Histone deacetylase inhibitors (HDACi) induce growth arrest and apoptosis in colon cancer cells and are being considered for colon cancer therapy. The underlying mechanism of action of these effects is poorly defined with both transcription-dependent and -independent mechanisms implicated. We screened a panel of 30 colon cancer cell lines for sensitivity to HDACi-induced apoptosis and correlated the differences with gene expression patterns induced by HDACi in the five most sensitive and resistant lines. A robust and reproducible transcriptional response involving coordinate induction of multiple immediate-early (Fos, Jun, egr1, egr3, atf3, arc, nr4a1) and stress response genes (Ndrg4, Mitb, Mit1E, Mit1F, Mit1H) was selectively induced in HDACi sensitive cells. Notably, a significant percentage of these genes were basally repressed in colon tumors. Bioinformatics analysis revealed that the promoter regions of the HDACi-induced genes were enriched for KLF4/Sp1/Sp3 transcription factor binding sites. Altering KLF4 levels failed to modulate apoptosis or transcriptional responses to HDACi treatment. In contrast, HDACi preferentially stimulated the activity of Sp1/Sp3 and blocking their action attenuated both the transcriptional and apoptotic responses to HDACi treatment. Our findings link HDACi-induced apoptosis to activation of a Sp1/Sp3-mediated response that involves derepression of a transcriptional network basally repressed in colon cancer. Cancer Res; 70(2); 609–20. ©2010 AACR.
shown to sensitize colon cancer cells to TRAIL and Fas ligand-induced apoptosis (15, 16), suggesting activation of the two pathways may occur in parallel.

Whereas the downstream effectors of HDAci-induced apoptosis have been studied in detail (17, 18), the molecular events that initiate the process are less well defined. One postulated transcription-dependent mechanism is alteration in the balance in expression of proapoptotic and antiapoptotic genes in favor of apoptosis (2). For example, in colon cancer cells HDAci upregulate expression of the proapoptotic genes, Bax (19) and Bak (20), and downregulate expression of the antiapoptotic Bcl-XL gene (13).

Alternatively, HDAci can initiate apoptosis via transcription-independent mechanisms (21). For example, HDAci can promote apoptosis via hyperacetylation of HSP90 and subsequent depletion of prosurvival HSP90 client proteins (22, 23). HDAci-induced apoptosis has also been linked to aberrant mitosis (22, 24–27).

Therefore, whether HDAci-induced apoptosis is initiated primarily in a transcription-dependent or -independent manner is currently unclear. In the present study we identify a robust and reproducible transcriptional response selectively induced by HDAci in sensitive cell lines involving the induction and repression of 48 and 44 genes respectively. The induced genes were highly enriched for immediately (IE) and stress response (SR) genes. We show that this transcriptional response and subsequent apoptosis are coordinately induced by HDAci in a Sp1/Sp3-dependent manner. Notably, a significant percentage of the HDAci-induced genes are basally repressed in colon tumors, suggesting HDAci-induced apoptosis involves deregulation of a complex transcriptional network basally repressed in colon cancer cells.

Materials and Methods

Cell lines and cell culture. The 30 colon carcinoma cell lines used were Caco-2, Colo201, Colo205, Colo320, Dld-1, HCT116, HCT-15, HCT-8, HT29, LoVo, LS174T, RKO, SKCO-1, SW1116, SW403, SW48, SW480, SW620, SW837, SW948, T84, WiDr, HT29-Cl.16E, HT29-Cl.19A, LIM1215, LIM2405, HCC2998, KM12, RW2982, and RW7213. The source and methods of maintenance have been previously described (28). RKO-EcR and RKO-EcR-KLF4 (also called RKO-RG24) were kindly provided to us by Dr. Vincent Yang and used as previously described (29).

Drug sensitivity assays. Methods used to determine apoptosis, clonogenic survival, and response of xenografts to HDAci treatment in vivo are described in the supplementary text.

