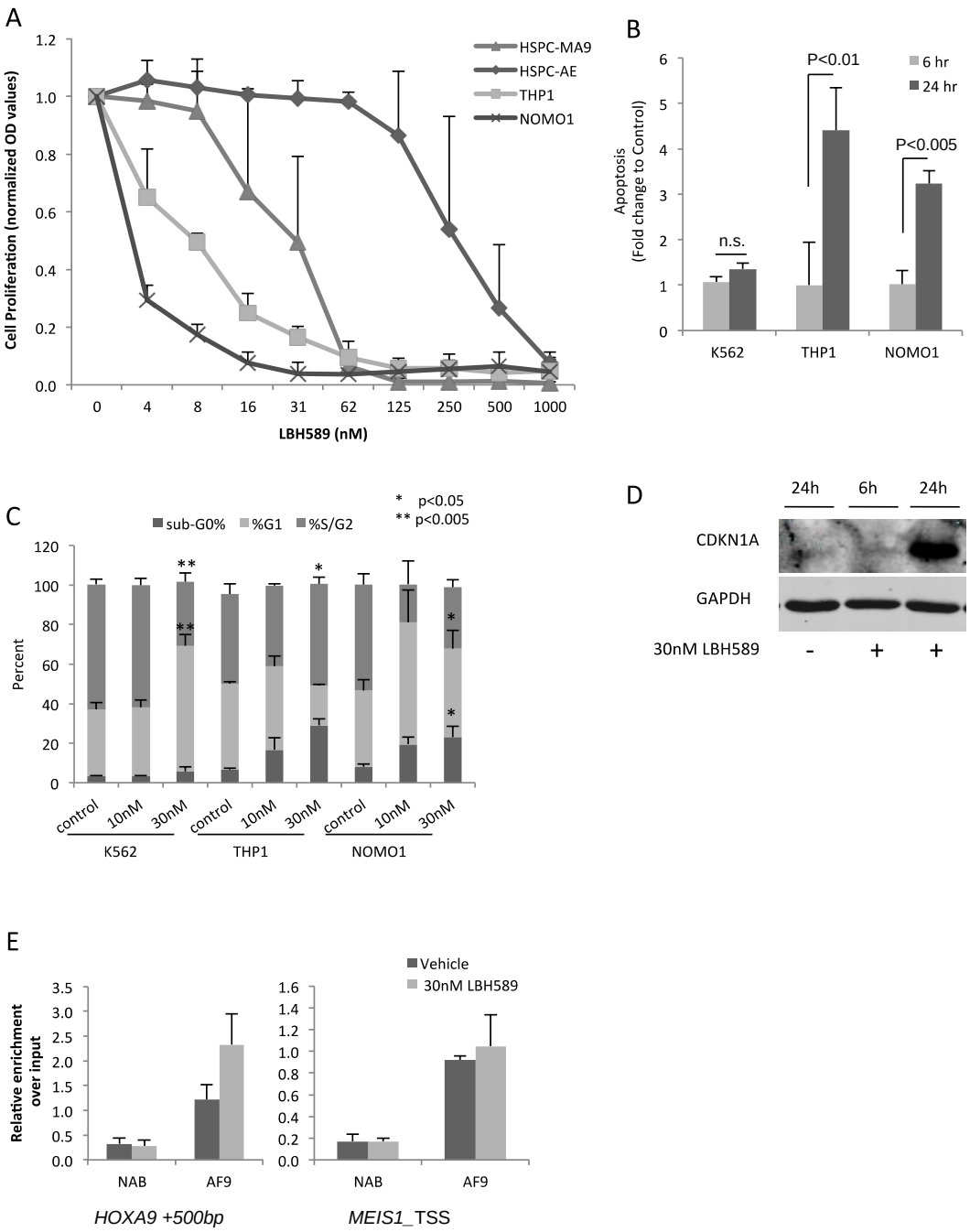


Figure 17. MLL-AF9 cells are highly sensitive to the HDAC inhibitor panobinostat. (A) HSPC-MA9, HSPC-AE, THP-1, and NOMO-1 cells were exposed for 72 hours to serial dilutions of panobinostat followed by addition of WST-1. Averaged normalized optical density (OD) values are shown compared to vehicle. (B) Cells exposed to 30 nM of panobinostat stained with annexin V for 6 and 24 hours were analyzed using flow cytometry. The plot shows the averaged fold changes of 3 independent experiments compared to vehicle. (C) PI incorporation of AML cell lines treated with vehicle and 10 or 30 nM of panobinostat for 24 hours. (D) Total CDKN1A immunoblotting of HSPC-MA9 cells exposed to 30 nM panobinostat for the indicated time points.

1.2. Analysis of MLL-AF9 fusion protein stability

HDAC inhibitors trigger protein degradation by deacetylation of key residues, a mechanism that has been described for AML1-ETO [108]. Thus, we checked whether deacetylation and MLL-AF9 degradation could also account for the sensitivity of HSPC-MA9 cells. Using qChIP with anti-AF9 C-terminal antibodies in THP-1, we found that MLL-AF9 was present on *HOXA9* and *MEIS1* promoters after treatment exposure (Figure 17E). These results indicate that MLL-AF9 fusion protein is able to bind its target genes after treatment with HDAC and led us to rule out protein degradation as the cell death–inducing mechanism.

1.3.Transcriptional changes induced by panobinostat exposure

We performed gene expression analysis to gain insight into the molecular consequences and mechanisms underlying the high sensitivity of HSPC-MA9 to panobinostat. We evaluated H4 acetylation to confirm the effectiveness of the drug and observed a global increase (Figure 18A). Significant upregulation of 90 and 249 genes (FC>2; FDR<0.05) was observed after 6 and 24 hours, respectively, of treatment with panobinostat (Figure 18B, Table S9); 60% of the early-activated genes (52 genes) were also activated at 24 hours. As previously reported using HDACi (ie, SAHA and depsipeptide) [109], we also observed significant transcriptional downregulation of 26 and 139 genes after 6 and 24 hours, respectively, with 27% of the early downregulated genes (7 genes) also silenced at 24 hours (Figure 18B).

We performed gene set enrichment analysis (GSEA) to determine whether our previous observations of cell cycle blockage and increased apoptosis in MLL-AF9 cells (Figure 17B-C) were mediated by downregulation of gene expression. A significant association was observed between upregulated genes and apoptosis-related gene set [110] (NES 1.66; FDR<0.01) (Figure 18C), and between downregulated genes and cell cycle genes [110] (NES 2.74; FDR<0.0001) (Figure 18D). Additionally, a significant association was also detected between upregulated genes and terminal myeloid differentiation [111] (NES 1.61; FDR<0.02) (Figure 18E), and THP-1 PMA induced monocytic differentiation signatures [112] (NES -2.19; FDR<0.0001) (Figure 18F). We performed morphological and flow cytometry analysis of THP-1 and the AML1-ETO–

derived cell line SKNO-1 after exposure to panobinostat. Both cell lines have the capacity to differentiate in vitro [113, 114]. A monocytic phenotype (Figure 18G) and a significant increase in the percentage of CD11b+ cells were observed in THP-1 without changes in SKNO-1 cells (Figure 11H).

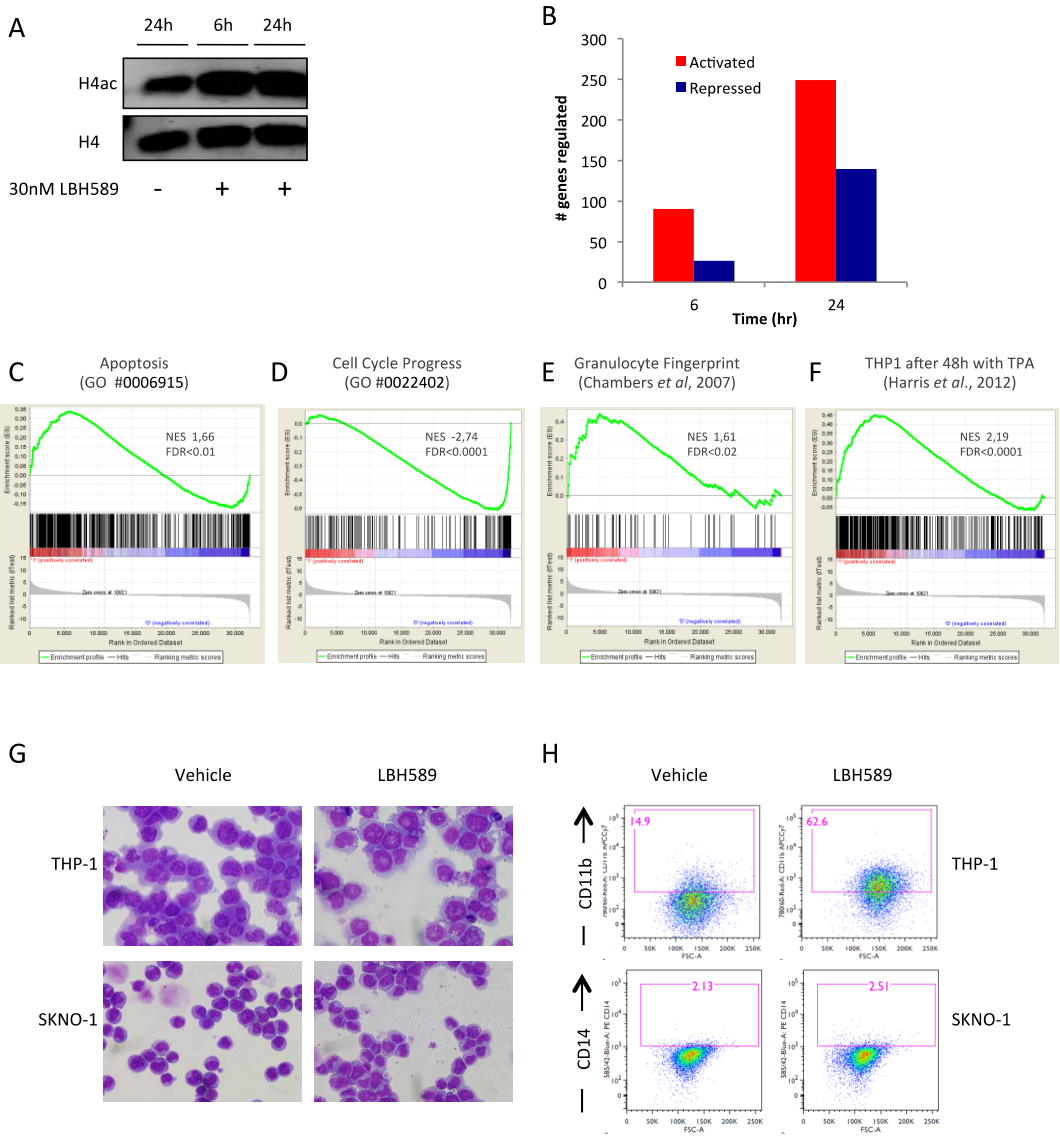


Figure 18. HDAC inhibition leads to transcriptional deregulation. (A) H4ac immunoblotting of HSPC-MA9 exposed to 30 nM of panobinostat at the indicated time points. (B) Total significantly modified genes at the indicated time points on 30 nM panobinostat-treated HSPC-MA9 cells compared to vehicle (FC>2; FDR<0.05). Three independent HSPC-MA9 clones were studied in duplicate. (C-F) GSEA plots using genes related to apoptosis (C), cell-cycle genes (D), granulocyte "fingerprint" signature genes (E), and gene signature of PMA-induced macrophage differentiation of THP-1 in panobinostat–treated HSPC-MA9 cells (F) (for gene sets see Table S10). (G) Wright-Giemsa staining of THP-1 and SKNO-1 cells treated with 10

nM of panobinostat for 24 hours. (H) Representative FACS analysis show CD11b+ THP-1 cells (top) or CD14+ SKNO-1 cells (bottom) after treatment with 10 nM of panobinostat.

