The gene signature in CCAAT-enhancer-binding protein α dysfunctional acute myeloid leukemia predicts responsiveness to histone deacetylase inhibitors

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ABSTRACT

C/EPBα proteins, encoded by the CCAAT-enhancer-binding protein α gene, play a crucial role in granulocytic development, and defects in this transcription factor have been reported in acute myeloid leukemia. Here, we defined the C/EBPα signature characterized by a set of genes up-regulated upon C/EBPα activation. We analyzed expression of the C/EBPα signature in a cohort of 525 patients with acute myeloid leukemia and identified a subset characterized by low expression of this signature. We referred to this group of patients as the C/EBPα dysfunctional subset. Remarkably, a large percentage of samples harboring C/EBPα biallelic mutations clustered within this subset. We hypothesize that re-activation of the C/EBPα signature in the C/EBPα dysfunctional subset could have therapeutic potential. In search for small molecules able to reverse the low expression of the C/EBPα signature we applied the connectivity map. This analysis predicted positive connectivity between the C/EBPα activation signature and histone deacetylase inhibitors. We showed that these inhibitors reactivate expression of the C/EBPα signature and promote granulocytic differentiation of primary samples from the C/EBPα dysfunctional subset harboring biallelic C/EBPα mutations. Altogether, our study identifies histone deacetylase inhibitors as potential candidates for the treatment of certain leukemias characterized by down-regulation of the C/EBPα signature.

Introduction

Acute myeloid leukemia (AML) is a malignant hematopoietic disease that accounts for over 90% of acute leukemias in adults, and is characterized by an accumulation of immature and non-functional blood cells in the bone marrow and blood. Despite this general definition, AML is a heterogeneous disease consisting of distinct blood disorders with different genetic abnormalities, clinical features, responses to therapy, and prognoses. Consequently, one of the research emphases of recent decades has been dedicated to the identification of biologically defined subgroups of AML with the ultimate goal of personalized treatment.

Traditionally, standard AML therapy relies on the use of chemotherapy, which targets leukemic cells as well as healthy cells resulting in significant side-effects. The use of drugs intended to differentiate leukemic cells into normal cells, without killing the healthy cell population, is therefore clinically very attractive. A precedent for this was found 40 years ago, when it was shown that dimethylsulfoxide (DMSO) differentiated murine virus-induced erythroleukemia cells into healthy normal cells in culture,[1] and since then numerous DMSO structural analogs have been developed. Two of these, vorinostat (also known as SAHA, Zolinza or suberoylanilide hydroxamic acid) and romidepsin (also known as FK228 or Istodaz), have been recently approved by the Food and Drug Administration. Vorinostat and romidepsin both target histone deacetylases (HDAC). HDAC are enzymes which deacetylate lysine residues in histones, allowing interactions between negatively charged DNA and positively charged histones, resulting in a closed chromatin conformation and frequently repressed transcription. However, the effect of HDAC is not restricted to epigenetic changes, and in fact there are several other proteins regulated by acetylation, including transcription factors (c-myc, YY1, E2F) and tumor suppressor genes (pRb, p53).[2] In recent years, there has been an increasing interest in the use of HDAC inhibitors in cancer treatment protocols given these inhibitors' apparent ability to preferentially target tumor cells in comparison to non-malignant cells. Despite the clinical use of these drugs and the large number of ongoing clinical trials, the molecular mechanisms of action remain far from being completely understood.[3,4]

Among the most common abnormalities in AML are defects in CCAAT/enhancer-binding protein alpha (C/EBPα). C/EBPα is a transcription factor that plays a crucial role in the commitment of multipotent progenitor cells into the myeloid

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lineage. In AML, two types of mutations have been described in C/EBPα: N-terminal and C-terminal mutations.[5,6] The N-terminal mutations introduce an early stop codon which prevents translation of the p42 C/EBPα isoform, while preserving translation of an inhibitory p30 C/EBPα isoform, whereas C-terminal mutations are mainly in-frame mutations or deletions which affect dimerization and DNA binding. The majority of AML patients with defects in C/EBPα harbor biallelic mutations, which combine C/EBPα N- and C-terminal mutations.[7,8]

In the present study, we identified a C/EBPα dysfunctional subset of AML patients characterized by down-regulation of the "C/EBPα signature". Patients with C/EBPα biallelic mutations demonstrated a low C/EBPα signature activation score, and predominantly clustered inside the C/EBPα dysfunctional subset. The connectivity map[9] predicted positive connectivity between the C/EBPα signature and HDAC inhibitors. Furthermore, we demonstrated that these small molecules could reactivate the C/EBPα signature and promote granulocytic differentiation of biallelic C/EBPα mutant samples in the C/EBPα dysfunctional subset.

Methods

Gene expression profiling, identification of the CEBPα signature, and data analysis

Gene expression profiling was performed in K562 p42-C/EBPα-ER expressing cells treated with β-estradiol (n=4) or a control ethanol vehicle (n=4) for 6 h using Affymetrix U133 arrays (GSE43998). Prediction analysis of microarrays (Stanford University) was used to identify probe sets which represent genes differentially expressed between two conditions. The heat map was generated using the 42 probe sets identified from the prediction analysis of microarrays, and hierarchical clustering was generated using JMP8 (*http://www.jmp.com/*). The Ward method was applied and data were standardized (i.e. converted to Z scores).

