c-Myc modulation & acetylation is a key HDAC inhibitor target in cancer

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Translational Relevance

Although HDACi have often been described as preferentially tumor-selective, the underlying mechanism(s) of action remain unclear. Furthermore, their use in the clinic has not always met expectations. By mechanistically addressing HDAC inhibitors preferential tumor-selectivity, our findings suggest c-Myc, and its acetylation and modulation, as markers of HDAC inhibitors response prediction in vitro, ex vivo, in mice and in patients undergoing clinical trials. The potential improved patient stratification could pave the way towards ‘personalized’ therapies.
Abstract

Purpose: Histone deacetylase inhibitors (HDACi) are promising anticancer drugs. Although some HDACi have entered the clinic, the mechanism(s) underlying their tumor selectivity are poorly understood.

Experimental Design/Results: Using gene expression analysis, we define a core set of 6 genes commonly regulated in acute myeloid leukemia (AML) blasts and cell lines. c-Myc, the most prominently modulated, is preferentially altered in leukemia. Upon HDACi treatment, c-Myc is acetylated at lysine 323 and its expression decreases, leading to TRAIL activation and apoptosis. c-Myc binds to the TRAIL promoter on the proximal GC box through Sp1 or Miz1, impairing TRAIL activation. HDACi exposure triggers TRAIL expression, altering c-Myc-TRAIL binding. These events do not occur in normal cells. Excitingly, this inverse correlation between TRAIL and c-Myc is supported by HDACi treatment ex vivo of AML blasts and primary human breast cancer cells. The predictive value of c-Myc to HDACi responsiveness is confirmed in vivo in AML patients undergoing HDACi-based clinical trials.

Conclusions: Collectively, our findings identify a key role for c-Myc in TRAIL deregulation and as a biomarker of the anticancer action of HDACi in AML. The potential improved patient stratification could pave the way towards personalized therapies.
Introduction

Disruption of cellular pathways controlling proliferation and cell death, caused by genetic or epigenetic alterations, is a fundamental event for initiation and progression of cancer (1,2). Alterations in balanced epigenetic networks have been identified in all types of human cancers and involve multiple genes and molecular pathways. Changes in the epigenome comprehend several types of covalent modifications of DNA and associated proteins. The best-studied changes include modifications of the amino-terminal ends of core histones and methylation of cytosines in DNA. The mechanisms that drive epigenetic silencing in pre-malignant cells are, however, still rather elusive. Recent studies have suggested that one of the first steps in tumorigenesis may be epigenetic inactivation of genes altering the normal pathways of cell proliferation, differentiation and death (3). Unlike genetic mutations, epigenetic modifications are potentially reversible allowing the malignant cell population to revert to its initial state. With the advent of numerous drugs acting on specific enzymes involved in the epigenetic regulation of gene expression, targeting the epigenome is emerging as an effective and valid approach complementary to chemotherapy as well as chemoprevention of cancer, raising the possibility of epigenetic therapies. The re-expression of epigenetically inactivated genes can result in the suppression of tumor growth or sensitization to anticancer therapies. It is now well established that epigenetically silenced genes may be reactivated pharmacologically by the action of small molecules (‘epidrugs’) able to correct epigenetic defects. Treatment with epidrugs may inhibit enzymes such as DNA methyltransferases or histone deacetylases, reactivating transcription of silenced genes and restoring normal cellular growth and differentiation (4-6). Epigenetic transcriptional repression of tumor suppressor genes, cell cycle and DNA repair, and activation of invasion- and metastasis-regulating genes has been demonstrated in a wide variety of tumors. Recently, the effects of DNA methyltransferase inhibitors (DNMTi) and/or histone deacetylase inhibitors (HDACi) in advanced cancers have been investigated in clinical trials (6-8); at least 15 HDACi are currently under clinical investigation either alone or in combination with other therapeutic modalities for the treatment of hematological and solid malignancies (9).
One key problem with anticancer drugs is their lack of tumor specificity. Identifying genes that are differentially expressed in cancer cells compared to normal cells is likely to provide valuable information for customized treatment. We (and others) have previously demonstrated that the treatment of acute myeloid leukemia (AML) with HDACi is linked to re-expression of TRAIL and induction of apoptosis (10-12). The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis via 2 death receptors, death receptor 4 (DR4/TRAIL-R1) and 5 (DR5/TRAIL-R2), expressed on the cell membrane. Upon binding, apoptosis is rapidly triggered in a variety of human cancer cell lines but not in normal cells. This property makes TRAIL one of the promising novel bio-therapeutic agents for cancer therapy (13). However, the potential applications of TRAIL are limited since many primary cancer cells are resistant, generally due to defects in transduction of apoptotic signal of TRAIL-R. Several studies have employed strategies to boost the sensitivity of tumor cells to TRAIL, based on the combined administration of TRAIL with different drugs such as proteasome inhibitors, HDACi, and kinase inhibitors (14-17).

In light of the vastly differential cell responsiveness to TRAIL, it is crucial to identify and characterize biomarkers for sensitivity to this ligand. Ashkenazi and colleagues found that post-translational modification of DR4 and, in particular, DR5 plays a role in modulating TRAIL sensitivity in cancer cells. Death receptor O-glycosylation by peptidyl O-glycosyltransferase, GALNT14, enhances the TRAIL-induced clustering of DR4 and DR5, leading to activation of caspase 8. mRNA expression of GALNT14 is correlated with TRAIL sensitivity in pancreatic carcinoma, non-small-cell lung carcinoma and melanoma cells, suggesting that GALNT14 might serve as an excellent biomarker for screening patients most likely to benefit from TRAIL-based therapy (18).