As a second approach, we also performed IPA (Ingenuity Systems) of the differential expression signatures. After 24 hours of pan-HDAC inhibition with panobinostat, we also identified significant changes in functions such as cell cycle, cell death, and DNA replication and repair (B-H p value<0.05), with abnormal upregulation of genes such as *CDKN1A* and *FOXO1* and repression of *E2F1*, *RAD51*, and *TERT* (Table 12). Thus, our data show that panobinostat mediates its effects by transcriptional deregulation by rapidly inducing aberrant expression of the genes involved in apoptosis and myeloid differentiation and by repressing genes implicated in the cell cycle.

TABLE 12. Functions significantly overrepresented on HSPC-MA9 hyperacetylated genes (B-H p-value <0.05)

Category	B-H p value	Molecules
Cell Cycle	1.08E-02-8.67E-02	FOXO1↑, CDKN1A↑, FOXM1↑, CHEK1↓
DNA Replication, Recombination, and Repair	1.08E-02-8.67E-02	RAD51↓, XRCC3↓, CHEK1↓
Cell-To-Cell Signaling and Interaction	1.59E-02-1.59E-02	FOXO1↑, E2F1↓
Cell Death and Survival	5.44E-02-6.57E-02	RAD51↓, CDKN1A, TERT↓, SLC19A1, GLI1↑

2. Integrative map of MLL-AF9 induced chromatin modifications.

2.1. ChiP-seq analysis of the histone acethylation pattern

As the acetylation profile associated with MLL-AF9-expressing cells that could explain their response to panobinostat was unknown, we compared HSPC-MA9 and normal HSPC ChIP-seq data using anti-H4 acetylation antibody. The histone acetylation regions were located primarily in the promoter regions defined as 1 kb around a transcription start site (TSS) and moderately span gene body regions (Figure 19 and S1). Among the peaks with a less than 5-kb distance from an annotated TSS, we

identified 3494 genes (including 44 miRNAs) with histone H4 hyperacetylation (20% of the total number of peaks) and 475 genes (including 8 miRNAs) with histone H4 hypoacetylation (17% of the total number of peaks) (FC>1.5, FDR<0.001) (Table S11 and S12). These observations showed that only expression of MLL-AF9 fusion protein in HSPC cells induced a global aberrant acetylation profile across the genome.

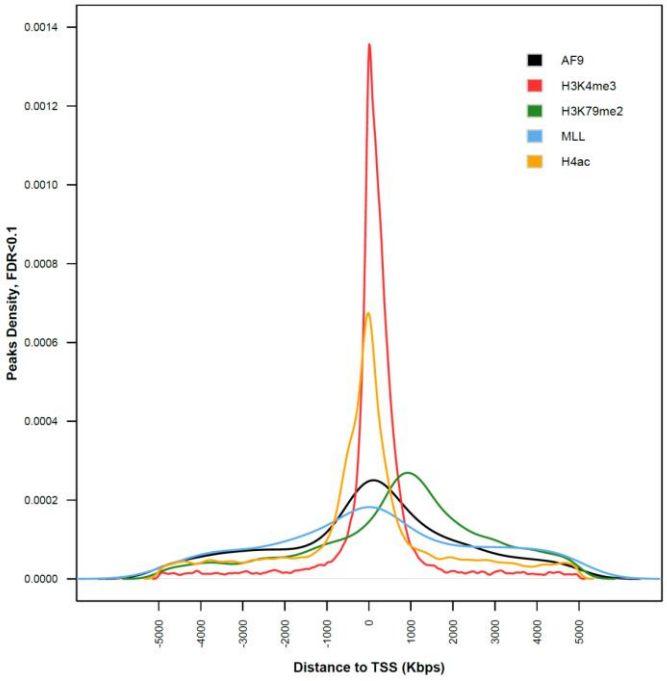
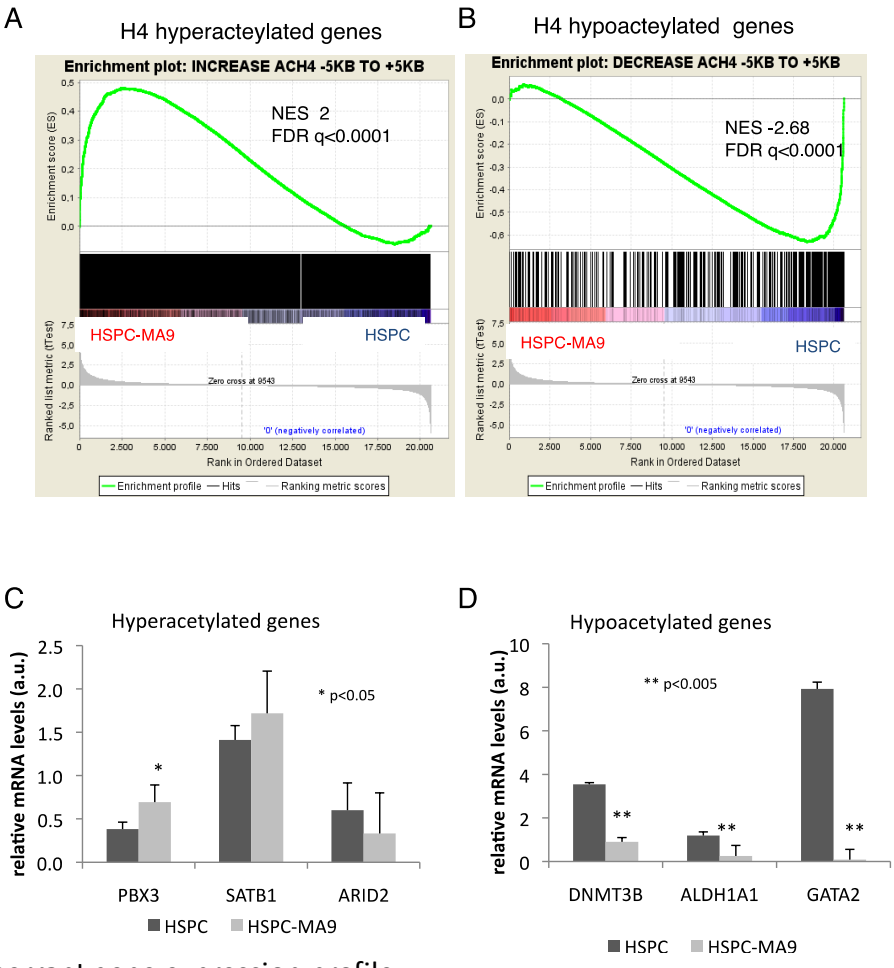


Figure 19. Genome wide ChIP-seq peak density. ChIP-seq peaks obtained for MLL, AF9 and all the chromatin modifications studied on HSPC-MA9 cells are plotted related to their position to a TSS (FDR<0.1). A 5kb window to TSS is shown

2.2. Histone acetylation and HSPC-MA9 transcriptional profile

The identified hyperacetylated gene set (n=3494) showed a significant enrichment among HSPC-MA9–transcribed genes, while the hypoacetylated gene set (n=475) was found to be negatively correlated with HSPC-MA9 active genes compared with HSPC controls (GSEA analysis, FDR<0.0001) (Figure 20A-B). Furthermore, quantitative mRNA expression of 3 hyperacetylated genes (*PBX3*, *SATB1*, and *ARID2*) showed maintained or higher expression in HSPC-MA9 cells than in normal HSPC, whereas hypoacetylated genes (ie, *GATA2*, *DNMT3B*, and *ALDH1A*) were significantly silenced (Figure 20C-D). Interestingly, the genes found to be hyperacetylated were significantly implicated in critical signaling pathways, such as ERK, B-cell receptor, Rho A, p38 MAPK, and mTOR (Table S13) (IPA, B-H–corrected p value <0.05), and no significant association with canonical pathways was found for the hypoacetylated gene set (data not shown). Taken

together, these data suggest involvement of H4 hyperacetylation in the MA9-HSPC a



aberrant gene expression profile.