Chromatin immunoprecipitation followed by sequencing or quantitative reverse transcriptase polymerase chain reaction

Chromatin immunoprecipitation followed by sequencing (ChIP-seq analysis) (GSE43998) was performed in K562 C/EBPα-ER-expressing cells treated with 1 μM β-estradiol or a control ethanol vehicle, and in K562 ER control-expressing cells treated with 1 μM β-estradiol ($100x10^6$ cells). ChIP followed by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was performed in K562 cells treated with trichostatin A (TSA) or ethanol control ($10x10^6$ cells). Cells were treated for 6 h prior to formaldehyde fixation. Immunoprecipitation was performed using Protein G Dynabeads (Invitrogen), and 20 μg rabbit polyclonal IgG anti-ERα (Santa Cruz sc543X), 20 μg normal rabbit IgG control, 2 μg H3K4me3 (Millipore, 17-678), or 2 μg normal mouse IgG control. For ChIP-seq the purified ChIP DNA was used to construct ChIP-seq libraries with the Illumina ChIP-seq sample preparation kit (Illumina), as indicated by manufacturer. Libraries were sequenced on GA IIx (Illumina) at 36 bp. ChIP-seq reads were aligned against the hg18 human genome reference (NCBI Build 36.1) using Bowtie[10] allowing two mismatches. Each ChIP-seq dataset was normalized to 10 million reads. Peak calling and annotation were performed using the Homer package[11] with default parameters. ChIP-seq and microarray expression datasets were integrated using R (*http://www.r-project.org/*).

Histone deacetylase inhibitor treatment

K562 cells were cultured for 6, 12, 24, and 48 h in the presence of TSA and SAHA at the indicated concentrations. Vehicle controls were 0.2% ethanol for TSA and 0.2% DMSO for SAHA. Samples from the AML patients were cultured for 7 days in X-vivo 10 (Lonza) medium supplemented with 25 ng/mL human stem cell factor, 10 ng/mL human interleukin-3, and 10 ng/mL human interleukin-6. HDAC inhibitors, consisting of 0.25 μM TSA, 1 μM SAHA, and 0.5 μM MERCK60, or vehicle control (0.2% ethanol) were added to the cultures as indicated. Medium was refreshed 48 h after culture and repeatedly as needed. RNA was isolated after 5 days of treatment, and flow cytometric analysis performed on days 4 and 7 after treatment.

Study approval

Informed consent was obtained from the patients in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Boards of Beth Israel Deaconess Medical Center (Boston, USA), and the Erasmus University Medical Center (Rotterdam, the Netherlands).

Statistical analysis

We used a two-sided, unpaired Student *t*-test to determine the statistical significance of experimental results. When indicated, a Fisher exact test or Pearson χ^2 test analysis was applied. *P* values <0.01 are considered statistically significant.

Further information is provided in the *Online Supplementary Design and Methods* section.

Results and Discussion

C/EBPα activation up-regulates expression of 33 genes to define a C/EBPα signature

C/EBPα is a master regulator of granulocytic differentiation, and K562 cells stably transfected with an inducible C/EBPα-estrogen receptor fusion protein (C/EBPα-ER) have been used as a model for human granulocytic differentiation.[12] When stimulated with β-estradiol (E2) to induce nuclear translocation of C/EBPα, these cells differentiate towards neutrophils within 3-4 days of culture.[12] To identify genes up-regulated upon C/EBPα activation, and therefore involved in granulocytic development, K562 C/EBPα-ER-expressing cells were stimulated with 1 μM E2 or ethanol vehicle control, and RNA was isolated at different time points. Quantitative RT-PCR showed expected changes in well-known C/EBPα direct target genes as early as 4 h after stimulation, reaching optimal expression at 6 h after stimulation (*data not shown*). Based on this result, K562 C/EBPα-ER-expressing cells were induced for 6 h with E2 or ethanol and gene expression profiles were determined using microarrays (GSE43998). Using prediction analysis of microarrays, we identified 42 probe sets, corresponding to 33 genes, significantly up-regulated in the E2-stimulated cells in comparison to ethanol-treated control cells (Table 1). Figure 1A shows a heat map and hierarchical clustering according to expression of the 42 probe sets in E2-treated (n=4) or ethanol-treated (n=4) cells. Fourteen of the 33 genes identified were analyzed by quantitative RT-PCR, and gene expression up-regulation was verified using the same cell system (Figure 1B). Based on these results, we defined a C/EBPα signatures characterized by the up-regulation of 33 genes upon C/EBPα activation.

Next, we determined whether C/EBPα binds to regulatory elements of the C/EBPα signature genes upon C/EBPα activation. K562 C/EBPα-ER-expressing cells and control K562 ER cells (stably expressing the estrogen receptor moiety only) were stimulated with E2 or vehicle control for 6 h and ChIP-seq was performed using an ER-specific antibody. ChIP-seq analysis showed significant enrichment of C/EBPα in the proximity of the transcriptional start site (± 30 Kb, according to the Affymetrix gene annotation) of all genes from the C/EBPα signature, with the exception of *TRDV3* and *TRD@*, upon E2 treatment compared to vehicle treatment of K562 C/EBPα-ER cells (Figure 1A and *Online Supplementary Figure S1A*) (GSE43998). Similar binding was observed when comparing C/EBPα-ER-expressing cells to ER control cells treated with E2 cells (Figure 1A and *Online Supplementary Figure S1A*). The two probe sets, (*TRDV3* and *TRD@*) with no significant enrichment of C/EBPα in the proximity of the transcriptional start site, target the T-cell receptor δ locus. Notably, T-cell receptor δ locus is a large genomic region known to host several gene segments, and included within the T-cell receptor α.[13] Interestingly, when we looked at the entire T-cell receptor α/δ region, we found significant peaks located at the 3' end of the locus (*Online Supplementary Figure S1B*), suggesting that C/EBPα might regulate expression of certain gene segments within the T-cell receptor δ locus possibly through 3' regulatory enhancer elements. These results demonstrate a significant overlap between microarray analysis and ChIP-seq data in this cell culture model of granulocytic differentiation following C/EBPα activation. Overall, combining gene expression profile analysis and ChIP-seq data, to determine genes regulated by C/EBPα and genomic regions where C/EBPα binds, respectively, provides a better understanding of how C/EBPα controls granulocytic differentiation.