The Myc oncogene is another potential marker for TRAIL sensitivity in cancer cells (19-21), including those with a defective intrinsic pathway signaling route for biomarker determination (22,23). Here we demonstrate that c-Myc binds to TRAIL promoter region and is essential for its silencing in AML cells. The HDACi SAHA (Vorinostat) and MS27-275 (Entinostat) induce c-Myc...
acetylation at lysine 323, leading to c-Myc down-regulation both at mRNA and protein levels, thus
unlocking TRAIL expression. Finally, we demonstrate that c-Myc-K323 acetylation and c-Myc
down-regulation also occur ex vivo in primary AML samples treated with HDACi.
Our findings indicate that c-Myc acetylation (at K323) and down-regulation might therefore
represent a useful biomarker for HDACi responsiveness and follow-up in AML.

Materials and Methods

Cell Lines and Cultures
AML (ATCC, DSMZ for NB4) cells were cultured in RPMI with 10% fetal bovine serum (FBS)
(Sigma-Aldrich, St Louis, MO), 2 mM L-glutamine (Euroclone, Milan, Italy) and antibiotics (100
U/mL penicillin, 100 µg/mL streptomycin and 250 ng/mL amphotericin-B). Both DSMZ and
ATCC authenticate cell lines by short tandem repeat (STR) DNA typing, developing a database
online for DNA of STR loci. For this study, NB4 and U937 cells have been validated by CGH
array. All cell lines used have been validated for the presence and expression of characteristic
fusion proteins and or molecular details. MePR-2B and human endometriosis stromal cells (hESC)
were cultured and validated as in (24) and in (25), respectively. For other cell lines identity and
details see Supplementary Methods.

Primary blasts, CD34+ progenitors and ex vivo culture
Procedures are described in Supplementary Methods.

RNA extraction and RT-PCR in vivo
Total RNA was extracted with Trizol (Invitrogen) by 23 AML patients in phase II of a clinical trial
at the M.D. Anderson Cancer Center (ClinicalTrials.gov Identifier: NCT00656617) and converted
into cDNA using VILO (Invitrogen). In vivo evaluation of relative expression of c-Myc on GAPDH
was evaluated.
Mammary tissue dissociation

Normal and tumoral mammary tissue by patients of breast cancer were dissociated by collagenase Type II (Invitrogen, Carlsbad, CA) in according to the manufacturers’ protocol.

Purification, culture and FACS analysis of mouse HSPCs

Procedures are described in Supplementary Methods.

Ligands

SAHA (Merck, Readington, NJ, USA), MS27-275 (Bayer-Schering AG, Berlin-Wedding, Germany), and EX527 (Selisistat) (Alexis Biochemical, Nottingham, UK) were dissolved in DMSO (Sigma) and used at 5x10^{-6} M.

Gene expression

Hybridizations were performed with Illumina HumanRef-8 V2 Expression BeadChips. Data have been deposited and are accessible through GEO accession number GSE55154 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55154). See also Supplementary Methods.

RNase protection assay (RPA)

RPA was carried out as in (12).

Western blotting

Western blotting analyses were performed following recommendations of antibody suppliers. Antibodies used were: c-Myc (N-262) (Santa Cruz, CA, USA), c-MycK323ac (Diagenode, Liège,
Belgium, custom-made), pan-acetylated lysines (Millipore, Heidelberg, Germany), α-tubulin (Sigma-Aldrich), ERKs (Santa Cruz).

**Chromatin immunoprecipitation (ChIP)**

H3K4me1, H3K4me2, H3K4me3, H3K9ac, H3K18ac, H3K9me, H3K16ac, H3K9,14ac, K9H3me1, K9H3me2, H4K16ac, H4K20me, H4K20me3, H3S10ph-K9ac (Abcam, Cambridge, UK), RNApoII and RNApoII5ph active (Covance, Princeton, NJ, USA), Sp1 and Sp3 (Millipore, Heidelberg, Germany) antibodies were used. ChIP assays were performed as previously described (12). See also Supplementary Methods and Table S2.

**Luciferase reporter assay, siRNA and shRNA**

pTRL were previously described (12). Plasmids for c-Myc and its phosphorylation mutants were kindly provided by H. Gronemeyer; MYC-K323 mutants (Q or R) were produced for this study. In MCF7 cells, siRNA was obtained from Dharmacon (Ladayette, CO, USA). MISSION shRNA target set by Sigma-Aldrich was used. pLKO.1-puro and pLKOshMyc plasmids were used according to manufacturer’s instructions. See also Supplementary Methods.

**Apoptosis and cell cycle**

Apoptosis and cell cycle analysis were performed as in (26).

**Immunoprecipitation assay**

Immunoprecipitation assay was performed as in (27).

**In vivo mouse studies**

PML-RARα APLs in the C57Bl/6 background were described previously (28) and in Supplementary Methods.
Results

Modulation in c-Myc expression is a signature of HDAC inhibition in AML cells

In order to identify a core set of HDACi-regulated genes and to provide a rationale for novel biomarkers useful in clinical practice, gene expression analyses were performed (Fig. 1). K562, U937 and NB4 AML cell lines were treated with two clinically advanced HDACi, SAHA and MS27-275, and gene expression profiles were analyzed in response to 6-hour treatment. Bioinformatic analysis revealed overlap in the genes altered in expression following treatment with the two HDACi within each cell line (Fig. 1A) as well as cell line-specific responses. In the three AML cell lines, 319 and 215 genes were commonly modulated by SAHA and MS27-275, respectively, and 154 genes were modulated by both HDACi. Importantly, a comparative gene expression analysis between the 154 common set derived from AML cell lines and 3 primary blasts of AML patients treated \textit{ex vivo} with SAHA showed a set of 6 commonly modulated genes, which we refer to as the core (Fig. 1B). It includes increased CRISPLD2 and TUFT1, and decreased MYB, MYC, PHFIS and RGS19. The well-known oncogenic properties of c-Myc, including its ability to recruit HAT/HDAC complexes (29), make it the most intriguing. Expression levels of the 6 core regulated genes were also evaluated in 2 CD34$^+$ normal progenitors treated with SAHA and then compared with those of AML cell lines and blasts. This comparison showed that all genes were similarly regulated by SAHA in CD34$^+$ except for c-Myc, which was only marginally affected by the treatment in the normal progenitors (Fig. 1B, right panel).