Microarray experiments. Exponentially growing cells were treated with 5 mmol/L butyrate for 24 h, and total RNA was extracted using the RNeasy kit (Qiagen). For cDNA microarray analysis, 50 μg of RNA was labeled with Cy3 dUTP (control) or Cy5 dUTP (butyrate-treated). Probe preparation, hybridization conditions, and array scanning procedure were as previously described (5, 6). Probes were hybridized to 27,000 feature cDNA microarrays generated by the Albert Einstein College of Medicine microarray facility (30), and images were analyzed using Genepix Pro software (Axon Instruments, Union City, CA) and Lowess normalized. For each cell line, two independent butyrate treatment experiments were performed, and the mean fold change in expression following butyrate treatment relative to control was computed for each sequence. Microarray experiments using the Nimblegen platform are described in Supplementary Methods.

To identify genes differentially regulated (induced or repressed) in response to HDACi treatment in sensitive and resistant cell lines, the mean fold change in expression (butyrate relative to control) across the five sensitive and five resistant lines was computed for each gene. Differentially expressed genes were selected upon their ability to satisfy each of the following three criteria: (1) changed 2-fold or greater relative to control in either the sensitive or resistant panel; (2) differentially regulated by a factor of 1.5-fold or greater between sensitive and resistant cells; (3) significantly differentially regulated between sensitive and resistant cells by an unpaired t-test, with a P-value of <0.05 considered statistically significant.

To identify genes not changed in response to butyrate treatment, we first identified all genes for which the median fold induction in response to butyrate treatment was <10% (within the range of +1.1 to −1.1), for both the sensitive and resistant panel. From this list we then selected genes that were also not significantly differentially expressed between sensitive and resistant lines (P value resulting from a t test between sensitive and resistant is >0.05). Clones (2,751) that meet the above criteria were identified. From these, a random number generator was used to select 48 genes that were used for comparative analyses with the HDACi-induced gene list.

Promoter analysis. Promoter sequences for the 48 HDAci-induced genes as well as an equal number of control genes not changed by HDAci were obtained from the University of California Santa Cruz genome browser.4 The promoter region encompassing 1.5 kb upstream of the transcription start site (TSS) through 200 bp downstream was systematically searched for the presence of transcription factor binding sites using the MATCH tool (BIOPASS Biological Databases GmbH, Wolfenbüttel, Germany) on the TransFac public site. For more detailed guanine-cytosine (GC) content, Sp1, Sp3, and KLF4 binding site analyses, each promoter was subdivided into 1-kb windows beginning 10 kb downstream of the TSS and ending 10 kb upstream of the TSS. The GC content of each 1-kb window was computed using the freak tool in the EMBOSS suite accessed on the Washington State University Bioinformatics server5 (31). The frequency of Sp1, Sp3, and KLF4 binding sites in these windows were determined as above.

4 http://genome.ucsc.edu/
5 http://www.bioinformatics2.wsu.edu/emboss/
Transcriptional Basis of HDACi-Induced Apoptosis

Quantitative reverse transcription–PCR, Western blotting, transient transfection, siRNA knockdown, chromatin immunoprecipitation analysis. Detailed methods are described in supplementary text. Primers used in quantitative reverse transcription–PCR (qRT-PCR) and chromatin immunoprecipitation (ChIP) analysis are described in Supplementary Tables S1 and S2, respectively.

Immunohistochemistry. We used the Protein Atlas online immunohistochemistry resource6 for images of metallothionein and activating transcription factor 3 (ATF3) staining in normal colon and colon tumor sections (32, 33).

Results

Identification of colon cancer cell lines sensitive and refractory to HDACi-induced apoptosis. To identify colon cancer cell lines sensitive and refractory to HDACi, a panel of 30 colon cancer cell lines was screened for HDACi-induced apoptosis by treatment with low (1 mmol/L), intermediate (5 mmol/L), or high (10 mmol/L) dose of the HDACi, sodium butyrate, for 72 hours. As shown in Fig. 1, a continuum response to butyrate-induced apoptosis was observed. The five cell lines with the highest apoptotic response to low-dose butyrate treatment were defined as butyrate "sensitive" (SV400, HCT116, HCT8, HCC2998, and SW948), and the five cell lines with the lowest apoptotic response to high-dose butyrate treatment defined as butyrate "resistant" (LIM2405, Colo320, RKO, HCT15, and HT29 cl.19A) for subsequent studies.