C/EBPα is a key transcription factor which regulates expression of target genes controlling myeloid differentiation, such as PU.1[14] and IL-6rα.[15] Here, we defined a C/EBPα signature which is up-regulated upon C/EBPα activation, and we hypothesize that up-regulation of these genes will orchestrate granulocytic differentiation. In fact, several of the genes belonging to the C/EBPα signature, such as *FOS*, *GPR109B*, and *ADFP*, were previously shown to be up-regulated upon C/EBPα activation in $CD34^+$ cells.[16] Since binding of C/EBPα to the C/EBPα signature genes and gene up-regulation occurs as early as 6 h after C/EBPα translocation, we might assume that C/EBPα directly regulates gene expression. In support of this hypothesis, we and others have shown direct binding of C/EBPα to the promoter of several genes identified in the C/EBPα signature. For instance, binding of C/EBPα has been described in the proximal promoter of *Trib1*,[17] *ANXA1*,[18] and *Id1*.[19] Our ChIP seq data corroborated C/EBPα binding in the close proximity to the transcriptional start site of several genes, including *Trib1* and *ANXA1*. Additionally, binding of C/EBPα was also observed in further upstream and downstream regions from the transcriptional start site, suggesting binding of C/EBPα to distal regulatory regions or enhancer elements. In summary, our ChIP-seq data indicated C/EBPα binding to genes identified in the C/EBPα signature, suggesting that they are direct C/EBPα target genes. However, we cannot exclude the possibility that some genes are secondary targets, although the fact that our gene expression profile was performed after 6 h of stimulation might argue against this hypothesis.

We also hypothesize that genes from the C/EBPα signature are functionally involved in commitment and differentiation of myeloid cells. Accordingly, expression of several C/EBPα signature genes, such as *Id1*,[20] *Fos*,[21] *GPR109B*,[22] *ANXA1*,[23] *TNSF10*,[24] and *C1orf38*,[25] has been reported through granulocytic development and in mature neutrophils. Moreover, several of the C/EBPα signature genes, such as *Id1*,[26,27] *GPR109B*,[22] *ANXA1*,[23] and *IL18RAP*,[28] have been directly related to granulocyte differentiation or function. Collectively, these results indicate that C/EBPα activation up-regulates the expression of 33 genes, defining a C/EBPα signature, which may be directly implicated in neutrophil differentiation and function.

Table 1. List of 33 genes, corresponding to 42 probes, significantly up-regulated in K562 C/EBPα-ER expressing cells upon 6 h of treatment with E2 in comparison to treatment with ethanol vehicle control. These genes were selected by prediction analysis of microarrays. *Indicates genes further analyzed by quantitative RT-PCR (See Figure 1).

Gene name	Gene symbol
1-acylglycerol-3-phosphate O-acyltransferase 9	*AGPAT9*
Acyl-CoA synthetase long-chain family member 1	*ACSL1**
Adducin 3 (gamma)	*ADD3**
Adipose differentiation-related protein	*ADFP**
Annexin A1	*ANXA1**
Chemokine (C-C motif) ligand 3	*CCL3*
Chromosome 1 open reading frame 115	*C1orf115*
Chromosome 1 open reading frame 38	*C1orf38**
Ecotropic viral integration site 2B	*EVI2B**
Endothelial PAS domain protein 1	*EPAS1**
Epiregulin	*EREG*
Family with sequence similarity 117, member A	*FAM117A*
Family with sequence similarity 129, member A	*FAM129A*
G protein-coupled receptor 109B	*GPR109B**
Guanylate binding protein 2, interferon-inducible	*GBP2**
Hydrogen voltage-gated channel 1	*HVCN1**
Inhibitor of DNA binding 1	*ID1**
Interleukin 18 receptor accessory protein	*IL18RAP**
Lipase, endothelial	*LIPG**
Mex-3 homolog B (C. elegans)	*MEX3B**
MOCO sulphurase C-terminal domain containing 2	*MOSC2**
Nuclear receptor coactivator 7	*NCOA7*
K voltage-gated channel, Isk-related family, member 3	*KCNE3*
Regulator of G-protein signaling 18	*RGS18*
Solute carrier family 26 (sulfate transporter), member 2	*SLC26A2*
Spermidine/spermine N1-acetyltransferase 1	*SAT1**
T-cell receptor alpha locus	*TRA@*
Thrombospondin 1	*THBS1*
Trafficking protein, kinesin binding 2	*TRAK2*
Tribbles homolog 1 (Drosophila)	*TRIB1**
Tumor necrosis factor (ligand) superfamily, member 10	*TNFSF10**
V-fos FBJ murine osteosarcoma viral oncogene homolog	*FOS**
Zinc finger and BTB domain containing 16	*ZBTB16*

Identification of a C/EBPα dysfunctional subset of acute myeloid leukemia patients characterized by down-regulation of the C/EBPα signature

Since mutations in C/EBPα occur in approximately 10% of AML patients, we next used the 33 genes selected by prediction analysis of microarrays to cluster 525 newly diagnosed AML patients (GSE14468).[29] We identified a subset of 110 AML patients' samples that clustered together and showed predominantly down-regulation of the 33 genes (Figure 2A). The 33 genes in our signature are expressed during induction of differentiation by C/EBPα in a manner (up-regulation) opposite to that in this cluster of AML patients (down-regulation). These AML patients do, therefore, have defective activation of the C/EBPα signature, and we refer to this cluster as the dysfunctional C/EBPα subset (Figure 2A). Our cohort of 525 newly diagnosed AML patients included 26 with biallelic mutations in C/EBPα, and 12 with monoallelic mutations in C/EBPα. Remarkably, 17 out of the 26 samples with C/EBPα biallelic mutations were included in the dysfunctional C/EBPα subset, whereas only two out of the 12 samples with monoallelic mutations in C/EBPα were present in the dysfunctional C/EBPα subset (Figure 2A). Moreover, we analyzed the 525 AML patients' samples based on an activation score of the C/EBPα signature. We observed that a significant number of samples with C/EBPα biallelic mutations (21 out of 26, P=0.00319) had an activation score <0, indicating low activation of the C/EBPα signature in these patients (Figure 2B). No significant relationship was observed between monoallelic mutation status and activation score (P=0.571) (Figure 2B).