These data indicate that c-Myc might play a role in the tumor-specific action of HDACi in AML (11,12).

c-Myc binds the TRAIL promoter region

We and others have previously demonstrated that HDACi-induced apoptosis is mediated by TRAIL activation and represents a tumor-selective mechanism sparing normal cells (12). Past studies
established that c-Myc sensitizes cells to TRAIL (22,23). Although no evidence has been provided to date as to whether TRAIL and c-Myc affect distinct or the same apoptotic signaling pathways, their action may be interdependent in regulating the apoptotic signaling pathway activated by HDACi. Thus, we hypothesized that modulation of c-Myc itself might have a causal role in TRAIL activation and that tumor cell death or survival may depend on the acetylation status of c-Myc. In line with our hypotheses, ChIP experiments using U937 cells followed by semi-quantitative and quantitative PCR (Fig. 2A-B) revealed the presence of c-Myc on the TRAIL promoter in untreated conditions and its displacement after only 30 minutes and 4 hours of treatment with SAHA and MS27-275, respectively. Given that non-canonical E-boxes are found on the TRAIL promoter regulatory region and that 2 GC boxes on the TRAIL promoter are essential in its regulation by HDACi (12), c-Myc binding might be mediated by multi-protein complexes targeting GC boxes. Moreover, both HDACi displayed a strong inhibitory effect on c-Myc expression at mRNA (Fig. 2C and Supplementary Fig. S1A) and at protein levels (Fig. 2D). Notably, kinetics of c-Myc down-regulation correlated with the TRAIL mRNA induction by HDACi previously reported (12). Interestingly c-Myc reduction by HDACi is only partially proteasome-mediated as shown by the incomplete c-Myc restoration after treatment with MG-132, a known proteasome inhibitor, already after 3 hours, without significantly modifying cell cycle progression (Supplementary Fig. S1B). Furthermore, the addition either of cycloheximide or actinomycin D did not counteract SAHA-mediated c-Myc down-regulation, suggesting that a complex level of transcriptional, translational and post-translational regulation is occurring (Supplementary Fig. S1C-D). Notably, in the leukemia cell lines also c-FLIP mRNA levels resulted down-regulated, supporting the role of c-FLIP to potentiate the HDACi-mediated apoptosis pathways as also reported in other cell systems (30,31) (Supplementary Fig. S1E).

**c-Myc is a critical regulator of TRAIL levels**
To explore the regulation of TRAIL expression by c-Myc, transient transfection assays with chimeric luciferase reporter genes driven by TRAIL promoter were performed in U937 cells, revealing HDACi-inducible elements within 165 bases upstream of the transcription initiation. SAHA-inducibility of TRAIL was attenuated by cotransfection of c-Myc, suggesting that c-Myc is a negative regulator for TRAIL activation in AML (Fig. 2E). In agreement with our previous study (12), mutations in the promoter proximal GC box of TRAIL completely abrogated SAHA and c-Myc responsiveness, but did not affect basal levels, whereas mutations in the distal GC box reduced basal activity, but maintained substantial SAHA induction and c-Myc responsiveness (Fig. 2E, upper right). The fact that the two phosphorylation mutants c-Myc\textsuperscript{T58A} and c-Myc\textsuperscript{S62A} were still able to block TRAIL activation suggests that the role of phosphorylation of c-Myc at the conserved residues serine 62 (S62) and threonine 58 (T58) is marginal in these settings (Fig. 2E, lower right).

Further supportive evidence for c-Myc-induced repression of basal levels of TRAIL promoter as well as its induction by HDACi was obtained by Western blot analyses. A negative correlation between c-Myc and TRAIL expression was observed in three different cell lines (NB4, HL60 and U937) (Fig. 2F, upper panel). Moreover, siRNA-mediated reduction of c-Myc in MCF7 cells resulted in an increase in TRAIL protein levels (Fig. 2F, lower panel). In accordance, we observed a higher induction of TRAIL in stably infected sh-c-Myc than control U937 cells after HDACi treatment and an increased TRAIL basal expression level (Fig. 2G). In agreement and as expected, the growth rate of cells expressing sh-c-Myc was visibly altered when compared to sh-CTRL (Fig. 2H). Conversely, c-Myc overexpression increased both the capability to form colonies in semisolid medium and SAHA responsiveness (Supplementary Fig. S2A). c-Myc down-regulation and TRAIL induction by HDACi were not dependent on p21 expression (Supplementary Fig. S2B). Notably, TRAIL\textsuperscript{shRNA} showed reduced basal level of c-Myc and the HDACi-mediated down-regulation was no longer detected (Supplementary Fig. S2C). Furthermore, DR5 and p21\textsuperscript{WAF1} expression levels and their induction after HDACi treatment were also increased, demonstrating that these
HDACi-responsive genes with similar promoter architecture share similar c-Myc-responsive features (Supplementary Fig. S3) (32,33). Collectively, the data show that c-Myc binds to TRAIL promoter via the proximal GC box, supporting our hypothesis. Furthermore, it may be speculated that responsiveness to SAHA is influenced by c-Myc-TRAIL modulation.