Figure 20. The H4ac profile is involved on the MLL-AF9 aberrant gene expression profile GSEA plots (A) hyper-acetylated and (B) hypo-acetylated genes were compared to HSPC-MA9 expression profiles. (C-D) qRT-PCR was performed by triplicate on HSPC and HSPC-MA9 cells (C) Relative mRNA levels on hyper-acetylated genes and (D) hypo-acetylated genes. PGK gene was used for normalization. Error bars represent standard error

2.3. ChiP-seq analysis of MLL-AF9 target genes

In order to define the direct contribution of MLL-AF9 fusion protein to induction of aberrant histone acetylation, we performed ChIP-seq using antibodies against AF9 C-terminus and MLL N-terminus. We found MLL and AF9 bound to 188 and 260 promoter regions (5000 nt upstream and 200 nt downstream of a TSS), respectively. By combining both, we identified 66 MLL-AF9 bound promoters (FDR<0.01), including

well-known MLL-AF9 targets, such as *HOXA9* and *MEIS1*, and other less well-defined genes, such as *PBX3, JMJD1C*, and *MEF2C* (Table S14). qPCR analysis of ChIP DNA (qChIP) enabled us to validate selected targets on independent samples (Figure 21A-B). Moreover, the MLL-AF9 target genes identified were significantly associated with genes overexpressed in HSPC-MA9 (NES 1.18; FDR< 0.21) and in t(9;11) primary cases [96] (NES 1.90; FDR<0.0001) (Figure 22A-B)

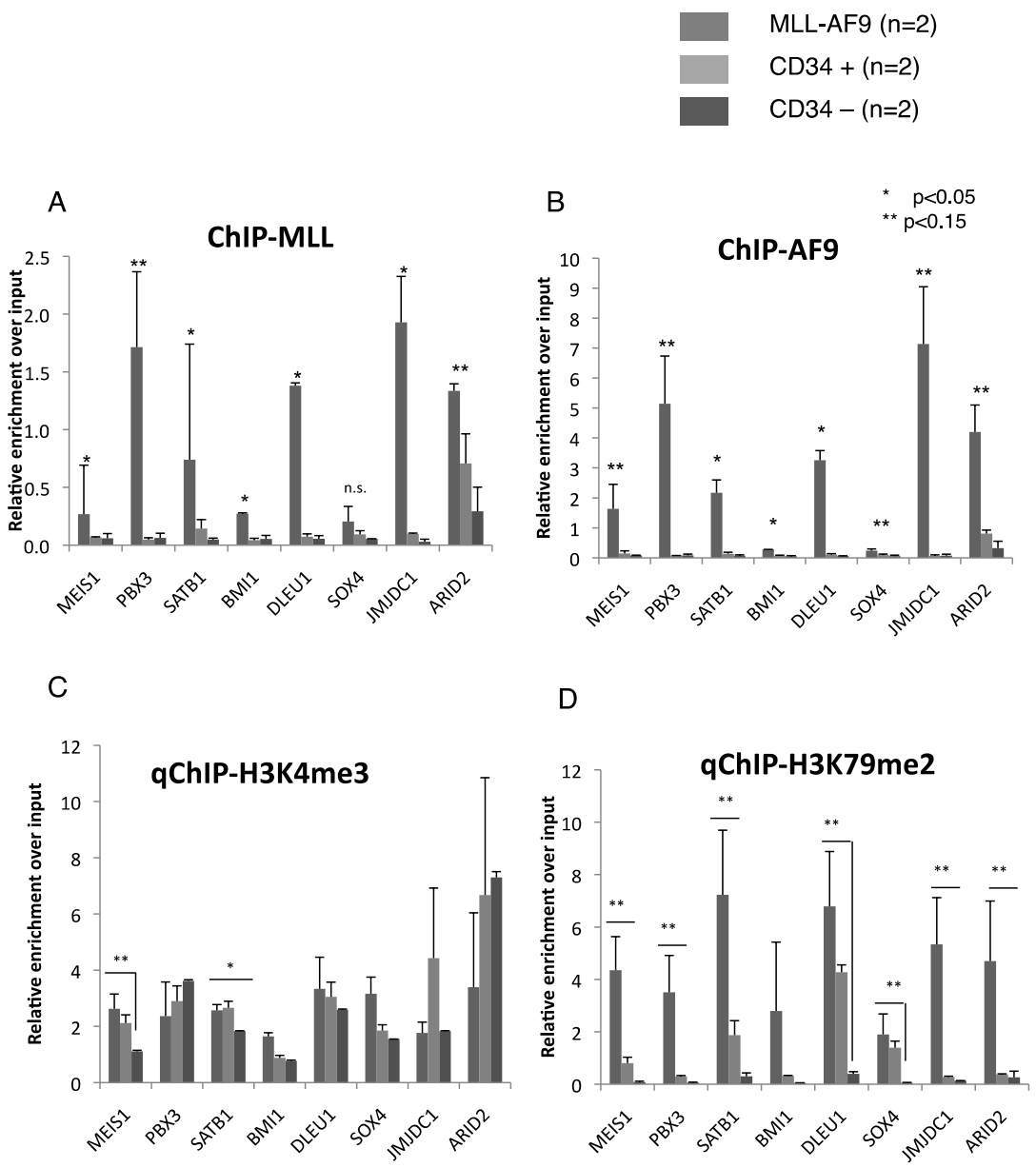


Figure 21. **qChIP validation of selected targets.** HSPC-MA9, HSPC (CD34+) and CD34- cells using antibodies agains (A) MLL N-terminal, (B) AF9 C-terminal, (C) H3K4me3 and (D) H3K79me2 on selected regions. The average of two experiments is shown. Error bars represent standard error.

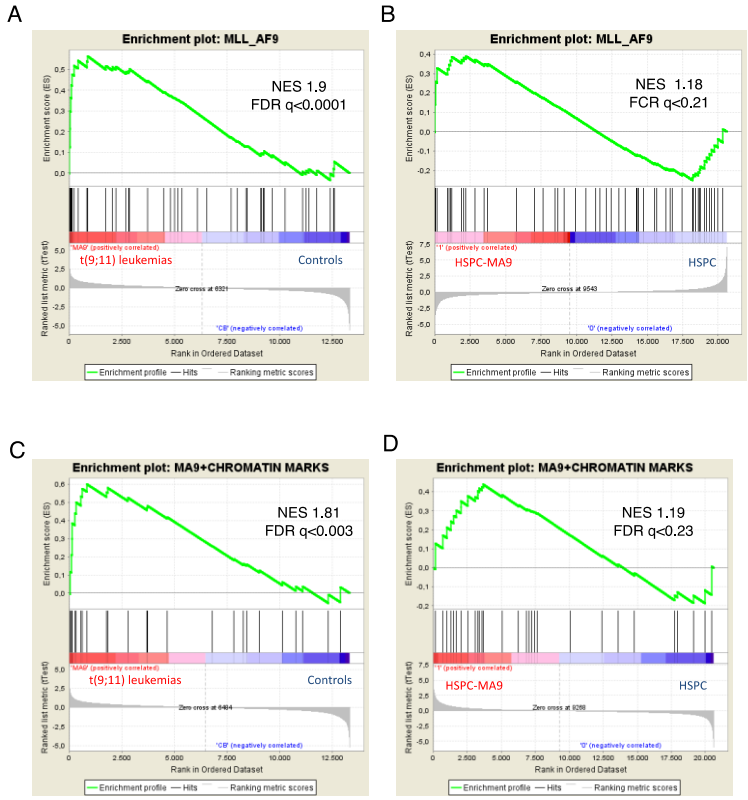


Figure 22. MLL-AF9 targets are associated with active transcription. GSEA plots of (A-B) the identified MA9 target genes on (A) t(9;11) primary samples and (B) HSPC-MA9 expression profiles. (C-D) those MA9 target showing all the studied chromatin marks (H3K4me3, H3K79me2 and H4ac) on (C) t(9;11) primary samples and (D) HSPC-MA9 expression profiles (for gene sets see Table S10).

2.4. Histone acetylation presence on MLL-AF9 targets

More than 70% of the MLL-AF9 bound regions (48 genes) identified showed a significant H4ac presence (Table S6, Figure 22). However, when comparing only the differentially acetylated regions with the HSPC normal cells, we found H4 hyperacetylation at 50% of MLL-AF9 target genes (31 genes) and H4 hypoacetylation at 6% of MLL-AF9 target genes (4 genes) (Figure 23A). These observations were confirmed by qChIP (Figure 23B). Furthermore, at *HOXA9* and *MEIS1* promoters, the H4ac increase correlated with an increase in RNA polymerase II binding (Figure 23D-E). These results indicate an aberrant acetylation pattern directly associated with fusion protein DNA binding