Taken together, these data identify a C/EBPα dysfunctional subset of AML patients characterized by down-regulation of the C/EBPα signature. A significant number of C/EBPα biallelic mutants, but not monoallelic mutants, clustered inside this dysfunctional C/EBPα subset. We hypothesize that C/EBPα biallelic mutants clustering outside the dysfunctional C/EBPα subset may harbor additional, not yet identified defects, which might affect the gene expression profile leading to their differential clustering. Furthermore, patients with C/EBPα monoallelic mutations retain one wild-type allele, which could explain why these patients tend to cluster outside the dysfunctional C/EBPα subset. In line with these findings, patients with C/EBPα monoallelic mutations have a different gene expression profile and prognosis than biallelic cases.[29]

Histone deacetylase inhibitors up-regulate expression of the C/EBPα signature

Since we identified a C/EBPα dysfunctional subset of AML patient samples characterized by down-regulation of the C/EBPα signature genes, we hypothesized that these patients could benefit from a treatment intended to re-activate expression of the C/EBPα signature. In order to identify molecules positively connected to the C/EBPα activation signature, we made use of the connectivity map (Cmap).[9] Table 2 lists small molecules that demonstrated positive connectivity to C/EBPα activation and, therefore, negative connectivity to C/EBPα dysfunction. Among the compounds with the highest correlation, we