To validate these findings using an independent method, clonogenic survival assays were performed following 24 h butyrate treatment on the adherent-sensitive and -resistant cell lines. Consistent with the apoptosis data, colony formation was reduced to a significantly greater extent in sensitive compared with resistant lines (Fig. 1B and C; P = 0.01, unpaired t test). To confirm the differential response of colon cancer cells to HDACi in vivo, two sensitive (HCT116 and HCT8) and two resistant (LIM2405, HCT15) cell lines were grown as xenografts in severe combined immunodeficient mice and treated with the HDACi, VPA, for 2 weeks. VPA was used in these studies as it is structurally similar to butyrate, for 72 hours. As shown in Fig. 1, a continuum response to butyrate-induced apoptosis was observed. The five cell lines with the highest apoptotic response to low-dose butyrate treatment were defined as butyrate "sensitive" (SV400, HCT116, HCT8, HCC2998, and SW948), and the five cell lines with the lowest apoptotic response to high-dose butyrate treatment defined as butyrate "resistant" (LIM2405, Colo320, RKO, HCT15, and HT29 cl.19A) for subsequent studies.

To confirm that the apoptotic effects of butyrate were due to the inhibition of HDAC activity, the cell lines were re-screened for apoptotic response to VPA, and to the structurally distinct hydroxamic acid–based HDAC inhibitors, Trichostatin A (TSA), and SAHA. As shown in Supplementary Fig. S1, cell lines sensitive and resistant to butyrate-induced apoptosis were likewise differentially sensitive to each of the other HDACi. Notably, the differential sensitivity of these cell lines to HDACi was specific to this class of agent and was not due to an inherent differential sensitivity of these cell lines to apoptosis in general, as a number of cell lines refractory to HDACi-induced apoptosis were sensitive to apoptosis induced by the mechanistically distinct chemotherapeutic agents, 5FU, CPT, and oxaliplatin (Supplementary Fig. S1B).

Differential sensitivity of cell lines to HDACi-induced apoptosis is not linked to the degree of HDAC inhibition or HDACi-induced growth arrest. Interestingly, HDACi treatment resulted in robust induction of histone H3 and histone H4 acetylation to a similar extent in both sensitive and resistant cell lines (P = 0.44 and P = 0.37 for Ac.H3 and Ac.H4, respectively; Supplementary Fig. S2), indicating differential sensitivity to HDACi was not due to differences in the degree of HDAC inhibition.

Consistent with this finding, HDACi treatment resulted in significant growth inhibition in all 10 cell lines (Supplementary Fig. S3). The percentage of cells in S phase was decreased by 73 ± 8.2% and 62 ± 1.8% in resistant and sensitive cell lines, respectively (P = 0.59), and the percentage of cells in G2-M was induced 359 ± 95% and 388 ± 112% in resistant and sensitive cell lines, respectively (P = 0.85) 24 h after HDACi treatment. Consistent with HDACi-induced growth inhibition, expression of p21 (WAF1/CIP1), a regulator of cell cycle progression and known HDACi target gene, was consistently induced by butyrate treatment in each of the 10 cell lines (Supplementary Fig. S4A and B).

HDACi-induced apoptosis is inhibited by actinomycin D. To determine the importance of transcription in HDACi-induced apoptosis, HCT116 cells were cotreated with HDACi and the RNA polymerase inhibitor, actinomycin D. Actinomycin D significantly attenuated HDACi-induced apoptosis, showing HDACi-induced apoptosis in colon cancer cells is dependent upon de novo transcription (Supplementary Fig. S5A and B).

IE and SR genes are preferentially induced by HDACi in sensitive colon cancer cell lines. Having identified sensitive and resistant cell lines and established the dependency of HDACi-induced apoptosis upon de novo transcription, the transcriptional basis for this differential sensitivity was determined. The five sensitive and five resistant cell lines were treated with 5 mmol/L butyrate for 24 hours and changes in gene expression profiled using 27,000-feature cDNA microarrays (entire database provided as Supplementary Table S3). Notably, the overall number of genes changed in response to HDACi treatment (P > 0.05, t test) and the range of transcriptional changes in terms of fold change was similar for sensitive and resistant cell lines.