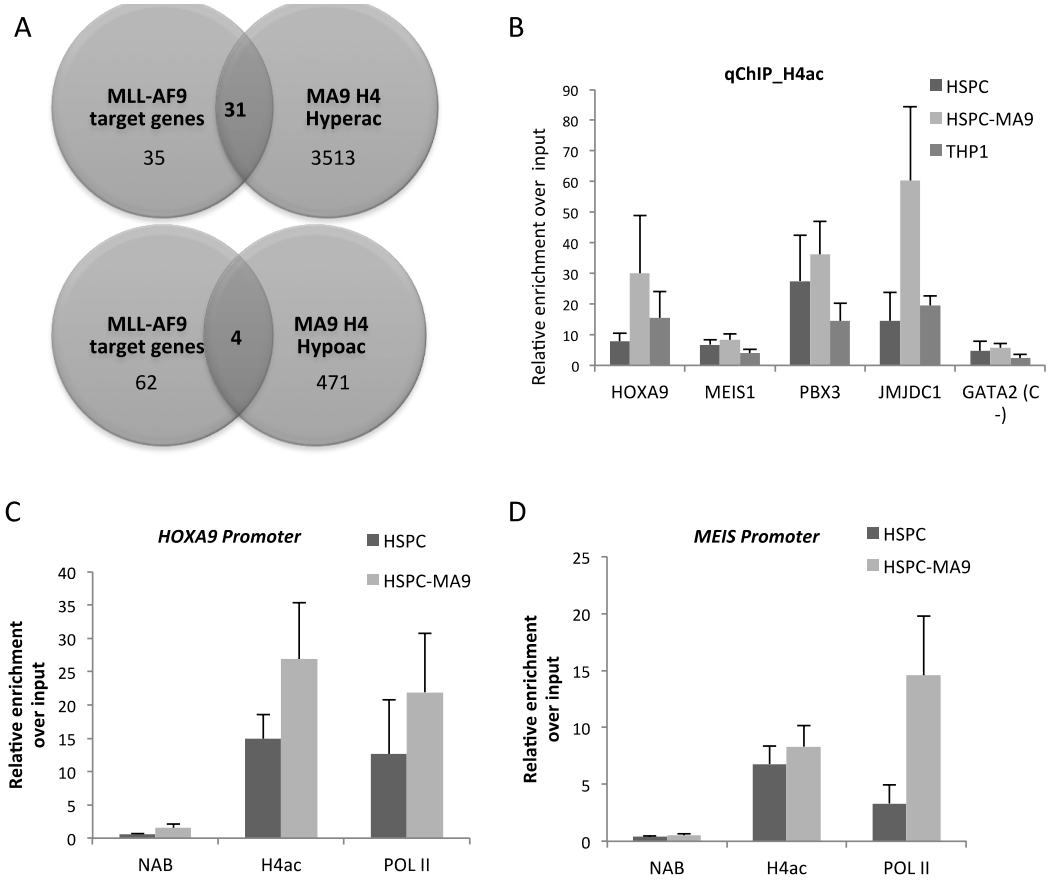


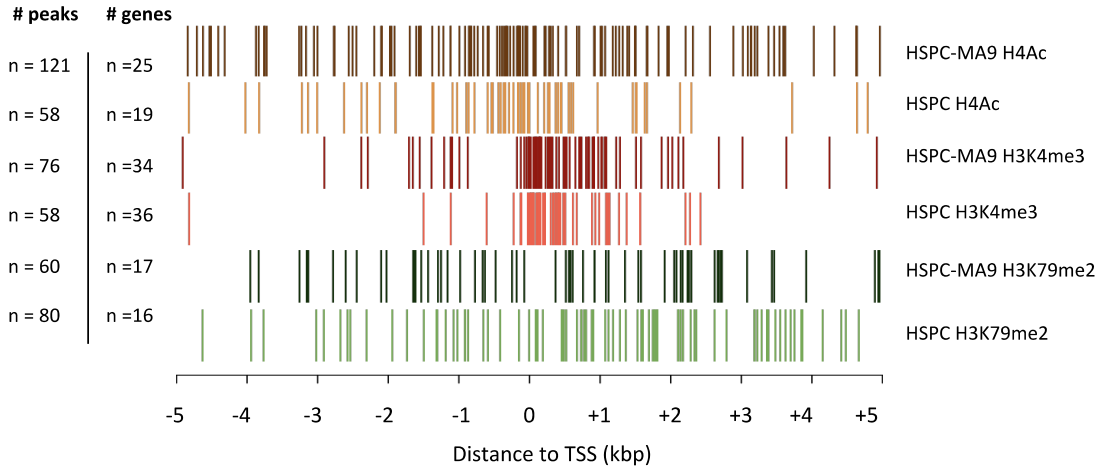
Figure 23. MLL-AF9 expression induces an aberrant histone acetylation pattern that correlates with transcription. (A) Venn diagrams of the overlapping MA9 target genes indentified with H4 hyperacetylated (top) or H4 hypoacetylated (bottom) genes on HSPC-MA9 cells (FC>1.5; FDR<0.001). (B) qChIP fold enrichment of H4ac in HSPC, HSPC-MA9, and THP-1 cells in selected regions. GATA2 was hypoacetylated on MA9 cells and thus used as a negative control. The average of at least 3 experiments is shown. (C-D) H4ac and RNA Pol II qChIP fold enrichment on MA9 target genes. Average of 3 experiments is shown. Error bars represent standard error

2.5. ChiP-seq analysis of the chromatin methylation marks

MLL-AF9 fusion protein causes an epigenetic lesion defined by an abnormal pattern of H3K4me3 and H3K79m2 on its target genes [21]. We then investigated whether the histone H4 acetylation pattern observed on HSPC-MA9 cells correlated with H3K4 and H3K79 methylation marks at MLL-AF9 target loci. ChIP-seq analysis was performed using H3K4me3 and H3K79me2 antibodies in HSPC controls and HSPC-MA9 cells. The genome-wide distribution of H3K4me2 was found mainly in promoter regions, while H3K79me2 occupied the gene bodies (Figure 22). On MLL-AF9 target

genes, an abnormal amount and distribution of H3K79me2 was also found along the gene body, and no severe changes were observed in H3K4me3 (Figure 24 and S2, Table S14). These findings were further validated by qChIP (Figure 14C-D). Interestingly, 36 of the 66 identified MLL-AF9 target genes presented the three studied chromatin marks (Table S14). GSEA revealed a high correlation with overexpressed genes on t(9;11) primary cases [96] and HSPC-MA9 (Figure 22C-D). Together, these findings indicate that histone H4 acetylation at MLL-AF9 target loci coincides with H3K79me2 and H3K4me3 chromatin marks.

Figure 24. Schematic representation of ChIP-seq peaks obtained on HSPC-MA9 (MA9)



and HSPC (CB) cells with the antibodies shown on the right in MLL-AF9 target genes. Each bar represents a peak (FDR<0.01) and is plotted according to its distance from a TSS. The total number of peaks and genes for each condition is shown on the left.

3. Integrative analysis of chromatin modifications and expresion signatures induced by HDAC inhibition

GSEA was performed to determine the transcriptional status of genes upregulated after treatment with panobinostat. We observed that the 249 genes upregulated after exposure to panobinostat were significantly associated with genes previously shown to be silenced in *MLL*-leukemia patients compared with controls [96] (NES –1.57; FDR<0.012) (Figure 25A), on HSPC-MA9 cells compared with normal HSPC (NES –1.2; FDR<0.25) (Figure 25B), and on the previously described MLL-AF9 signature of leukemic stem cells (NES –1.47; FDR<0.02) [79](Figure S3). These results indicate that

transcriptional changes occurring after exposure to panobinostat mainly affect genes silenced in the presence of MLL-AF9 fusion protein, including genes associated with the stemness properties of MLL-AF9 leukemia.

Unexpectedly, MLL-AF9 binding regions showed a significant association with panobinostat upregulated genes (NES 1.59; FDR<0.002) (Figure 25C). This finding was further confirmed using the MLL-AF9 target gene set identified by Bernt *et al*. in murine AML (NES 1.73; FDR<0.002) [21] (Figure S3). Moreover, a significant correlation was identified between the panobinostat-upregulated genes and the set of genes downregulated upon withdrawal of *MLL-AF9* expression in murine MLL-AF9;NRas^{G12D} AML cells [115] (NES –2.56; FDR<0.0001) (Figure S3). Taken together, these data suggest that after exposure to panobinostat, MLL-AF9 target genes, which are already overexpressed in MLL-AF9 leukemia, are boosted to further increase their expression levels.

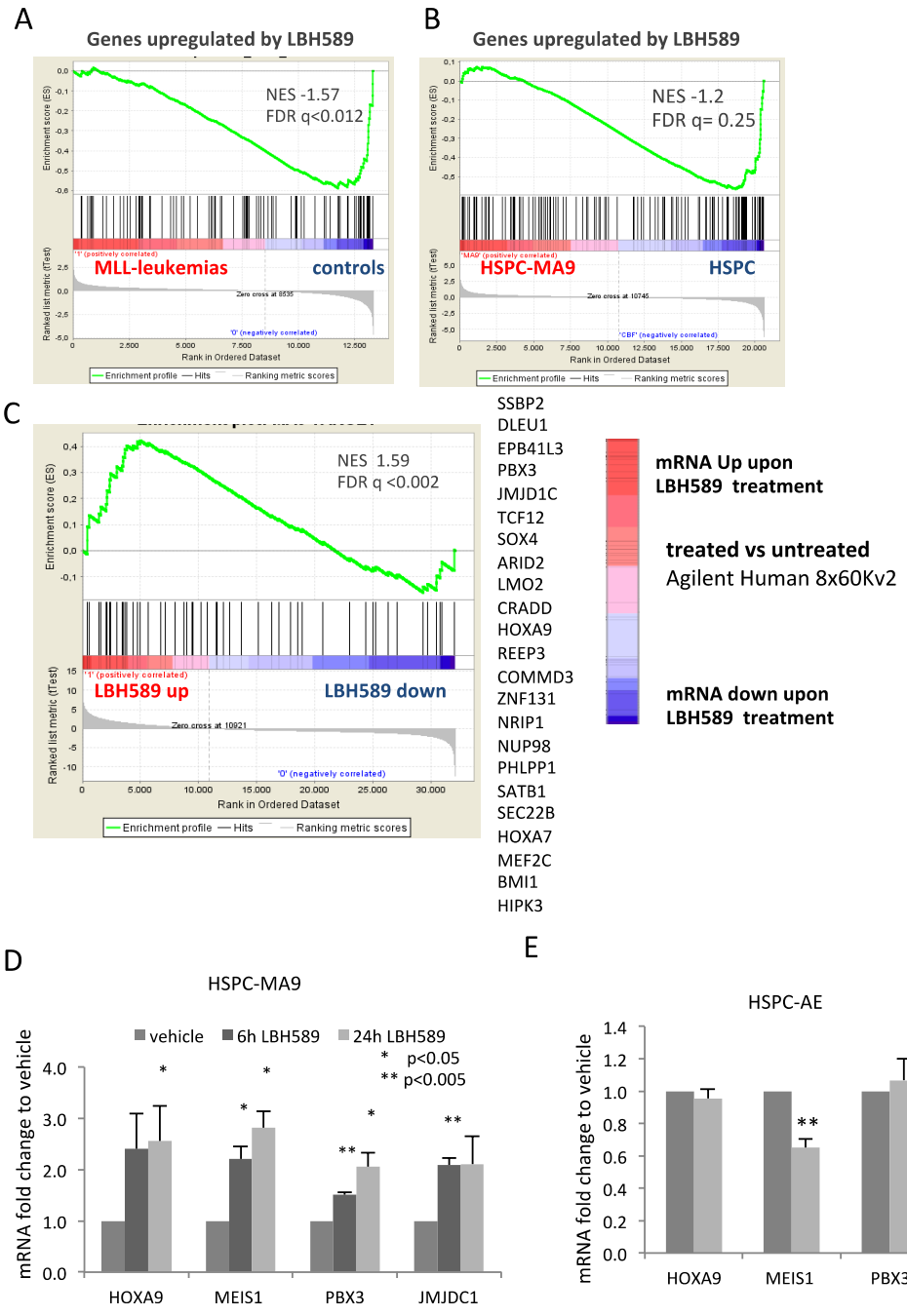


Figure 25 Panobinostat leads to increased expression of MLL-AF9 indirect silenced genes and active direct targets. GSEA plots of panobinostat-upregulated genes (FC<2; FDR<0.05) compared to (A) primary MLL leukemia and (B) HSPC-MA9 expression profiles. (C) MA9 target gene list (n=66) associated with the expression profile of HSPC-MA9 exposed to 30 nM of panobinostat for 24 hours (FC<2, FDR<0.05). The list of genes defining the core enrichment is shown on the right (for gene sets see Table S2). (D) HSPC-MA9 cells or (E) HSPC-AE cells cultured with vehicle or 30 nM of panobinostat for 6 and 24 hours were lysed for RNA, and qRT-PCR was performed for the indicated genes. The PGK gene was used for normalization. Error bars represent standard error.