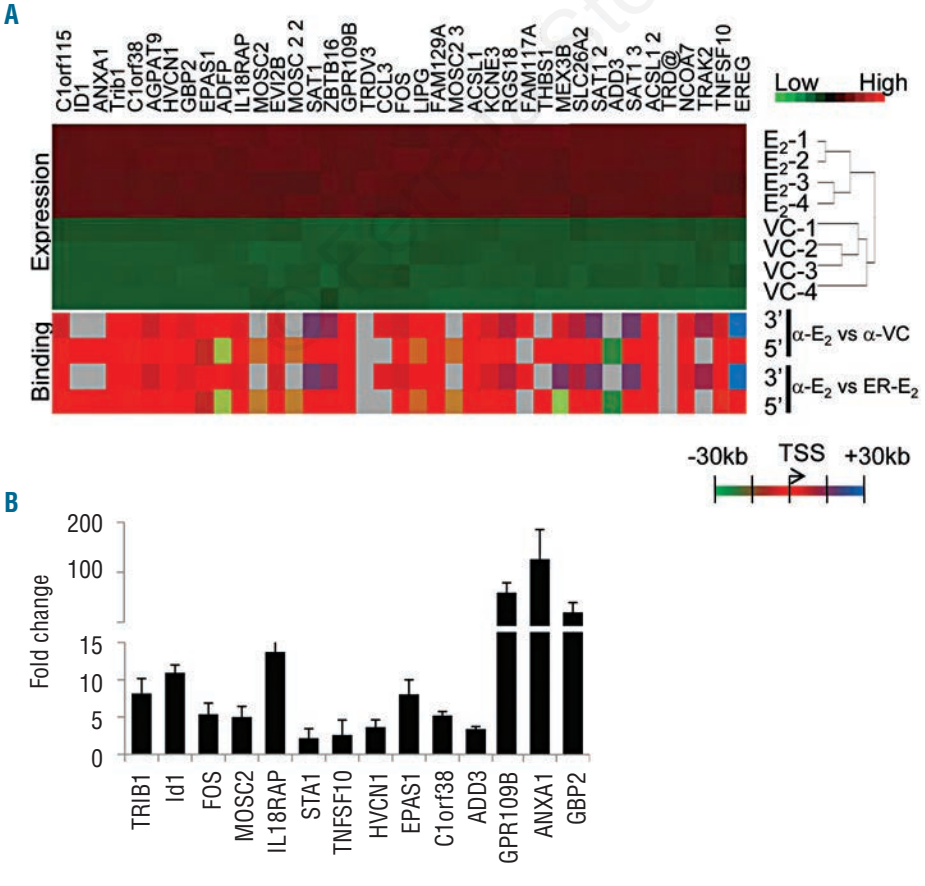


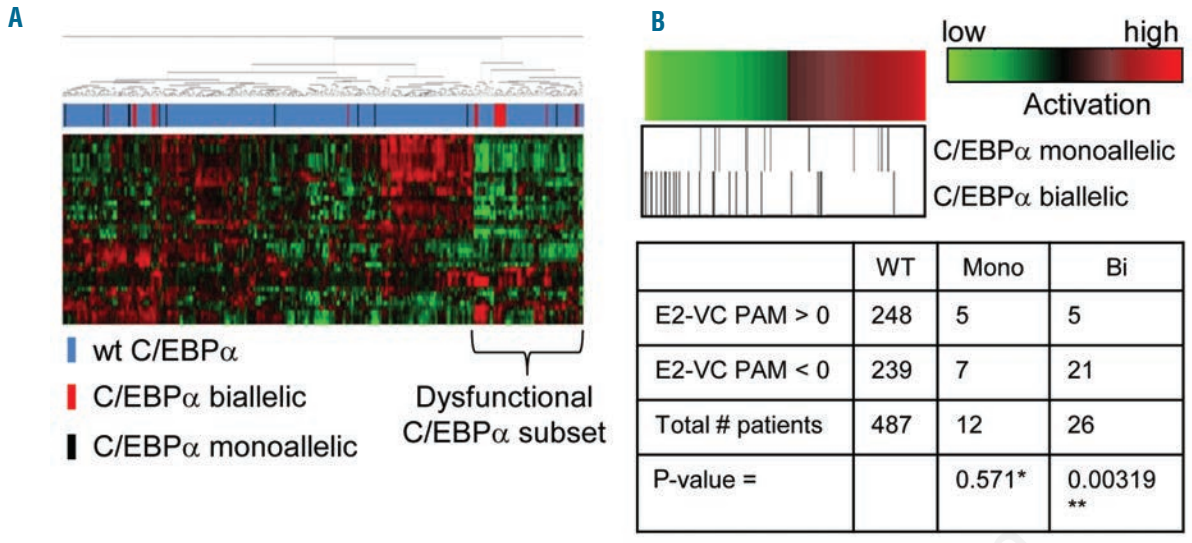
Figure 1. C/EBPα induction results in up-regulation of the C/EBPα signature. (A) Upper panel: heat map and hierarchical clustering based on gene expression of 42 probe sets identified using prediction analysis of microarrays (PAM). K562 C/EBPα-ER cells were treated with either vehicle control (VC-1, VC-2, VC-3, VC-4) or 1 μM β-estradiol (E_2-1, E_2-2, E_2-3, E_2-4) for 6 h. Data were normalized to z-scores for each gene. Red/green indicate an increase/decrease in gene expression relative to the universal mean for each gene. Lower panel: heat map (ChIPseq) shows the distance between the nearest downstream (5') and nearest upstream (3') significant CEBPα binding site from the transcriptional start site (TSS) of corresponding genes. α-E_2 (K562 C/EBPα-ER cells treated with 1 μM β-estradiol), α-VC (same cells treated with vehicle control), and ER-E_2 (K562 ER cells treated with 1 μM β-estradiol). Red indicates close proximity to the TSS. Gray represents regions without C/EBPα binding sites. (B) Quantitative RT-PCR in K562 C/EBPα-ER cells treated with either vehicle control or 1 μM β-estradiol for 6 h. Expression of 14 of the 33 genes identified is shown. Y-axis indicates fold change upon E2 treatment relative to the vehicle control. The experiment was performed using biological quadruplicates. Error bars indicate the standard deviation.

identified three HDAC inhibitors with significantly positive enrichment: TSA, SAHA, and valproic acid. We reasoned that HDAC inhibitors negatively correlate to C/EBPα non-activation, a condition of the C/EBPα dysfunctional subset, and could, therefore, be used to re-activate the C/EBPα signature for these specific AML patients. Furthermore, we observed that an additional six HDAC inhibitors were present in the Cmap, and that four out of these six had positive enrichment scores (*Online Supplementary Table S1*). However, their correlation with the C/EBPα signature was not significant; a possible explanation for this observation could be that those six HDAC inhibitors are under-represented in the Cmap (n=23 for these 6 HDAC inhibitors *versus* n=251 for TSA, SAHA, and valproic acid).

We next determined whether these small compounds could up-regulate expression of the C/EBPα signature genes. K562 cells were treated with TSA (0.25 μM and 1 μM), SAHA (0.5 μM and 2 μM), or vehicle control, and RNA was isolated at different time points. RT-PCR demonstrated up-regulation of several genes, such as *FOS*, *C1orf38*, and *SAT1*, as early as 6 h after HDAC inhibitor treatment (*data not shown*). At 12 h we analyzed the expression of 19 genes belonging to the C/EBPα signature and observed dose-dependent increases in the expression of 16 of them: *Trib1*, *ID1*, *FOS*, *IL18RAP*, *SAT1*, *TNSF10*, *HVCN1*, *EPAS1*, *C1orf38*, *ADD3*, *ANXA1*, *GBP2*, *ACSL1*, *ADFP*, *LIPG*, and *MOSC2* (Figure 3A and *Online Supplementary Figure S2A*). Interestingly, *Evi2b* expression was up-regulated at later time points (24 h and 48 h), whereas *GPR109B* expression was increased after 6 h of stimulation and decreased at later time points (*Online Supplementary Figure S2B*). Of note, only *MEX3* did not show changes of expression at any time after HDAC inhibitor treatment (*Online Supplementary Figure S2B*). The results indicate that the effect of HDAC inhibitors on the C/EBPα signature genes does not occur at a unique time point, suggesting that there might be different mechanisms of gene activation/regulation following HDAC inhibition. For example, HDAC inhibitors could have a direct effect on the expression of certain C/EBPα signature genes, and these up-regulated genes could induce a second wave of gene up-regulation. Alternatively, the effect of HDAC inhibitors could depend on the chromatin state of each gene, and modifications allowing up-regulation might be gene-specific, possibly depending on additional factors. We, therefore, sought to investigate whether there was a change in chromatin state due to treatment with HDAC inhibitors, which could thereby affect gene regulation. ChIP followed by quantitative RT-PCR indicated that K562 cells treated for 6 h with TSA had an enrichment of the active histone modification mark H3K4me3 in the proximal promoter of *FOS*, *TRIB1*, *ID1*, and *C1orf38* (Figure 3B), indicating that chromatin modifications can occur following HDAC inhibitor treatment favoring transactivation of the C/EBPα target genes. K562 cells do not endogenously express C/EBPα, suggesting that re-activation of the C/EBPα signature occurs in a C/EBPα–independent manner. One possibility is that HDAC inhibitor treatment induces activation of other transcription factors with redundant functions. In support of this hypothesis, we previously showed that C/EBPβ can be induced in the absence of C/EBPα to restore differentiation.[30,31] Altogether, these observations indicate that HDAC inhibitors re-activate the