**HDACi treatment unlocks TRAIL transcription**

Given that TRAIL promoter region may be repressed by c-Myc, we decided to investigate its epigenetic status. We performed quantitative ChIP assays to identify changes in histone H3 and H4 modifications at various loci across the TRAIL promoter region in U937 cells (Fig. 3, Supplementary Fig. S4 and Table S1). HDACi treatment induced an active epigenetic state of TRAIL by increasing H3K9 acetylation levels in the presence of high H3K4me3. The repressive transcriptional mark H3K9me3 was detected in basal conditions, and after 24-hour exposure to HDACi decreased significantly. In agreement with previous reports (34), H4K16ac decreased upon HDACi treatment, being negatively correlated with H3K9ac and positively correlated with H3K18ac. Analysis of RNA polymerase II and its active serine 5 phosphorylated form showed their recruitment to the TRAIL promoter region after only 30 minutes and 4 hours of SAHA and MS27-275 treatment, respectively. Notably, the different kinetics triggered by SAHA and MS27-275 positively correlated with TRAIL mRNA induction in U937 cells (data not shown and (10)). These findings indicate that TRAIL transcription is triggered by HDACi treatment.

**HDACi modulate c-Myc:Max binding to Sp1 and Miz1 on the TRAIL promoter region**

To better address the repressive role of c-Myc on TRAIL induction, we investigated the interactions between c-Myc and putative positive and negative transcriptional regulators (Fig. 4), and their expression levels (Supplementary Fig. S5). DNA binding and transcriptional activity of c-Myc requires its dimerization with Max (35). As shown in ChIP experiments, c-Myc:Max binding
occurred in basal conditions and was abolished upon HDACi treatment. Notably, Max was downregulated both at mRNA and protein levels, showing to be similarly regulated by HDACi as c-Myc (Supplementary Fig. S5). We previously reported that Sp1 resides on the TRAIL promoter region in basal conditions (12), and, then others provided evidence that Sp1 is responsible for HDACi-mediated TRAIL activation (36). Furthermore, c-Myc is reported to repress promoters with multiple Sp1-binding sites through Sp1/Sp3 (37). In agreement, our ChIP experiments revealed a strong Sp1:Myc binding tentatively explaining the repressed state of the TRAIL promoter region despite a highly H3K4me-marked chromatin. The pleiotropic action of the c-Myc-Max complex is further highlighted by its ability to repress another transcriptional activator, Miz1 (38), which we here show to reside on the TRAIL promoter (Fig. 4). Interestingly, corroborating the role of these interactions on TRAIL induction, Sp1 and Miz1 expression levels increased upon HDACi treatments (Supplementary Fig. S5).

Taken together our findings suggest a model (Supplementary Fig. S5) in which under basal conditions in tumor cells, TRAIL activation is blocked by binding of Myc-Max dimers to Sp1 or Miz1 at GC boxes. HDACi treatment triggers TRAIL activation by altering transcriptional factor binding. c-Myc-Max dimer disassembly may cause loss of c-Myc repressive activity.

**HDACi induce c-Myc acetylation at K323**

We then investigated whether HDACi treatment affected the acetylation status of c-Myc since multiple acetylation sites have been reported for c-Myc (39,40). Immunoblotting analyses with c-Myc-ac lysine-specific antibodies against several epitopes (Supplementary Fig. S6A) showed that c-Myc was hyperacetylated on K323 (Fig. 5A). The specificity of anti-MycK323ac antibody was validated by Western blot: the signal was abolished in the presence of the immunogenic but not control peptide. The antibody did not recognize acetylated histones (Supplementary Fig. S6B, upper left panel ‘HE’), and specifically detected acetylated c-Myc (Supplementary Fig. S6B, upper right panel ‘TE’). Pan-Kac antibody recognition was used as control (Supplementary Fig. S6B, lower panel).
important, the SAHA-induced increase in c-Myc acetylation was not cell type dependent as it was detected in many other cancer cell lines (Supplementary Figs S5, S6, S7). Furthermore, it was induced only by HDACi but not the SIRTi EX527 (41) (Supplementary Fig. S6C). Furthermore, c-MycK323ac was readily detectable already after 1 hour of SAHA treatment in U937 cells (Fig. 5A).

Interestingly, c-MycK323 acetylation was inversely proportional to HDACi-mediated down-regulation by c-Myc (Fig. 5B and Supplementary Figs S5 and S7). ChIP experiments (Fig. 5C) using c-Myc and c-MycK323 antibodies corroborated and extended the observation that whereas c-Myc is rapidly lost upon HDACi administration, c-MycK323ac transiently increases at the TRAIL promoter (Supplementary Table S2). Furthermore, c-MycK323ac is inversely proportional to c-Myc occupancy at the TRAIL promoter regulatory region and correlates with HDACi-mediated HDAC1 displacement (or its masking) previously reported by us and others (10,42). To corroborate and strengthen the functional role of c-MycK323ac in triggering TRAIL expression, transient transfection assays with chimeric luciferase reporter genes driven by the TRAIL promoter were performed in U937 cells in presence of SAHA and/or c-MycK323 mutants. SAHA-inducibility of TRAIL was strongly reduced by c-MycK323R and not c-MycK323Q (Fig. 5D), suggesting the importance of c-Myc acetylation in HDACi-mediated TRAIL induction. Moreover, ChIP experiments performed in MCF7 cells transiently transfected with c-Myc wt and mutant constructs and treated with HDACi, showed as c-MycK323R was retained on the TRAIL promoter region, blocking RNApolII binding and, thus, TRAIL activation (Fig. 5E).