Finally, the qRT-PCR of MLL-AF9 selected target genes confirmed boosted expression in HSPC-MA9 cells after treatment with HDACi (Figure 25D). We also observed overexpression, in THP-1 and NOMO-1 cells treated with a concordant increase in H4ac and RNA polymerase II binding in *HOXA9* and *MEIS1* promoters (Figure 26A-D). We did not observe such transcriptional changes in HSPC-AE cells or in the AML SKNO-1 and PML-RARα–derived NB4 cell lines (Figure 25E and 26A-B). Our results show that, on MLL-AF9 cells, panobinostat leads to massive overexpression of the oncogenic targets of MLL-AF9, which could cooperate with cell death by inducing oncogenic stress

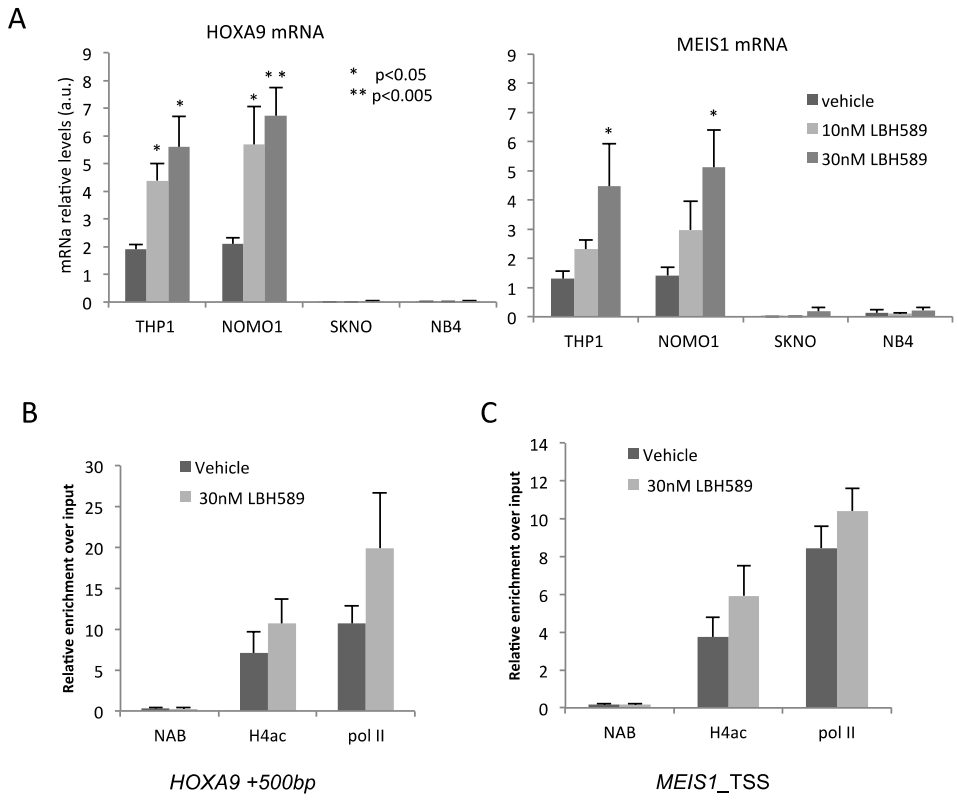


Figure 26. Upregulation of MLL-AF9 target genes after exposure to panobinostat is restricted to MLL-AF9 AML models. (A) In AML cell lines cultured for 24 hours with the indicated concentrations of panobinostat, RNA was extracted and quantitative PCR performed for expression of (left) HOXA9 and (right) MEIS1. (B) THP-1 cells exposed to 30 nM of panobinostat for 24 hours underwent qChIP. HOXA9 and MEIS1 promoters showed increased H4ac and RNA polymerase II binding with the treatment. The average of 3 experiments is shown. Error bars represent standard errors.

DISCUSSION

The rapid development of high-throughput technologies over the last decade has made a huge contribution on the understanding of AML biology. Initially, expression-profiling studies revealed the presence of unique expression signatures associated with balanced AML chromosomal translocations [96]. Lately, ChIP-chip, ChIP-seq and global methyl-DNA analysis (array- or sequencing-based methylation analysis) in AML models harboring different chromosomal translocation lead to a global characterization of epigenetic modifications, such as DNA methylation and histone tail modifications. These epigenetic modifications are nowadays known to play a major role in the AML expression signatures associated to the different AML subtypes [116]. Although, this knowledge has contributed to generate important information of AML biology, integrative studies that may have a real impact in the AML management are still sparse. The understanding of leukemogenesis, which includes among others the cellular, genetic and epigenetic mechanisms, is mandatory for the development of novel so-needed pharmacological strategies for these patients.

We made use of a powerful cellular system developed from human hematopoietic stem/progenitor cells (HSPC) stably transduced with either *AML1-ETO* or *MLL-AF9* cDNAs. The cellular effects, both *in vitro* and *in vivo,* induced by these oncoproteins expressed on human HSCPc have been described in depth over the years [44, 47, 77, 95, 117, 118]. These models allowed us to perform an integrative approach to better characterize the chromatin modifications associated to leukemia development and pharmacological response.

PROJECT I: AML1-ETO fusion protein: DNA Binding Map and Chromatin modifications

Identification and characterization of chromatin-associated AML1-ETO target genes

AML1-ETO fusion protein has been shown to recruit repressive complexes, altering the chromatin landscape of specific target genes. However, genome wide studies of the chromatin-modified AML1-ETO target regions have never been assessed.

AML1-ETO is known to repress transcription and several studies have been performed to better understand the epigenetic mechanisms leading to this silencing. We have previously showed that the presence of this fusion protein was not sufficient to induce the specific DNA methylation pattern observed in t(8;21) primary patient samples [119]. These data prompted us to hypothesize that the contribution of chromatin modifications on the specific expression signature observed on this leukemia subtype may target the leukemic initiation process.

To determine the chromatin modifications associated with AML1-ETO we focused on chromatin H4 deacetylation, previously characterized at specific target genes, due to the interaction of AML1-ETO wit the NCoR/SMRT/HDAC complex [36]. Furthermore, we also investigated the role of histone H3 lysine 9 methylation (H3K9me3) that may also contribute to the transcriptional repression. This histone modification is catalyzed by the methyltransferase SUV39H1, which interacts with AML1 wt protein through the AML1 Runt domain. This domain is retained in AML1-ETO and therefore has been hypothesize to interact also with the oncoprotein [52]. A first example of these interactions was the c-FMS locus in which the presence of a repressive histone deacetylation and histone H3 lysine 9 trimethylation was associated with AML1-ETO binding [51].

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 cellular model [44, 47]. 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 [120], and of ERK/MAPK, a key pathway in the self-renewal process of hematopoietic stem cells [121]. 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 development induced by t(8;21). Such as *MLLT3,* a positive regulator of the erythroid and megakaryocytic cell fate decision [122]; RPS19, a ribosomal protein which silencing decreases the proliferative capacity of hematopoietic progenitors and leads to a defect on erythroid development [123]; SIRT1, downregulated during neutrophil differentiation of acute promyelocytic leukemia cells [124] and CTCF which knock-down inhibited differentiation into erythroid lineage of the K562 cell line [125].

The 264 genes identified as AML1-ETO targets with an increased H3K9me3 were found to be significantly involved in the Wnt/b-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/b-catenin pathway required for the development of leukemic stem cells and for the maintenance of self-renewal on AML1-ETO patients [119]. Thus our study reveals another level of complexity identifying the H3K9me3 modification as an initial event targeting this pathway (i.e. *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 with DNA methyltransferase 1 (DNMT1) [126]. However, the interaction of SUV39H1 and AML1-ETO remains unclear and we had not found an enrichment of SUV39H1 binding among the AML1-ETO studied targets. Therefore further studies are needed to determine the mechanism underlying the observed increase of H3K9me3.

Although the presence of both H3K9me3 and histone H4 deacetylation has been reported associated with AML1-ETO binding at the c-FMS locus [51], we found that the simultaneous presence of these chromatin modifications 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.

DNA binding sequence analysis of AML1-ETO targets

In our study, only a small proportion of AML1-ETO bound regions (30% of total) were predicted to present an AML1 TFBS. AML1 and AML1-ETO were originally characterized to recognize *in vitro* the conserved DNA sequence TGT/cGGT [34], with a preferential binding of the fusion protein to DNA sequences containing multiple AML1 TFBS [35]. Our results support previous findings of multiple transcription factors, apart from AML1 wt, as HEB (HeLa E-box binding factor) [41] and the ETS (E-twenty-six) factors ERG and FLI1 [43], bound to nearly all AML1-ETO binding sites. The localization of AML1-ETO to regions containing other TFBS may indicate that other proteins facilitate the binding of the oncoprotein to different regions on the genome. Furthermore, previous reports using ChIP-seq analysis of AML1 target genes also identified DNA binding that was not associated with canonical AML1 response elements [127].

The detailed sequence analysis of AML1-ETO binding sites on HSPC-AE cells revealed a non-previously reported significant enrichment of the Sp1 TFBS present in over 50% of the target genes. The presence of an Sp1 TFBS was independent of the associated silencing chromatin mark. We also found a significant representation of PBX5 and E2F1 TFBS on approximately 15% and 30% sites respectively. Sp1 transcription factor is essential in hematopoietic differentiation [128], and is able to interact with the RUNT domain of AML1 and AML1-ETO [129]. These results highlights the importance of performing integrated analysis using genome-wide binding profiles, in order to obtain a comprehensive view of combinatorial interactions between fusion proteins and key regulators as Sp1.

It is important to mention that different cellular models (mice vs human, cell lines vs cellular models) or the techniques and analytical methods used (ChIP-chip vs ChIP-seq) may produce different and sometimes contradictory results. During the last years, new studies using ChIP-seq reveal that AML1 and AML1-ETO are bound preferentially to inter- and intragenic regions and to few promoter regions (1kb around TSS) [43, 127]. Less than 10% of AML1-ETO ChIP-seq peaks identified on the t(8;21)-derived Kasumi-1 and SKNO-1 cell lines were present on promoters, suggesting that AML1-ETO

could have a role in regulating enhancers [43]. On the contrary, another ChIP-seq study on Kasumi-1 showed that AML1-ETO binding sites distributed within 1.5 kb of TSS, and found an AML1 TFBS on 70% of AML1-ETO bound sequences [130]. We have performed a ChIP-chip proximal promoter analysis, covering 5.5 kb upstream to 2.5 kb downstream of the represented genes (17 000 in total), in a model with abundant AML1-ETO protein presence. It is reasonable to think that both the promoter-biased study we made and the different cellular background may explain the different percentages of binding sites obtained. However, all these studies provided essential elements to better understand the complex and heterogeneous DNA binding pattern of this fusion protein, and new functional studies are needed to better clarify these discrepancies.