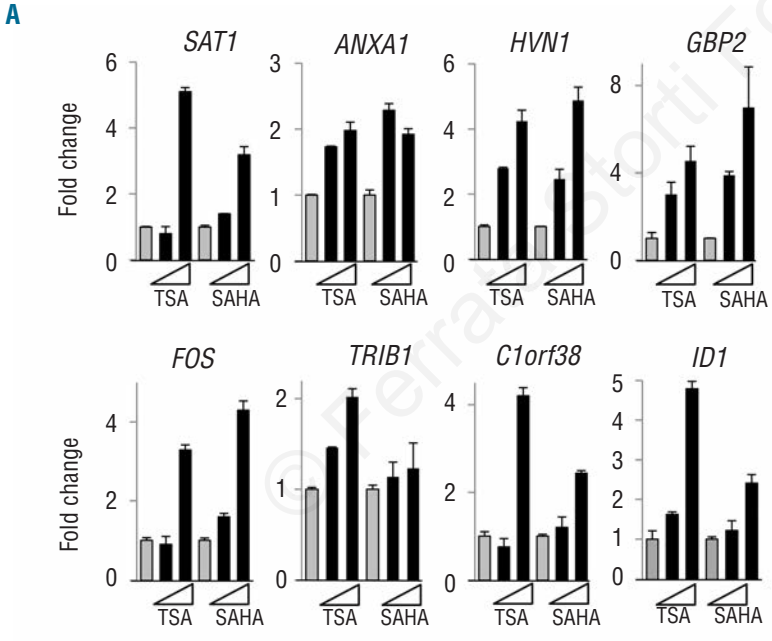
Table 2. List of the top 20 compounds obtained from the connectivity map using the C/EBPα signature (33 genes up-regulated in the K562 C/EBPα-ER cells treated with E2 *versus* ethanol control). Enrichment values are the result of permutation testing combining different treatment instances of the same compound. Positive enrichment values indicate positive connectivity to C/EBPα-activation and negative connectivity to C/EBPα-NON-activation. Three of the top four positively enriched small molecules are HDAC inhibitors: trichostatin A, vorinostat, and valproic acid.

Rank	Small molecule name	Mean	N.	Enrichment	*P* value	Specificity	Percent non-null
1	trichostatin A	0.398	182	0.617	0	0.1185	53
2	sirolimus	0.335	44	0.445	0	0.1024	50
3	vorinostat	0.447	12	0.656	0.00004	0.2412	58
4	ginkgolide A	-0.836	4	-0.916	0.0001	0	100
5	luteolin	-0.799	4	-0.882	0.00048	0.0057	100
6	valproic acid	0.261	57	0.258	0.00078	0.1053	54
7	gliclazide	-0.74	4	-0.833	0.00145	0.005	100
8	metamizole sodium	-0.409	6	-0.703	0.00159	0.0526	50
9	methacholine chloride	-0.776	3	-0.876	0.00385	0.0105	100
10	proglumide	-0.554	5	-0.71	0.00443	0	80
11	levonorgestrel	-0.631	6	-0.656	0.00467	0.0546	83
12	trifluoperazine	0.332	16	0.407	0.00644	0.3942	56
13	amitriptyline	0.342	6	0.637	0.00691	0.1019	50
14	dosulepin	0.725	4	0.752	0.00724	0.0205	100
15	sanguinarine	-0.827	2	-0.941	0.00742	0.0125	100
16	apigenin	-0.559	4	-0.752	0.00762	0.0598	75
17	ivermectin	0.634	5	0.687	0.00765	0.0914	100
18	norethisterone	0.685	4	0.742	0.00851	0.0203	100
19	pyrvinium	0.326	6	0.615	0.01005	0.2558	50
20	harmalol	-0.664	3	-0.827	0.01028	0	100



	WT	Mono	Bi
E2-VC PAM > 0	248	5	5
E2-VC PAM < 0	239	7	21
Total # patients	487	12	26
P-value =		0.571*	0.00319 **

Figure 2. AML patients clustered according to C/EBPα signature. (A) Thirty-three genes selected by prediction analysis of microrarrays (PAM) were used to cluster 525 newly diagnosed AML patients. The dysfunctional C/EBPα subset clusters 110 patients, and includes 17 of the 26 patients with C/EBPα biallelic mutations. Patients with wild-type C/EBPα (wt) are indicated in blue, C/EBPα biallelic mutants in red, and C/EBPα monoallelic mutants in black. (B) Five hundred and twenty-five newly diagnosed AML patients were sorted based on the activation a score of the C/EBPα signature. Green indicates a score <0 and red indicates score >0. C/EBPα monoallelic and biallelic mutants are indicated with a stripe. The table shows the C/EBPα status and number of patients in each group (wt: patients with wt C/EBPα, mono: C/EBPα monoallelic mutants, Bi: C/EBPα biallelic mutants). The *P* value was calculated using the Fisher exact test (*) for monoallelic mutations and the Pearson χ^2 test (**) for biallelic mutations.