Genes differentially induced by HDACi in sensitive versus resistant cell lines were identified using a stringent supervised analysis as described in Materials and Methods. Satisfying these criteria, 48 sequences were identified as significantly and preferentially induced by butyrate in sensitive cell lines (Fig. 2; Supplementary Table S4). Notably, 7 of these 48 genes, Fos, Jun, Afb3, Arc, Nr4a1 (Nur77), Egr1, and Egr3, are IE genes whereas an additional 7 genes have previously been classified as SR genes: Gadd45b, Ndrg4, Mti1B, Mti1E, Mti1F, Mti1H, and Mtix (Fig. 2A).

Forty-four genes preferentially repressed by HDACi in sensitive lines were also identified (Supplementary Table S4). These included several genes involved in organization

6 http://www.proteinatlas.org
of microtubules and the actin cytoskeleton (TRIP6, SRHML, PLXNB1, MAP7, LASP1, and LAD1), cell adhesion (OCLN, DSC2), transcriptional repression (NCoR2, SET), and apoptosis (FLIP, DAXX).

Validation of transcriptional changes. To validate the differential induction of HDACi-induced genes in sensitive and resistant lines, we performed qRT-PCR and Western blot analyses. As shown in Fig. 2B, Fos, EGR1, EGR3, and IGFBP3 mRNA induction by butyrate showed strong concordance with the microarray data, with robust and preferential induction in the sensitive cell lines. Preferential induction in sensitive cell lines of three additional genes, ATF3, Jun, and syntaxin, was confirmed at the protein level (Fig. 2C). To confirm that the transcriptional changes induced by butyrate were largely due to HDAC inhibition, gene expression changes induced in response to butyrate were compared with changes induced by VPA and SAHA in HCT116 cells by microarray analysis. Examination of the gene expression changes across the entire microarray revealed a marked similarity in the transcriptional changes induced by each of these agents (Supplementary Fig. S6A and B), whereas specific examination of the genes comprising the apoptosis signature revealed an even stronger correlation ($r = 0.83$ or greater), indicating the butyrate-induced changes are highly likely to be the consequence of HDAC inhibition.

Induction of IE genes is specific to HDACi-induced apoptosis. To confirm that the transcriptional response observed was specific to HDACi-induced apoptosis and not a nonspecific reflection of cells undergoing apoptosis, HCT116 cells were treated with HDACi or the mechanistically distinct chemotherapeutic agents 5FU and oxaliplatin for 24 hours. As shown in Supplementary Fig. S7, expression of Fos, EGR1, EGR3, and IGFBP3 mRNA and ATF3, Jun, and syntaxin protein expression were induced primarily in response to HDACi treatment. In contrast, p21, which is induced both by HDACi and in a p53-dependent manner in response to DNA damage, was induced by all agents tested (Supplementary Fig. S7).
Promoters of genes selectively induced by HDACi in sensitive cell lines are GC rich and are enriched for KLF4/Sp1/Sp3 binding sites. IE and SR gene expression has previously been shown to be coordinately induced in response to serum, 12-O-tetradecanoylphorbol-13-acetate, and growth factor treatment (34), suggesting that a common transcription factor or a set of transcription factors likely regulates their expression. To determine the identity of such a factor(s), a

![Figure 2](image-url)

Figure 2. A, heat map of the 48 genes selectively induced by butyrate in sensitive cell lines following 24 h of treatment. B, QPCR validation of select gene expression changes following 24 h of treatment with 5 mmol/L butyrate (But 5). C, Western blot validation of differential induction of select proteins in sensitive (HCT8 and HCT116) and resistant cell lines (LIM2405).
systematic bioinformatic search of the promoter regions of each of these genes was performed. As a control, the promoters of an equal number of genes not induced by HDACi were also analyzed. First, a nucleotide composition analysis of the region 5-kb upstream through 5-kb downstream of the TSS, measured in 1-kb windows, showed that the GC content of HDACi-induced gene promoters was significantly higher than that of the control set. As shown in Fig. 3A, promoter GC content increased significantly in both groups with increasing proximity to the TSS; however, the magnitude of this increase was significantly higher in HDACi-induced genes. To determine whether the promoters of HDACi-induced genes were enriched for specific transcription factor binding sites, the frequency of occurrence of all established transcription factor binding sites in the 1.5-kb promoter region upstream and 200-bp downstream of the TSS was computed using the MATCH database. As shown in Supplementary Table S5, the frequency of occurrence of multiple transcription factor binding sites was significantly different between HDACi-induced versus uninduced genes, with the vast majority of sites underrepresented in the promoters of genes induced by HDACi. The transcription factor binding sites most significantly enriched in HDACi-induced genes were the related GC-rich KLF4, Sp1, and Sp3 binding sites. To further confirm this finding and to examine the distribution of KLF4/Sp1/Sp3 binding sites within the promoters of these genes in more detail, we computed the frequency of occurrence of these sites in 1-kb increments beginning 10-kb upstream through 10-kb downstream of the TSS. As shown in Fig. 3B, the frequency of KLF4 and Sp1/Sp3 sites was significantly higher in HDACi-induced genes.