Integrative analysis of AML1-ETO functional targets and transcriptional regulation

Regardless of the TFBS present, a significant and reversible silencing of most AML1-ETO target genes was confirmed in the AML1-ETO knock out model and primary samples. We found repression on genes that present an AML1 (e.g. *YES1, AML1*) and/or an Sp1 TFBS (e.g. *CTCF, SIRT1)*. On the contrary, Sp1 inhibition lead only to the activation of those genes with an Sp1 binding site at their promoter (i.e. CTCF and SIRT1) but no effect was seen on those presenting an AML1 TFBS (i.e. 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 repression takes place when the fusion protein directly binds DNA through an AML1 TFBS. All together these data supports the interaction between AML1-ETO and Sp1, which would lead to Sp1 transitivity inhibition [129], and identify this event as a genome wide mechanism involved in t(8;21) leukemia.

Role of Sp1 transcription factor in AML1-ETO AML

The finding of Sp1 transcription factor as an important driver of AML1-ETO binding prompted us to study its role in t(8;21) leukemia. Sp1 is a ubiquitous zinc finger that binds GC-rich regions in the promoters of a variety of genes involved in hematopoiesis (e.g. DNMT1) [131]. It is involved in hematopoietic differentiation, specifically found associated with the transcriptional profile of the erythroid lineage [128], and has been

described as part of the Sp1/NF-kb/HDAC1/mir-29b network that deregulates KIT gene transcription. This network is controlled by a feedback loop that is altered by the proteasomal inhibitor Bortezomib, inhibiting KIT gene transactivation [106].

We found that Sp1 protein is essential for AML1-ETO cells, but not for other leukemia subtypes as those expressing the MLL-AF9 oncofusion. Removal of Sp1 leads to a complete abrogation of HSPC-AE self-renewal, and induces apoptosis and growth arrest in SKNO-1 cell line. These result are very interesting taking into account that Bortezomib, which targets Sp1 complexes and induced Sp1 protein degradation, have shown promising results *in vitro* using different AML cell lines [106]. Thus, the use of Bortezomib in studies including t(8;21) seems a reasonable next step.

Furthermore, we observed an increase of the Sp1 protein levels in the presence of AML1-ETO, whereas mRNA levels remain unchanged. The same effect has been reported for HEB, another transcription factor associated to AML1-ETO DNA binding [41]. All together these results may indicate that AML1-ETO expression is associated with protein stabilization through post-translational mechanisms. The characterization of this novel role of AML1-ETO needs further investigation.

PROJECT II. Targeting Histone Acetylation in MLL-AF9 Leukemia

Efficacy of HDAC inhibition in MLL-AF9 cells

Most preclinical studies of HDACi focus on patients with AML harboring fusion proteins that aberrantly interact with HDACs (such as AML1-ETO). However, the effectiveness of HDACi in other AML contexts, such as rearrangements of *MLL*, has been poorly studied. We identified and characterized histone acetylation as a therapeutic target in MLL-AF9 leukemia. By studying the effects of exposure to panobinostat in a human model of HSPCs expressing either AML1-ETO or MLL-AF9 fusion proteins, we showed that HDAC function is essential for the maintenance of MLL-AF9 leukemia cells *in vitro*, since HSPC-MA9 cells were the most sensitive model.

The toxic effects of HDACis could be mediated by regulation of non-histone protein acetylation leading to protein degradation. In various cellular models, such as those based on AML1-ETO–expressing cells, fusion protein degradation has been postulated as the mechanism of action [108]. However, the binding of MLL-AF9 oncoprotein to *HOXA9* and *MEIS1* promoters upon exposure to panobinostat enabled us to rule out degradation as the mechanism of action in this cellular context. Moreover, the acetylation of AML1-ETO protein by the coactivator p300 was reported to be essential for its activity [54], but no such mechanism has been reported so far for MLL-AF9 oncoprotein.

Instead, we found that treatment with panobinostat was followed by rapid transcriptional activation and histone acetylation even before cell toxicity was observed. The changes observed in the expression signature led to deregulation of multiple molecular mechanisms, such as apoptosis, cell cycle, and differentiation, which can make transformed HSPC-MA9 cells succumb to disease. Furthermore, these changes precede increased apoptosis, G1-cell cycle blockage, the increase in CDKN1A protein levels, and the differentiation induced. Thus, our data imply that transcriptional modifications are responsible—albeit partially—for the cellular response to HDAC inhibition, which is highly similar to that observed in other AML subtypes [132]. However, we may not rule out that other non-histone protein acetylation are also involved on the observed effects.

Integrative map of MLL-AF9 induced chromatin modifications

Given the high sensitivity of panobinostat and the rapid effects on transcription, we hypothesized that the abnormal epigenetic profile induced by the presence of MLL-AF9 could be involved in the response to panobinostat. ChIP-seq analysis revealed a massive hyperacetylation of HSPC-MA9 samples compared to HSPC controls, with 7 times more genes harboring a histone H4 acetylation mark in HSPC-MA9 cells than genes that lost this chromatin mark. This aberrant acetylation pattern was highly concordant with the gene expression profile not only in HSPC-MA9 [77], but also in primary samples with t(9;11) [96]. Interestingly, we identified significant involvement of acetylated genes in signaling pathways known to be aberrantly activated and implicated in AML, such as mTOR, ERK, B-cell receptor, Rho A, and p38 MAPK [133]. Our data support that expression of MLL-AF9 fusion protein alone induces a global aberrant acetylation profile across the genome that correlates with the overexpression of essential genes in the maintenance of leukemic cells.

In order to better characterize the direct association between MLL-AF9 fusion protein and H4 acetylation profile, we mapped the MLL-AF9 DNA binding sites. Among the 66 MLL-AF9 target genes, we identified well-known MLL-AF9 targets such as the *HOXA* locus, as well as newly described targets and observed 27% coincidence with the previous MLL-AF9 binding map from a murine leukemia model [21]. The differences between our findings and those of Bernt *et al.* may partially reflect divergences between human and mouse leukemia models. Interestingly, H4ac was present in more than 70% of the identified targets, with H3K4 and H3K79 methylation marks present in over 50% of MLL-AF9 targets. We therefore showed, that MLL-AF9 target loci acquire both an aberrant histone methylation pattern and an aberrant histone acetylation pattern. The finding that the H4ac mark is a recurrent event at MLL-AF9 targets supports previous findings showing the contribution of Tip60 histone acetyl transferase to maintenance of MLL-AF9 leukemia and expression of *HOXA9 [76]*. Further experiments are needed to clarify the level of interaction between MLL-AF9 and histone acetyltransferases.

Role of chromatin modifications in expression profile changes upon HDACi

In the context of this newly characterized MLL-AF9 chromatin modification, we observed that most of the genes over-transcribed after treatment with the HDACi were silenced in HSPC-MA9 cells. However, MLL-AF9 target genes, which had already been actively transcribed, were also found over-transcribed in HSPC-MA9 cells exposed to panobinostat. These results are consistent with the findings of Wang *et al.*, who showed increased histone acetylation in already active genes after HDAC inhibition [26]. Furthermore, these data suggest an important role for HDACs in transcriptional regulation of MLL-AF9. Since HDAC1 has been reported to interact with the *MLL* wt [69], it may be also present in MLL-AF9 targets. Further studies are needed to better characterize the role of HDACs in the aberrant transcriptional regulation induced by MLL-AF9 fusion protein, given that the aberrant epigenetic profile found on MLL-AF9-binding sites is significantly associated with changes in gene expression induced by panobinostat.

We demonstrated that over-expression of MLL-AF9 target genes after treatment with panobinostat occurred exclusively in MLL-AF9-expressing cells and not in other AML contexts characterized by the repression of its target genes (ie. AML1-ETO or PML-RARa). As several clinical trials with panobinostat are currently under way, the association between clinical response and the presence of specific fusion proteins should be evaluated (http://clinicaltrials.gov).

CONCLUSIONS

1. Our study has demonstrated that the presence of AML1-ETO or MLL-AF9 fusion proteins on hematopoietic/stem progenitor cells (HSPC) lead to chromatin modifications, which via their effect on the global expression profile are important initial steps towards transformation of these leukemia subtypes as well as good therapeutically targets.

2. The main conclusions regarding the analysis of AML1-ETO binding sites and induced chromatin modifications are:

- The loss of H4 acetylation and the presence of H3K9me3 induced by the DNA binding of AML1-ETO fusion protein are independent epigenetic events. These chromatin modifications affect signaling pathways essential for the differentiation and self-renewal of hematopoietic progenitors.

- A significant enrichment for transcriptionally repressed genes in AML1-ETO-expressing cells compared to the control hematopoietic stem/progenitor cells (HSPC) was observed at AML1 target genes characterized by the loss of H4 acetylation or the presence of H3K9me3 chromatin mark.

- We revealed the presence of an AML1 transcription factor-binding site (TFBS) on a third of the identified target genes. However, a significant enrichment for other TFBS, mainly Sp1 TFBS, was observed in more than 50% of AML1-ETO target genes.

- The AML1-ETO fusion protein directly and reversibly silenced target genes independently of the TFBS that determines its promoter DNA binding. Interestingly, on primary samples the level of transcriptional repression varies depending on the TFBS used by AML1-ETO to bind DNA.

- Sp1 protein is critical for leukemia cells driven by AML1-ETO. We propose Sp1 as a good therapeutical candidate to evaluate in this leukemic subtype.

3. The main conclusions regarding the analysis of MLL-AF9 binding sites and induced chromatin modifications are:

- MLL-AF9-expressing cells are highly sensitive to the inhibition of HDACs by panobinostat. The rapid changes in gene expression induced by panobinostat exposure cooperate to produce the observed cell toxicity.

- The presence of MLL-AF9 oncoprotein induces an aberrant histone acetylation pattern, mainly characterize by a global hyperacetylation of histone H4. This aberrant acetylation pattern is highly concordant with the gene expression profile, not only in HSPC-MA9 cells, but also in primary samples with t(9;11)(p22;q23).

- More than 70% of the MLL-AF9 identified targets present the H4ac chromatin mark, which was found to be coincident with H3K4 and H3K79 methylation in over 50% of the targets. This novel finding allows a better understanding of the complexity of chromatin modifications at the MLL-AF9 target loci.

- Chromatin modifications induced by MLL-AF9 not only lead to transcriptional deregulation but also cooperate in the HDACi response. Panobinostat leads, on MLL-AF9 cells, to an increase acetylation and a massive over-expression not only of the indirectly silenced genes but also of MLL-AF9 target genes, which were already actively being transcribed. This effect, which could cooperate with cell death by inducing oncogenic stress, was related to MLL-AF9 presence.