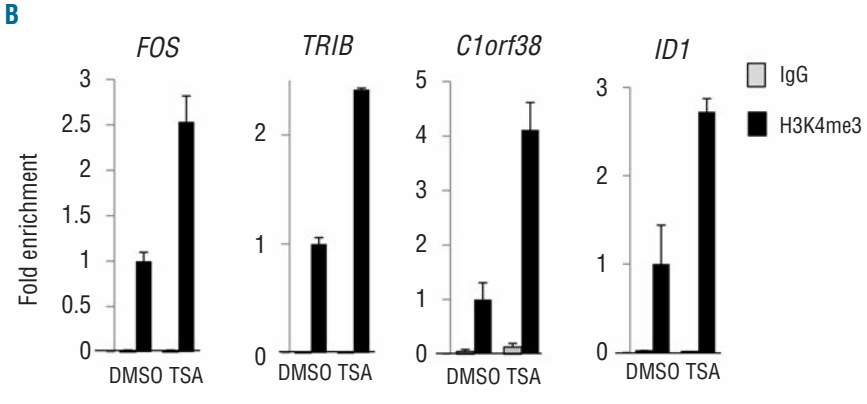


Figure 3. HDAC inhibitors upregulate the C/EBPα signature. (A) Quantitative RT-PCR in K562 cells treated with TSA (0.25 μM or 1 μM), SAHA (0.5 μM and 2 μM), or vehicle control (gray) for 12 h. Y-axis indicates fold change relative to vehicle control. This is one representative experiment out of three. (B) ChIP was performed using H3K4me3 or IgG antibodies on K562 cells treated with TSA (1 μM) or vehicle control (DMSO) for 6 h. Enrichment upon TSA treatment was determined in the proximal promoter of *FOS*, *TRIB1*, *C1orf38*, and *ID1*. The Y-axis represents fold enrichment relative to input.

C/EBPα signature, probably in a time-dependent manner involving transcriptional and epigenetic changes.

Histone deacetylase inhibitors promote granulocytic differentiation of biallelic C/EBPα mutant acute myeloid leukemia

The results presented above led us to investigate whether patients with biallelic C/EBPα mutations whose samples clustered within the C/EBPα dysfunctional subset, which lacks wild-type C/EBPα, would benefit from treatment with HDAC inhibitors, intended to reactivate C/EBPα target genes and promote granulocytic differentiation. Several HDAC inhibitors are currently being tested in clinical trials for solid tumors and hematologic malignancies. Our results indicate that only some AML patients, the C/EBPα dysfunctional subset, might benefit from treatment with HDAC inhibitors. We investigated whether biallelic C/EBPα mutant samples clustering inside the C/EBPα dysfunctional subset would respond to treatment with HDAC inhibitors. The HDAC inhibitors TSA and SAHA were titrated in these patients' samples using cell culture, and the effect on CD15 expression was determined by flow cytometric analysis (Figure 4A and *data not shown*). We determined that 0.25 μM TSA and 1 μM SAHA were optimal concentrations for further experiments. Of note, cultures with 2 μM SAHA were not viable due to the high percentage of cell death. Patients' samples with biallelic mutations in C/EBPα from the dysfunctional group (n=4) cultured in the presence of TSA or SAHA showed up-regulation of cell surface granulocytic markers such as CD15 and CD11b (Figure 4B). In contrast, patients' samples with biallelic mutations in C/EBPα, but clustering outside the dysfunctional group (n=4), had no significant changes under the same conditions (Figure 4B). It is inter-