**c-Myc (and its acetylated K323 form) tumor-specifically regulates TRAIL expression in *ex vivo* primary breast cancer & AMLs and *in vivo***

To corroborate our data, c-Myc levels and their correlation with TRAIL expression was also evaluated in 21 primary blasts derived from AML patients (Fig. 6A). In all cases the relative protein levels of TRAIL and c-Myc were inversely correlated. In untreated conditions, c-Myc and TRAIL
levels were high and low, respectively. Already after 24 hours of SAHA treatment, c-Myc mRNA levels were strongly decreased, while TRAIL transcript levels were up-regulated. TRAIL induction led to strong apoptosis, revealed as a percentage of cells in pre-G1 phase.

Excitingly, c-Myc deregulation by SAHA treatment was accompanied by its acetylation in ex vivo blasts, as representatively illustrated (Fig. 6B). Levels of c-Myc and its acetylated K323 form were not modulated by HDACi treatment in normal myeloid CD34+ progenitors in which basal levels of c-MycK323ac were present (Supplementary Fig. S8A-B). Similarly, c-FLIP mRNA levels resulted unaltered, supporting HDACi incapability to elicit cell death in normal systems (Supplementary Fig. S8B). Also in murine normal Lin+ cells, enriched up to 60% for their CD34+ component, c-Myc levels appeared constant after SAHA treatment, with cMycK323ac stably present (Fig. 6B and Supplementary Fig. S8B). This finding is in line with our previous published data highlighting the different response of AML blasts and normal counterparts to HDACi (12). Excitingly, ChIP experiments revealed that, in primary AML blasts, c-Myc resides on the TRAIL promoter region and gets displaced upon SAHA treatment. An inverse behavior was observed in CD34+ progenitors (Fig. 6C and Supplementary Fig. S8C). These data strongly suggested that a different regulation between, c-Myc, its acetylated form and TRAIL exists between normal and cancer cells. Several evidences further supported this notion. Indeed, in the immortalized (not normal, but not tumoral) cell systems, MePR-2B and hESC, neither modulation of TRAIL, nor c-Myc was observed, despite SAHA induced c-Myc hyperacetylation (Fig. 6D), suggesting that the Myc-TRAIL module deregulation is cancer specific and that deregulation of c-Myc acetylation may occur at an early step of neoplastic transformation.

Further supportive evidence derived from human ex vivo breast cancer cells. SAHA was able to induce c-Myc down-regulation and acetylation as well as TRAIL up-regulation in tumoral cells, but failed to do it in the neighboring normal counterpart derived from the normal tissue of the same patient (Fig. 6E). Thus, c-Myc regulation and its acetylation might represent an early event during malignant transformation, which can be reverted by HDACi treatment.
To corroborate and extend our data, c-Myc expression levels were evaluated in a group of 23 AML patients included in a phase II trial of SAHA with idarubicin and cytarabine (Fig. 6F). An evaluation of safety and efficacy of the study was previously published (2). The aim of this trial, the so-called IA program, was to test the efficacy of SAHA treatment in combination with standard chemotherapy for AML patients at the MD Anderson Cancer Center. Specifically, SAHA was administered orally 3 times a day at a dose of 500 mg on days 1 to 3; on day 4 idarubicin was administrated at a dose of 12 gm/m2/day for 3 days with cytarabine at a dose of 1.5 gm/m2/day as continuous infusion for 4 days. One cycle of therapy lasted 28 days.

Notably, in 14 patients, c-Myc expression decreased after 3 days of treatment with SAHA alone, providing a response prediction. The majority of patients displayed c-Myc down-regulation at day 7, although the greatest response was obtained at cycle end (day 28). Importantly, one therapy non-responder was a patient harboring chromosome 8 mutations on which the c-Myc gene resides.

Taken together, these data support the hypothesis that c-Myc (and its acetylated K323 form) tumor-specifically regulates TRAIL expression \textit{ex vivo} and in AML patients, predicting epi-treatment response. Strengthening the predictive role of c-Myc to HDACi responsiveness, c-Myc, c-MycK323ac and TRAIL expression levels were evaluated in an APL mouse model (28) (Fig. 6G). Survival studies on the mice after 27-days exposure to VPA were previously reported (28). VPA treatment resulted in a decreased signal of c-Myc and in concomitant increased signals both for c-MycK323ac and TRAIL. The confirmed opposite regulation of c-Myc and TRAIL and the contextual hyper-acetylation of c-Myc upon HDACi treatment \textit{in vivo} widen the key role of c-Myc in AMLs responsiveness.

**Discussion**

Deregulated apoptosis is one of the mechanisms contributing to tumorigenesis. Consequently, considerable efforts are being focused on finding coherent ‘apo-targets’ for potential therapeutic intervention. Pro-apoptotic pathways are often silenced in cancer cells; hence, the exciting...
possibility of driving tumor cells into death using drugs able to re-activate these pathways. A growing body of evidence has demonstrated the ability of epi-drugs such as HDACi to stimulate apoptosis in many tumor systems (43). Moreover, we (and others) have demonstrated that HDACi action is indeed tumor-selective, impacting on pro-apoptotic pathways in cancer but not in normal cells (12,44). Specifically, our and other groups have shown that HDACi-induced cancer-selective apoptosis involves TRAIL re-expression in AMLs (12,45).