**KLF4 is not a key regulator of HDACi induction of the IE/SR gene signature and apoptosis.** To address the role of KLF4 in mediating the HDACi-induced transcriptional and apoptotic response, we first determined the effect of KLF4 overexpression on HDACi-induced apoptosis and IE gene expression in the HDACi-resistant RKO cell line. RKO cells have low levels of KLF4 due to hemizygous deletion of a portion of the KLF4 locus as well as promoter methylation (35). RKO-EcR-KLF4 cells are an isogenic derivative of RKO cells containing a stably integrated ponasterone-inducible KLF4 gene (29). Ponasterone treatment induced robust induction of KLF4 expression in RKO-EcR-KLF4 cells after 24 h; however, no change in the magnitude of HDACi-induced apoptosis or HDACi induction of IE gene expression was observed (Supplementary Fig. S8). Second, KLF4 mRNA was selectively downregulated in HDACi-sensitive HCT116 cells. Despite >70% knockdown of KLF4 mRNA expression, no change in HDACi-induced apoptosis was observed (Supplementary Fig. S8B), collectively indicating KLF4 does not play a central role in HDACi induction of IE/SR gene expression or in HDACi-induced apoptosis in colon cancer cells.

**Activity of Sp1 and Sp3 is functionally implicated in HDACi induction of the IE/SR gene signature and apoptosis.** Next, we determined the role played by Sp1 and Sp3 transcription factors in HDACi-induced gene expression and apoptosis using multiple independent approaches. First, to determine whether Sp1 and Sp3 were physically localized to the promoters of HDACi-responsive genes, we performed ChIP analyses in HCT116 cells interrogating the Sp1/Sp3 sites within the proximal promoter regions of the *ATF3*, *EGR1*, and *JUN* promoters. Pulldowns performed using anti-Sp1 or anti-Sp3 antibodies resulted in significant enrichment of the proximal promoter fragments of these genes, relative to the immunoprecipitations performed using No Ab or IgG controls (Fig. 3C). In comparison, for each of these genes, no enrichment of distal promoter regions devoid of Sp1/Sp3 sites was observed (Fig. 3D). To determine the functionality of these sites in terms of facilitating transcription in response to HDACi treatment, we also examined AcH3 levels at these sites before and after HDACi treatment by ChIP analysis. As shown in Fig. 3E, AcH3 levels were increased ∼2-fold 24 hours after HDACi treatment consistent with the transcriptional induction of these genes. Notably, enrichment of Sp1 or Sp3 or of the class I HDAC, HDAC3, at the Jun promoter was not significantly altered in response to HDACi treatment (data not shown), indicating these factors are retained at the promoter during transcription of the gene.

Second, HDACi-sensitive and -resistant colon cancer cell lines were transfected with wild-type or mutant Sp1/Sp3-luciferase reporter constructs and treated with HDACi for 24 hours. As shown in Fig. 3F, the magnitude of induction of Sp1/Sp3 reporter activity was 2- to 10-fold higher in HDACi-sensitive lines compared with HDACi-resistant lines, indicating preferential induction of Sp1/Sp3 driven transcription in sensitive cells. Minimal effects were observed on the mutant Sp1/Sp3-luciferase reporter construct in both sensitive and resistant lines (data not shown).