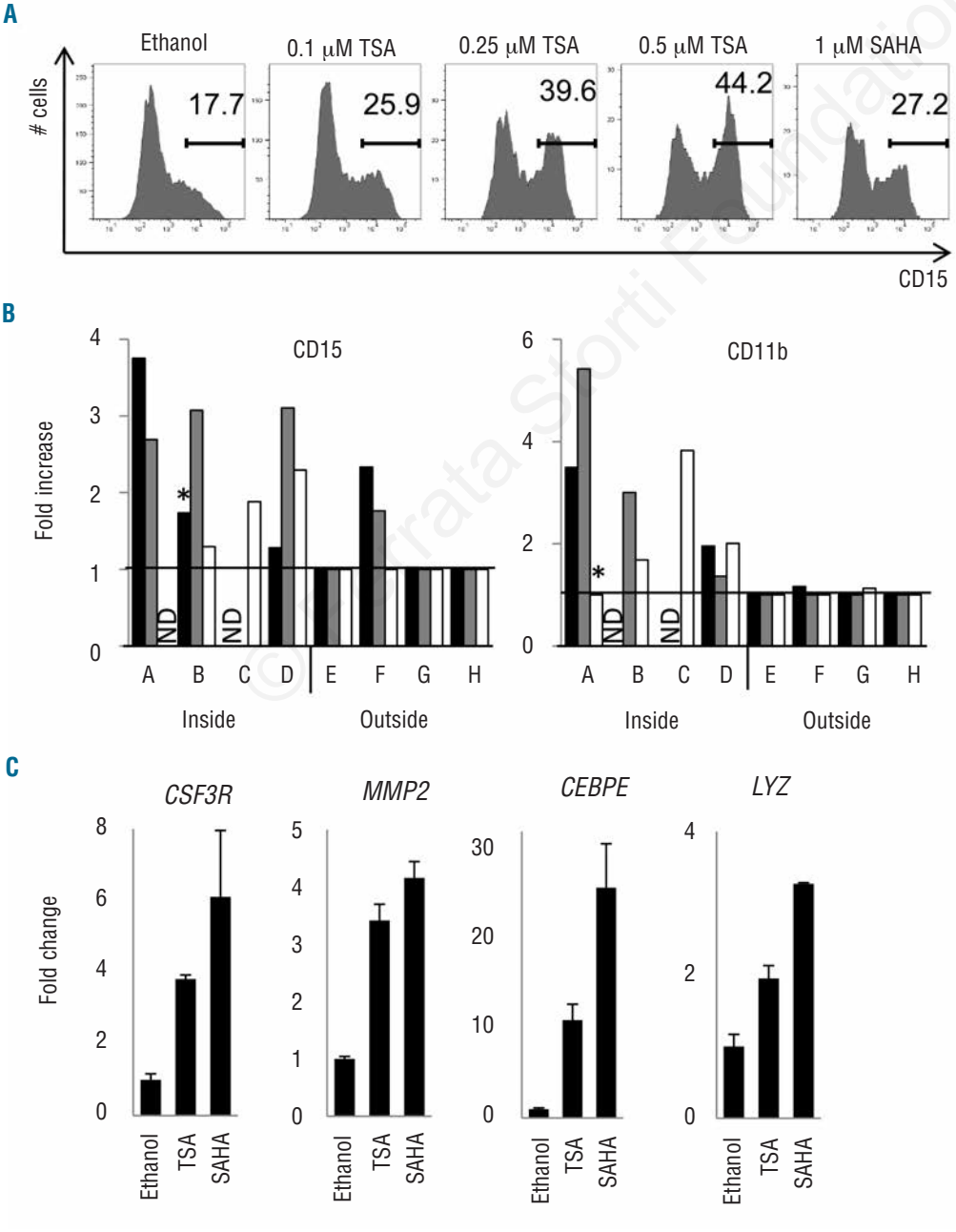


Figure 4. HDAC inhibitors promote granulocytic differentiation. (A) Dose-dependent effect of TSA (0.1 μM, 0.25 μM, 0.5 μM) and SAHA (1 μM) on primary AML samples from the C/EBPα dysfunctional subset with C/EBPα biallelic mutations (patient A). X-axis indicates fluorescence intensity of CD15 expression (logarithmic scale) and Y-axis the relative numbers of CD15 positive cells by flow cytometric analysis at day 4 of treatment. Numbers indicate the percentage of $CD15^+$ cells in the histograms. (B) CD15 (left panel) and CD11b (right panel) expression measured by flow cytometric analysis 7 days after treatment (*indicates 4 days). Patients' samples with C/EBPα biallelic mutations inside (A to D) and outside (E to H) the dysfunctional group were treated as indicated. Y-axis represents fold change in comparison to vehicle control, set at value 1. Black bars indicate 0.25 μM TSA, gray bars 0.5 μM SAHA (except 1 μM for patient A), and white bars 0.5 mM Merck60. ND: not determined. (C) G-CSF-R (*CSF3R*), gelatinase A (*MMP2*), C/EBPε (*CEBPE*) and lysozyme (*LYZ*) mRNA expression was determined by quantitative RT-PCR in a primary AML sample (patient A) with C/EBPα biallelic mutations from the dysfunctional group. Analysis was performed after 5 days of treatment with ethanol control, 0.25 μM TSA, or 1 μM SAHA. Y-axis indicates fold change relative to control treatment.

esting to note that, as predicted by the connectivity map, patients' samples with biallelic mutations in C/EBPα clustering outside the dysfunctional C/EBPα subset did not respond to HDAC inhibition. We hypothesize that C/EBPα mutations are not a driving force in the development of this particular AML, or that other abnormalities (which cannot be reversed by HDAC inhibitors) contribute to the leukemic phenotype. At this point of the study, a novel HDAC inhibitor became available, MERCK60, and we determined its effects on granulocytic differentiation in culture. We observed that MERCK60 had effects similar to TSA and SAHA on the expression of cell surface markers (Figure 4B). In addition, quantitative RT-PCR showed up-regulation of granulocyte-specific genes such as granulocyte colony-stimulating factor receptor (*CSF3R*), gelatinase A (*MMP2*), C/EBPε (*CEBPE*), and lysozyme (*LYZ*) in HDAC inhibitor-treated cells compared to cells treated with ethanol as a control in samples from within the C/EBPα dysfunctional group but not in those outside this group (Figure 4C and *Online Supplementary Figure S3*). Along with these results, we observed that *HDAC2*, *HDAC5*, *HDAC6*, and *HDAC8* were significantly up-regulated in the C/EBPα dysfunctional subset in comparison to the other AML samples (*Online Supplementary Figure S4*).

It has been reported that the most common hematologic adverse effects of HDAC inhibitors in clinical trials are neutropenia and thrombocytopenia.[32-35] However, the majority of the patients in these studies suffered from lymphoma or solid tumors. In AML patients, studies assessing HDAC inhibitors as monotherapy or in combination therapy, demonstrate evidence of neutrophilia and granulocytic differentiation in a small subset of patients.[36-39] Accordingly, our results indicate that only some AML patients might benefit from treatment with HDAC inhibitors.

In summary, our research identified a C/EBPα dysfunctional subset of AML patients characterized by inactivation of the C/EBPα signature. Using the connectivity map, we predicted that HDAC inhibitors could re-activate expression of the C/EBPα signature genes and promote granulocytic differentiation in patients in the C/EBPα dysfunctional subset. We demonstrated that biallelic C/EBPα patients clustering inside the dysfunctional subgroup, but not outside, could indeed benefit from this treatment. Similarly, several studies have suggested that HDAC inhibitors could promote differentiation of AML samples,[40,41] but so far the results obtained with HDAC inhibitors as a single agent or in combination therapies have not been encouraging.[37,42,43] Our data predict that only a subgroup of AML patients will respond to treatment with HDAC inhibitors. A precedent for this is found in acute promyelocytic leukemia, in which PML-RARα–positive cells, but not other AML cell-types, can be differentiated towards mature granulocytes in the presence of all-trans retinoic acid.[44] Furthermore, although HDAC inhibitors are currently used in clinical trials for the treatment of several malignancies, their mechanism of action is not understood. In the present study we showed that HDAC inhibitor treatment re-activates expression of certain C/EBPα target genes, and that this re-activation may occur in a C/EBPα–independent fashion. Altogether, our results suggest that HDAC inhibitors could represent a promising therapeutic approach in this particular subtype of AML. Though *CEBPA* mutations are present in only 10% of AML patients, by identifying a larger subset of patients who exhibit C/EBPα dysfunction, it can be postulated that HDAC inhibitors could represent a targeted intervention for a larger population of AML patients.

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Authorship and Disclosures

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