Here we address the key question of HDACi tumor selectivity. Although HDACi have often been described as preferentially tumor selective, the underlying mechanism(s) of their action remain unclear (8,46). To establish markers of HDACi response it is crucial to investigate their effect \textit{ex vivo} and \textit{in vivo} on patients in order to validate data from \textit{in vitro} experimentation. Our comparison of gene expression profiles of AML cell lines with AML primary blasts treated with HDACi revealed that they have 6 deregulated genes in common (Fig. 1). These 6 genes may represent the conserved signature between AML cell lines and blasts after HDACi treatment. Interestingly, when testing the responsiveness of these genes to HDACi in CD34\textsuperscript{+} normal cells, c-Myc gene expression was most differentially regulated. In normal progenitors c-Myc was slightly – if at all – deregulated while both in AML cell lines and primary blasts it was highly down-regulated.

c-Myc appears to play a repressive role in TRAIL induction in untreated AMLs since its overexpression (Fig. 2F) and silencing (Fig. 2F-G) resulted in inverse TRAIL expression. Similarly, c-Myc negatively regulates TRAIL promoter activation both at the steady state level and upon HDACi treatment (Fig. 2E). Supporting the role of c-Myc in marking HDACi response, c-Myc-silenced cells responded weakly to HDACi treatment, displaying an ‘initially impaired’ growth rate, likely due to TRAIL re-expression levels. Interestingly, although TRAIL expression was low in AML cells, active epigenetic marks at the TRAIL promoter (33), such as H3K4me1, 2, 3, are strongly present (Fig. 3, Supplementary Fig. S4 and Table S1), suggesting a potentially open chromatin structure. The puzzling finding that the TRAIL promoter did not contain canonical E-boxes for c-Myc binding (12) led us to hypothesize that the repressive binding of c-Myc on TRAIL
promoter likely occurs via the proximal GC box, which is a binding site for Sp1/Miz1. We previously showed that this cis-acting element is crucial for HDACi-mediated TRAIL activation (and other apoptotic genes) (12,32,33). As c-Myc resides on the TRAIL promoter regulatory region (Fig. 2A-B) and binds Sp1/Miz1 (Fig. 4), we may conclude that c-Myc mediates TRAIL regulation via the proximal GC box. In this scenario, the Myc-Max dimer strongly represses TRAIL and is displaced upon HDACi treatment, thus allowing transcriptional action of Sp1 (47). It is thus tempting to speculate that this type of (de)regulation is a common mechanism for HDACi-responsive genes, as supported by the similar behavior of DR5 and p21 (Supplementary Fig. S3).

Taken together, our findings strongly suggest that in cancer cells TRAIL is transcriptionally silenced by c-Myc and rapidly activated upon loss of c-Myc repression. Strikingly, the accumulation of TRAIL mRNA is inversely correlated with the displacement of c-Myc from the promoter.

This rapid de-repression is caused by the action of Sp1 and/or Miz1 protein, which remain associated with TRAIL promoter after c-Myc removal. TRAIL appears to be primed for rapid de-repression by Sp1 and/or Miz1, poised to begin transcription as soon as the removal of c-Myc from TRAIL promoter is initiated. RNApol-II enzyme is already recruited after 30 minutes of treatment. In a stable repressed state c-Myc is stalled on the TRAIL promoter region, effectively blocking transcription. Upon HDACi treatment the removal of c-Myc alone induces a rapid transcriptional response as the other factors are already engaged. A plausible model for such a mechanism is suggested by the model in Fig. 4.

Based on these data, several hypotheses may be formulated. Firstly, c-Myc deregulation in cancer acts both pro-proliferatively by direct binding to E-box-containing promoters, and as a suppressor of apoptosis via Sp1/Miz1-residing factors on GC boxes. Secondly, HDACi restore normal regulation by decreasing c-Myc levels and reactivating pro-apoptotic pathways in cancer. Thirdly, c-Myc acetylation triggered by HDACi alters c-Myc stability (Fig. 5D and Supplementary Fig. S1B-D). In support of the third hypothesis, K323, one of the predicted acetylation sites on c-Myc
was specifically acetylated after 1 hour (Fig. 5). c-MycK323ac occurs in many other cancer cell lines upon treatment with HDACi, but not with SIRTi (Supplementary Fig. S6B). Given that HDAC1 and 2 have previously been found to reside at the TRAIL promoter (12), c-Myc acetylation likely occurs by HDACi-mediated modification of the binding (and possibly stability) of the c-Myc-Max complex on the Sp1 and/or Miz1 factor present on TRAIL chromatin. Extending and corroborating this hypothesis, c-MycK323ac was found to be inversely proportional to c-Myc occupancy on the TRAIL promoter. Significantly, no c-Myc occupancy was found at the intron level (Fig. 5C). Overall, this model is consistent with the observation that active chromatin marks are associated with the majority of c-Myc target-promoters (49). Hence, c-Myc may block TRAIL expression in a steady state, which can be easily re-activated by HDACi. Inverse c-Myc/TRAIL gene expression was detected in a statistically valid number of ex vivo AML blasts and, as an example of solid cancer, in an ex vivo breast cancer, corroborating this hypothesis. Specifically, this finding shows that in AML blasts, c-Myc-dependent TRAIL silencing is a common event and that HDACi-induced apoptosis correlates with decreased c-Myc levels upon HDACi treatment (Fig. 6A).

We speculated that our findings (Fig. 1) suggest a differential action of HDACi on c-Myc expression. In turn, c-MycK323ac might serve as a marker of response prediction and explain the opposite effect of HDACi in cancer and normal cells. In CD34+ normal myeloid progenitors, levels of c-Myc (and c-MycK323ac) are present at a lower level, but are not modulated by HDACi (Fig. 6B and Supplementary Fig. S8), highlighting that c-Myc down-regulation and acetylation may contribute to the tumor specificity of HDACi in AMLs. Notably, K323 acetylation seems to affect stability of the c-Myc protein only when hyper-induced as in AMLs, suggesting that over-acetylation might cause reduced protein stability while preserving stability in normal conditions (50,51) (Fig. 6B).