CONCLUSIONES

1. Nuestro estudio ha demostrado que la presencia de las proteínas de fusión AML1-ETO o MLL-AF9 en el modelo celular de progenitores hematopoyéticos, dan lugar a modificaciones de la cromatina esenciales en la transformación leucémica, y por tanto excelentes dianas terapéuticas.

2. Las principales conclusiones del análisis de los sitios de unión de AML1-ETO y las modificaciones de la cromatina inducidas son:

- La desacetilación de la histona H4 y el aumento de la H3K9me3 en los sitios de unión al ADN de la proteína de fusión AML1-ETO son eventos epigenéticos independientes. Estas modificaciones de la cromatina afectan a vías de señalización esenciales en la diferenciación y auto-renovación de los progenitores hematopoyéticos.

- La comparación entre los niveles de expresión en los progenitores hematopoyéticos que expresan AML1-ETO y los control muestran que los genes diana de la oncoproteína que presentan la pérdida de la acetilación de H4 o la presencia de la marca H3K9me3 están significativamente silenciados

- Un tercio de los genes diana identificados presentan el motivo consenso de unión al ADN (TFBS) de AML1. Sin embargo, la presencia de motivos de unión de otros factores de transcripción es frecuente, siendo el TFBS de Sp1 el más recurrente, observado en más del 50% de los genes diana de AML1-ETO.

- La proteína de fusión AML-ETO induce la represión directa y reversible de sus genes diana, independientemente del TFBS que determine su unión al ADN. Los niveles de represión de estos genes varían en función del TFBS usado por AML1-ETO para su unión y reconocimiento del ADN en muestras primarias.

- Dado que hemos demostrado mediante estudios funcionales que el factor de transcripción Sp1 es crítico para el mantenimiento de las células leucémicas que presentan AML1-ETO, proponemos a Sp1 como una diana terapéutica a estudiar en este subtipo leucémico.

3. La caracterización de los sitios de unión al DNA de MLL-AF9 y de las modificaciones de la cromatina inducidas, se derivan las siguientes conclusiones:

- Las células que expresan MLL-AF9 son muy sensibles a la inhibición de las HDACs por panobinostat. Las rápidas modificaciones en la expresión génica inducidas por la exposición al panobinostat justifican la toxicidad celular observada.

- La presencia de la oncoproteína MLL-AF9 induce una alteración del patrón de acetilación de histonas, caracterizado por una hiperacetilación global de la histona H4. La presencia aberrante de esta marca se correlaciona con el patrón transcripcional observado, no solo de las HSPC-MA9 sino también en muestras primarias portadoras de la t(9;11)(p22;q23).

- La marca H4ac está presente en más del 70% de los genes diana de MLL-AF9 identificados, y además es coincidente en más del 50% de ellos con las marcas de metilación en H3K4 y H3K79. Por tanto, la unión de MLL-AF9 al ADN induce patrones aberrantes tanto de metilación como de acetilación de histonas.

- Las modificaciones de la cromatina inducidas por MLL-AF9 no solo colaboran en la desregulación transcripcional de HSPC-MA9, sino que además cooperan en su respuesta a la inhibición de las HDACs. La exposición a panobinostat de las células que expresan MLL-AF9 induce un aumento de la acetilación de histonas y una sobre-expresión masiva, no solo en genes indirectamente silenciados sino también en aquellos genes diana de MLL-AF9, que ya presentaban una activación transcripcional. Este efecto, que podría cooperar en la muerte celular mediante la inducción de estrés oncogénico, esta directamente relacionado con la presencia de MLL-AF9.

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APPENDIX I

Supplementary tables

Table S1, related to Figure 1. AML1- ETO target genes identified by ChIP-chip on the Agilent Human proximal promoter 244K array set. (see data on CD-room).

Table S2a, related to Figure 2. Functions significantly overrepresented on the 1168 AML1-ETO putative target genes (B-H p-value <0.05).

Category	Function Annotation	B-H pvalue	No. of Molecules
Cellular development	Differentiation of cells	0.0001	139
Cancer	Tumorigenesis	0.001	237
Cell death	Apoptosis of eukaryotic cells	0.0001	153
Hematological System	Maturation	0.03	13

Table S2b, related to Figure 2. Pathways significantly overrepresented on the 1168 AML1-ETO putative target genes (B-H p-value <0.05).

Canonical pathways	Genes	B-H p-Value	No. of Molecules
Wnt/β-catenin	AXIN1, AXIN2, CCND1, CDH3, CDKN2A, FZD7, FZD8, FZD10, GNAQ, GSK3A, KREMEN1, MAP3K7, MARK2, NLK, PPARD, PPP2R1A, PPP2R3B, RARA, SFRP5, SMO, SOX7, SOX18, TCF3, TCF7L2 (includes EG:6934), WNT4, WNT11, WNT2B, WNT9A, WNT9B	p<2.87E-5	29
Human embryonic stem cell pluripotency	AXIN1, BMP2, BMP6, FGF4, FGFR1, FZD7, FZD8, FZD10, GSK3A, S1PR5, SMAD7, SMO, SPHK1, TCF7, UTF1 (includes EG:8433), WNT4, WNT11, WNT2B, WNT9A, WNT9B	p<1.88E-3	20
TGF-β	BMP2, HRAS, INHA, INHBB, MAP3K7, MAPK1, MAPK6, PIAS4, RUNX2, RUNX3, SMAD7, SMURF1, ZFYVE9	p<0.03	13

Table S3, related to Figure 1. Genes found to be AML1-ETO and HDAC1 co-occupied and to present H4 deacetylation on HSPC-AE (see data on CD-room).

Table S4a, related to Figure 2. Functions significantly overrepresented on the 103 AML1-ETO/HDAC1 putative target genes (B-H p-value <0.05).

Category	Function Annotation	Molecules	No. of Molecules
Cancer	Tumorigenesis	ABCC4, AIM1 (includes EG:202), C19ORF2, DDX6, EXT1, FGFR1, FOXC2, FZD7, GNA13, H2AFX, INSIG1, MAPK1, NFKBIA, PDE3B, RHOG, RNF139, RPS19, RRM1, AML1, RUNX3, SEC63, SMAD7, TFAP2A, YES1, YWHAG	25
Cellular development	Differentiation of cells	CAND1, CTCF, EXT1, FGFR1, GNA13, IGFBP6, MAP3K7, MAPK1, NFKBIA, PCSK9, RPS19, AML1, SIRT1, SMAD7, TFAP2A, YWHAG	16
Hematological system development and function	Differentiation of erythroid cells	CTCF, MAPK1, RPS19	3
Gene Expression	Transcription	C19ORF2, CASK, CTCF, ELK3, FGFR1,FOXC2, HOXA7, JMJD1C,MAP3K7, MAPK1,NFKBIA, PEBP1, AML1, RUNX3, SIRT1, SMAD7, TFAP2A, UBTF	18

Table S4b, related to Figure 2. Functions significantly over-represented on the 103 AML1-ETO/HDAC1 putative target genes (B-H p-value <0.05).

Category	Function Annotation	Molecules	No. of Molecules
Cancer	Tumorigenesis	ABCC4, AIM1 (includes EG:202), C19ORF2, DDX6, EXT1, FGFR1, FOXC2, FZD7, GNA13, H2AFX, INSIG1, MAPK1, NFKBIA, PDE3B, RHOG, RNF139, RPS19, RRM1, AML1, RUNX3, SEC63, SMAD7, TFAP2A, YES1, YWHAG	25
Cellular development	Differentiation of cells	CAND1, CTCF, EXT1, FGFR1, GNA13, IGFBP6, MAP3K7, MAPK1, NFKBIA, PCSK9, RPS19, AML1, SIRT1, SMAD7, TFAP2A, YWHAG	16
Hematological system development and function	Differentiation of erythroid cells	CTCF, MAPK1, RPS19	3
Gene Expression	Transcription	C19ORF2, CASK, CTCF, ELK3, FGFR1,FOXC2, HOXA7, JMJD1C,MAP3K7, MAPK1,NFKBIA, PEBP1, AML1, RUNX3, SIRT1, SMAD7, TFAP2A, UBTF	18

Table S5, related to Figure 1. Genes with simultaneous presence of H3K9me3 and AML1-ETO occupancy (see data on CD-room).

Table S6a, related to Figure 2. Functions significantly overrepresented on the 264 AML1-ETO/H3K9me3 putative target genes.

Category	Function Annotation	Molecules	No. of Molecules
Cancer	Tumorigenesis	ADRA2A, AXIN2, BMP2, BNC1, C19ORF2, CDH3, CMTM8, COL18A1, COL6A2, CTNNAL1, CXXC5, DDX6, DYRK2, EIF4EBP1, FASN, FGF8, FGFR1, FGFR1OP (includes EG:11116), FZD7, GALR1, GNAS, GPC1, GRIN1, GRIN2D, HIC1, ICOSLG, IDH3A, IGF1R, INHBB, IRS2, LRP3, MICALL1, NFIL3, NPTX1, OBSCN (includes EG:84033), OCLN, PPARD, PPP1R3D, RAN, RGS2, RPS19, RYK, SCUBE2, SIAH1, SLC22A3, SMAD7, SQSTM1, SSTR3, STK35, TBC1D9, TCF3, TOMM34, TOP2B, UBE3A, VAMP2, VANGL1	56
Cell death	Apoptosis of eukaryotic cells	BMP2, COL18A1, CTBP2, DSP, EIF4EBP1, FANCA, FASN, FGF8, FGFR1, GNAS, GPX4, GRIN1, GSK3A, HOXA7, IGF1R, IRS2, MBTPS1, MLLT3, MYBL2, NFIL3, NPTX1, PIAS4, PPARD, PPP1R13B, PRKD1, RAI14, SEMA7A, SEPT4, SIAH1, SMAD7, SSTR3, TACC3, TCF3, TYMP, ZNF274	35
Cellular development	Differentiation of cells	AXIN2, BARX1, BMP2, COL18A1, DSP, EN2, FGF8, FGFR1, GNAS, ICOSLG, IGF1R, IRS2, LAMC1, METRN, MYBL2, NEUROD2, NME2, PBX3, PLCG1, PPARD, PRKD1, RGS2, RPS19, SDC3, SFXN1, SLC9A3, SMAD7, SMURF1, TCF3, WNT9A, ZIC2, ZIC5	32
Hematological system development and function	Differentiation of leukocyte cell lines	BMP2, IGF1R, IRS2, MYBL2	4

Table S6b, related to Figure 2. Pathways significantly overrepresented on the 264 AML1-ETO/H3K9me3 putative target genes (B-H p-value <0.05).