We therefore hypothesized a model to explain the differential response of normal and cancer cells to HDACi (Fig. 6H and Supplementary Fig. S8). In AMLs (or cancer in general) overexpressing c-
Myc, TRAIL expression is silenced; upon HDACi treatment, c-Myc acetylation and modulation derepresses TRAIL, leading to a strong apoptotic response. In normal cells expressing c-Myc and its acetylated K323 form at a steady state level, TRAIL is expressed; upon HDACi treatment, c-Myc and c-MycK323ac are unaltered, enhancing the repressive action of c-Myc on the TRAIL promoter and inhibiting the TRAIL apoptotic pathway. Confirming our hypothesis in vivo, AML patients in a clinical trial (ClinicalTrials.gov Identifier: NCT00656617) with SAHA followed by standard chemotherapy showed a similar decrease in c-Myc and an increase in TRAIL expression (Fig. 6F). Interestingly, non-response or rapid relapse correlated with the presence of mutations on chromosome 8 (on which c-Myc resides) or more in general with no expression followed by a reduction of c-Myc upon SAHA administration. Interestingly, corroborating and extending the modulation of c-Myc and its acetylation in AMLs, in the APL mouse model treated with HDACi (VPA) (28), c-Myc (and its acetylation levels) and, consequently, TRAIL expression correlated with HDACi responsiveness (Fig. 6G). In full agreement with this hypothesis, ex vivo primary human breast cancer cells displayed modulation of TRAIL, c-Myc and its K323 acetylation in response to SAHA, differently from the normal counterpart, increasing the evidences for a tumor specific effect.

Several lines of evidence support the important role of c-Myc. Firstly, c-Myc is one of the most frequently altered genes in cancer and is strongly modulated by HDACi. Secondly, clinical trials using HDACi are ongoing against all major cancer types including AMLs (6,8,52). Thirdly, to date, only one gene, HR23B, has been proposed as potentially predictive for HDACi response in cancer patients, although only in cutaneous T cell lymphoma (CTCL) (53,54) highlighting the desperate need for novel biomarkers. Despite positive outcomes with CTCL, improved patient stratification in clinical trials could pave the way towards ‘personalized’ therapies. Fourthly, c-Myc expression and/or its deregulation has been shown to be one of the key mechanisms which may explain differing rhAPO2L/TRAIL (Amgen/Genentech) sensitivity in several cancers. Taken together, our in vitro, ex vivo (AML and breast cancer) and in vivo (mouse, human) mechanistically-based data
strongly suggest that c-Myc may represent an important modulator for response to HDACi and further explain their preferentially tumor-selective action.

Disclosure of Potential Conflicts of Interest
The authors have no conflicting financial interest.

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References
Figure legends

**Figure 1. Gene expression analysis suggests that HDACi target c-Myc in AML systems.** (A) Venn diagrams showing the number of regulated genes in K526, U937 and NB4 cells treated with SAHA (left) and MS27-275 (right) for 6 hours. Venn diagram (center) showing genes common to both HDACi. (B) Venn diagram showing the number of regulated genes in cell lines and in AML blasts. Lower panel showing the 6 genes common to the 3 cell lines, AML patients and normal cells CD34⁺, and their relative fold-changes. Each gene found to be regulated met the criteria of a p-value < 0.05 and a fold-change of more than 2.

**Figure 2. c-Myc is a negative regulator of TRAIL expression.** (A and B) ChIP assays were performed in U937 cells treated with MS27-275 and SAHA at the indicated times using c-Myc antibody and a control IgG. c-Myc was present on the TRAIL promoter region and was displaced after HDACi treatment. (A) Conventional ChIP-PCR analysis showing c-Myc occupancy on the
promoter region. (B) ChIP-qPCR analysis showing the relative decrease of enrichment (data obtained on immunoprecipitated fractions were normalized to input chromatin (IP/Input)). Curves show the mean of at least two independent experiments with error bars indicating the standard deviation. (C and D) RNase protection (RPA) (C) and Western blotting analysis (D) showing gradual decrease of c-Myc transcriptional and protein levels. Both experiments were performed in U937 cells treated with MS27-275 and SAHA at the indicated times. L32 and GAPDH were used as loading controls for RPA and ERKs as loading control for Western blots. (E) Transient transactivation assays show the negative regulation on TRAIL activation by c-Myc via GC boxes. (Left) Expression vectors carrying a luciferase reporter followed by a TRAIL promoter region in their wild-type form (black bars) or mutated in GC boxes (proximal or distal) (top, right) were transfected in U937 cells in the presence of SAHA and/or c-Myc both wt and mutant in T58 or S62 (bottom, right). Normalized luciferase activity values from three independent experiments in triplicate are shown. (F) Opposite regulation between c-Myc and TRAIL expression levels. Representative Western blots of TRAIL and c-Myc in U937, HL60, and NB4 cells treated with SAHA for 24 hours. ERK signal was used as loading control. Western blots for TRAIL, BAX, and c-Myc in MCF7 cells after transfection with a scramble siRNA (siScramble), or with c-Myc-directed siRNA (siMyc) or GAPDH-directed siRNA (siGAPDH). GAPDH and actin were used as loading controls. (G) Knockdown of c-Myc led to a higher TRAIL up-regulation after HDACi treatment. qRT-PCR for TRAIL gene in U937 cells stably infected with a control shRNA (shCTRL) and a c-Myc-directed shRNA (shc-Myc) and treated with MS27-275 and SAHA at the indicated times. Normalized fold induction values were the average of experiments in triplicate with error bars indicating the standard deviation. Representative quantification of TRAIL (by qRT-PCR) and c-Myc (by Western blots) expression in shCTRL and shc-Myc stable cellular pools demonstrating the effects of c-Myc knockdown. (H) Proliferation curve of shCTRL and shc-Myc U937 cells after MS27-275 and SAHA treatment showing the ability of c-Myc to affect growth rate.
Figure 3. Epigenetic status of the TRAIL promoter region after HDACi exposure. qPCR analysis of ChIP enrichments calculated as IP/Input for the indicated histone modifications and RNA pol-II and its phosphorylated form on the TRAIL promoter region in U937 cells treated with MS27-275 and SAHA at the indicated times. Curves show the mean of at least two independent experiments with error bars indicating the standard deviation.

Figure 4. Modulation of c-Myc binding after HDACi exposure. qPCR analysis of ChIP and re-ChIP enrichments calculated as IP/Input for c-Myc, Max, Sp1 and Miz1.

Figure 5. c-Myc acetylation in K323: a key event in TRAIL activation by HDACi. (A) Western blotting analysis for c-Myc and pan-acetylated lysines from U937 extracts immunoprecipitated for c-Myc. Representative Western blots indicate the hyper-acetylation of c-Myc after 24-hour exposure to SAHA (upper, left). Western blots for c-MycK323ac and c-Myc from whole extracts of U937 cells treated with SAHA at the indicated times, showing the site-specific acetylation of the target. α-tubulin was used as loading control (bottom, left). (B) Western blottings for c-MycK323ac antibody on whole U937 cells treated with SAHA at the indicated times to validate antibody specificity. ERK signal was used as loading control. (C) qPCR analysis of ChIP calculated as IP/input on the indicated genomic region for c-Myc and its acetylated isoform in U937 cells treated with SAHA at the indicated times. Curves show the mean of experiments in triplicate with error bars indicating the standard deviation. (D) Transient transactivation assays show the key role of c-MycK323ac in TRAIL activation by SAHA. TRAIL-Luc promoter region was co-transfected in U937 cells with c-Myc or mutant in its K323 (Q or R) mutants. Normalized luciferase activity values from three independent experiments in triplicate are shown. (E) ChIP assays were performed in MCF7 cells transfected with Myc expression vectors both wt and mutant in K323R in the presence of SAHA at the indicated times. Normalized (to the wt plasmid) recovery values from two
independent experiments in triplicate are shown with error bars indicating the standard deviation. In the upper inset qPCR analysis for TRAIL was showed.

Figure 6. c-Myc regulation in primary cancer cells ex vivo, in mouse models and AML patients in vivo. (A) c-Myc and TRAIL expression levels by RT-qPCR and evaluation of the pre-G1 phase by FACS analysis in 21 primary blasts of AML patients showing the inverse relationship between c-Myc levels and TRAIL/apoptosis activation. All points were tested in triplicate and plotted as mean ± SEM using GraphPad Prism5 software. Student t-test (two-tailed) was applied to estimate the significance of gene expression changes: *** p<0.0001. (B) Representative Western blotting analysis for c-Myc and its acetylated form indicating that in AML blasts the regulation of c-Myc was the same as in cell lines, while in human normal progenitors CD34⁺ no c-Myc modulation was observed. In the insert Western blotting in murine Lin⁺ cells (C) qPCR analysis of ChIP for c-Myc in 2 representative samples of AML blasts and 2 human normal progenitor CD34⁺ showing the opposite regulation of c-Myc in the two cellular systems upon 24-hour treatment with SAHA. (D) Western blotting analysis for c-Myc, its acetylated form and TRAIL indicating that in indicated cellular systems the acetylation of c-Myc was maintained. (E) Representative Western blotting analysis for c-Myc, c-MycK323ac and TRAIL indicating that in breast cancer cells ex vivo the regulation of c-Myc recapitulates cell lines and AML blasts, while the normal counterpart does not display c-Myc modulation. (F) In vivo evaluation of relative expression of c-Myc in 23 AML patients in phase II of a clinical trial at the M.D. Anderson Cancer Center (see Materials and Methods). Therapy response was analyzed according to stage of therapy (x-axis, D, day) and % of c-Myc expression (y-axis). Gene expression was normalized to GAPDH sequentially during the first course of therapy of 28 days (D). Key colours represent patient identity. All points were tested in triplicate and plotted as mean ± SEM using GraphPad Prism5 software. Student t-test (two-tailed) was applied to estimate the significance of gene expression changes: ** p=0.0011. (G) Opposite regulation between c-Myc and TRAIL expression levels followed by an hyper-acetylation of c-Myc.
in cells derived from the spleen of untreated and treated leukemic mice with VPA for 48 h. α-
tubulin signal was used as loading control. (H) Representative model of HDACi-regulated TRAIL
transcription in normal and cancer cells. In normal cells expressing c-Myc (and its acetylated K323
form at a steady state level), TRAIL expression is present; upon HDACi treatment, c-Myc and its
acetylated K323 form remain unaltered, stabilizing the c-Myc repressive action on the TRAIL
promoter and repressing the TRAIL apoptotic pathway. In cancer, overexpressing c-Myc, TRAIL
expression is silenced; upon HDACi treatment, c-MycK323ac occurs, c-Myc is displaced from the
TRAIL promoter, reactivating TRAIL apoptotic pathways and leading to apoptotic response to
treatment.
Figure 1

**A**

Common genes

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**B**

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**Legend**

- **SAHA**: Red
- **MS27-275**: Blue
- **AML cell lines**: Orange
- **AML patients**: Green
- **CD34+**: Pink
Figure 3
Figure 5

A

B

C

D

E

TRAILp

Fold induction (Mut/wt)

Recovery % IP/Input

TRAILp

TRAILintron

TRAILpluc

TRAIL

Fold mut/wt

Recovery % IP/Input

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Figure 5
Figure 6
c-Myc modulation & acetylation is a key HDAC inhibitor target in cancer

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