Canonical Pathway	Genes	B-H p Value	No. of Molecules

Wnt/β-catenin	AXIN2, CDH3, FZD7, FZD8, GSK3A, KREMEN1, PPARD, SOX7, SOX18, TCF3, WNT2B, WNT9A	p<0.001	11
Human embryonic stem cells pluripotency	BMP2, FGFR1, FZD7, FZD8, GNAS, GSK3A, SMAD7, WNT2B, WNT9A	p<0.01	8
TGF-β	BMP2, INHBB, PIAS4, SMAD7, SMURF1	p<0.2	5

Table S7, related to Figure 4. Unsupervised sequence analysis of AML1-ETO data by PSCAN algorithm against the JASPAR database(see data on CD-room).

Table S8, related to Figure 4. Unsupervised sequence analysis of AML1-ETO data by PSCAN algorithm against the TRANSFACT database(see data on CD-room).

Table S9, related to Figure 11. Differential expressed genes (DEG) on HSPC-MA9 cells upon LBH589 treatment for 6 and 24 hr (see data on CD-room).

Table S10, related to Figure 11 and 15. Gene sets used for GSEA analysis (see data on CD-room).

Table S11, related to Figure 13. Hyper-acetylated regions on HSP-MA9 cells (5kb around TSS) (see data on CD-room).

Table S12, related to Figure 13. Hypo-acetylated regions on HSP-MA9 cells (5kb around TSS) (see data on CD-room).

Table S13, related to Figure 13. Gene lists of the hyper-acetylated genes significant associated with signaling pathways (IPA)

Table S14, related to Figure 14 and 17. MLL-AF9 target genes and associated chromatin marks

Supplementary Figures

Figure S1, related to Figure 12. USCS Genome Browser visualization of ChIP-seq enrichment peaks of one hyper-acetylated gene (JMJDC1) and one hypo-acetylated gene (GATA-2). Absolute values as well as the result of the comparative (HSPC-MA9 vs HSPC) is shown.

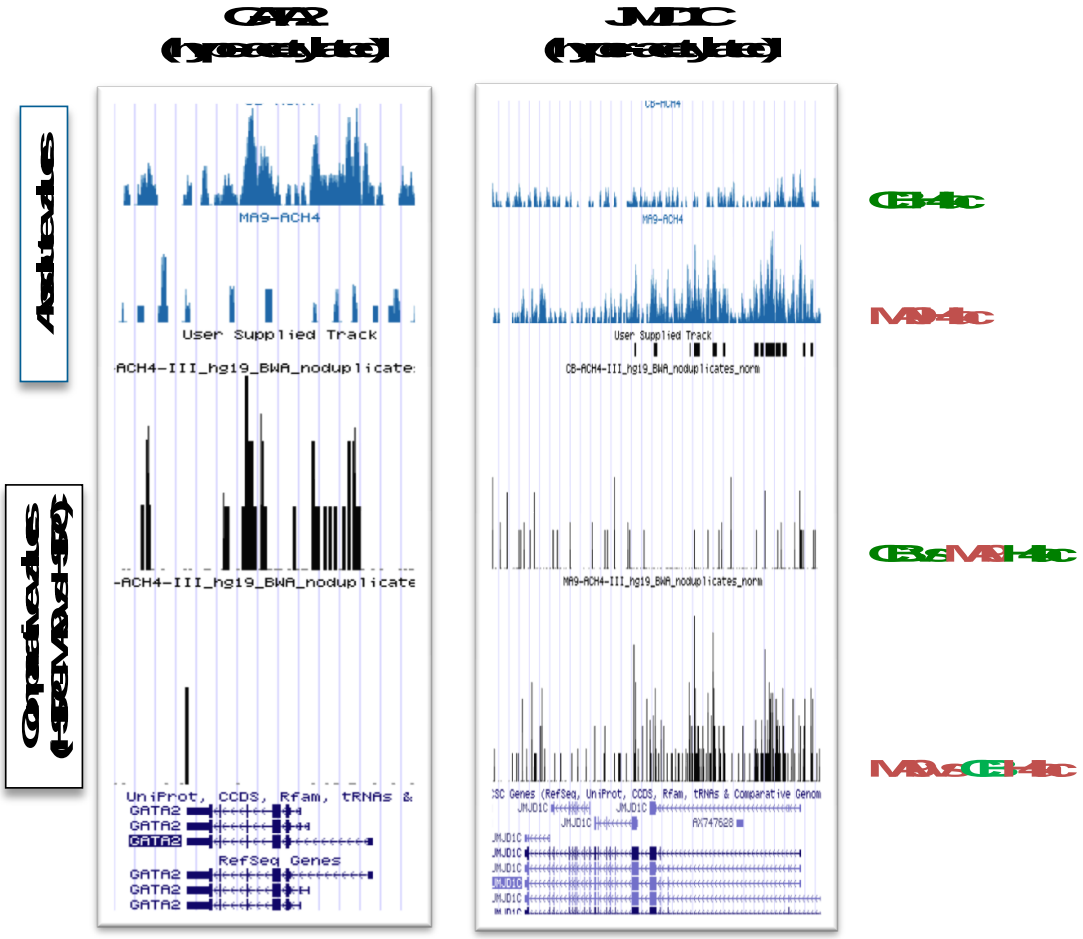


Figure S2, related to Figure 17. USCS Genome Browser visualization of the ChIP-seq enrichment peaks obtained with antibodies against MLL N-terminal, AF9 C-terminal, H4K4me3 and H3K79me2 on HSPC-MA9 and HSPC cells. Three examples of MLL-AF9 identified target genes are shown.

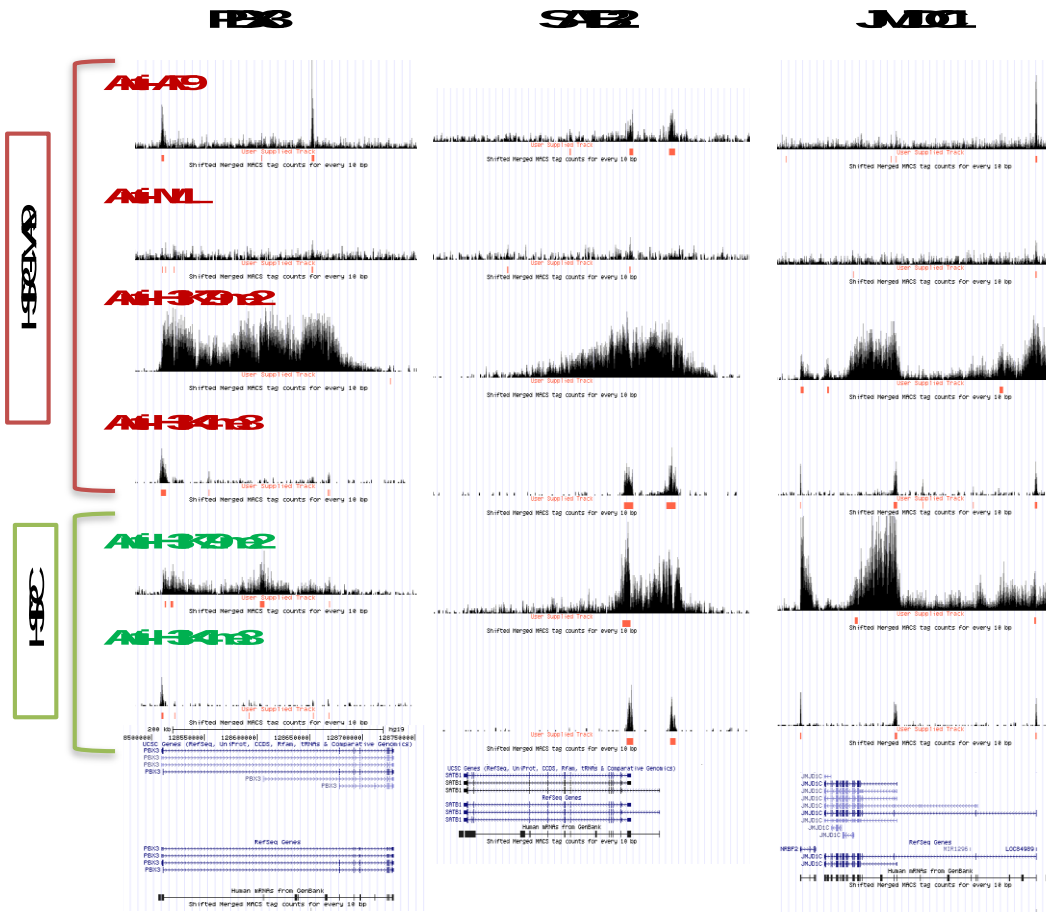
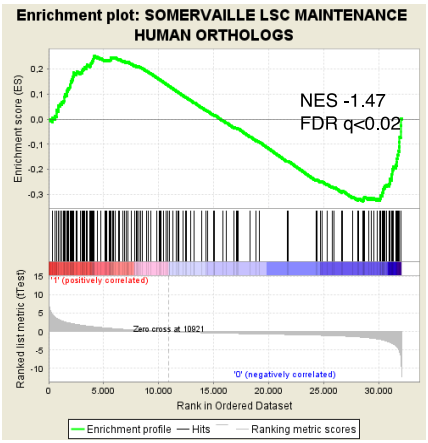
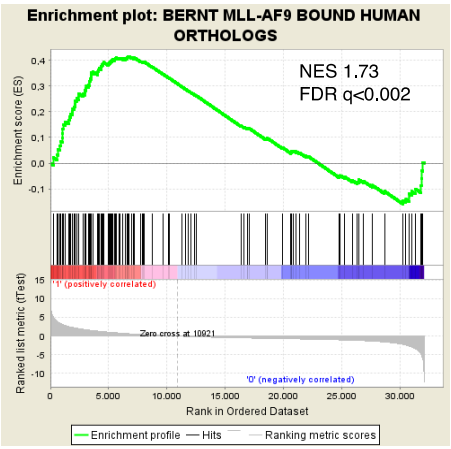


Figure S3, related to Figure 18. GSEA association of the LBH589 upregulated genes (n=249) with (A) MA9 signature of leukemic stem cells (LSC), (B) MA9 targets gene set identified by Bernt *et.al*. in murine AML, and (C) set of genes downregulated upon withdrawal of *MLL-AF9* expression in murine MLL-AF9;NRas^{G12D} AML cells

A



B



C