Third, whether pharmacologic inhibition of Sp1/Sp3 binding abrogated HDACi induction of IE genes and apoptosis was examined using the antibiotic mithramycin, which inhibits Sp1/Sp3 transcription by competitively binding to GC-rich elements (36). Cotreatment of HCT116 cells with mithramycin markedly attenuated butyrate and TSA-mediated induction of three of the four genes tested: *FOS*, *EGR1*, and *EGR3* (Fig. 4A). In contrast, mithramycin treatment failed to inhibit HDACi induction of *IGFBP3*, instead potentiating the HDACi effect (Fig. 4A). Likewise, mithramycin markedly attenuated HDACi-induced apoptosis in both HCT116 and HCT8 cells at the 24-hour time point as assessed by cell monolayer morphology, the percentage of cells with a subdiploid DNA content or poly(ADP-ribose) polymerase (PARP) cleavage (Fig. 4B–D). However, this effect was lost at later time points (48 and 72 hours) when mithramycin alone induced significant apoptosis (data not shown).

Finally, to directly determine the role of Sp1 and Sp3 in the HDACi-induced transcriptional and apoptotic response, Sp1 and Sp3 were downregulated in HCT116 cells using Sp1 and Sp3 targeting siRNAs. Effective downregulation of Sp1 and all three isoforms of Sp3 were confirmed by Western blot (Fig. 5A). As shown in Fig. 5B, downregulation of Sp1 and Sp3 resulted in a 17% and 46% inhibition of HDACi-induced apoptosis, respectively, and in the extent of HDACi-induced PARP cleavage (Fig. 5C). Downregulation of Sp1 and Sp3, alone and in combination, resulted in a heterogeneous pattern of effects on...
HDACi-induced gene expression, which fell into three categories. First, for a subset of the genes (FOS, IGFBP3), Sp1 and Sp3 silencing had no effect on HDACi-induced gene expression (Fig. 5D). For a second subset (EGR3, JUN), silencing of Sp3 but not Sp1 partially attenuated HDACi-mediated transcriptional induction (Fig. 5E). For the final subset (GADD45, EGR1), individual silencing of both Sp1 and Sp3 inhibited HDACi-mediated transcriptional induction (Fig. 5F). Collectively, these results indicate that Sp1 and Sp3 regulate a significant, although not the entire component of the HDACi-induced transcriptional response associated with promotion of apoptosis in colon cancer cells.

Genes selectively induced by HDACi in sensitive cell lines are basally repressed in colon tumors. To independently assess the biological significance of the subset of genes preferentially induced by HDACi in sensitive cell lines, we compared the relative expression of this gene subset in colon tumors and normal colonic mucosa. For this, we generated a microarray database comparing gene expression differences between 12 matched normal colon and colon tumor pairs. Of the 48 genes preferentially induced by HDACi in sensitive cells, corresponding gene expression data were available for 32 genes in the matched primary colon tumor/normal database. Notably, expression of 9 of these 32 genes (28%) was significantly repressed (>1.5-fold and \( P < 0.05 \)) in colon tumors compared with normal colonic mucosa. In contrast, only 2 of 32 (6.3%) were upregulated in colon tumors by these same criteria (Fig. 6A). As a control, the expression pattern in colon tumor and normal tissue of an equal number of genes not induced by HDACi treatment was also examined. As shown in Fig. 6D, 5.7% of the genes on this control list were upregulated and 5.7% were downregulated (>1.5-fold and \( P < 0.05 \)) in colon tumors relative to normal colonic mucosa (\( P = 0.004, \chi^2 \) test).

The HDACi-induced genes downregulated in colon tumors were ATF3, MT1B, MT1E, MT1F, MT1H, MT1X, NDRG4, PLEKHH2, and TGFβ3 (Fig. 6C). As shown in Fig. 6D, the downregulation of MT1F and ATF3 in colon tumors was independently validated using the protein atlas immunohistochemistry database (33, 37). These data show that the subset of genes identified as selectively induced during HDACi-induced apoptosis was enriched for genes that are basally downregulated in colon tumors. These findings are also consistent with a previous report indicating downregulation of a number of IE genes in colon tumors (38).

Discussion

HDAC inhibitors induce growth arrest, differentiation, and apoptosis in tumor cells of diverse origins, and their efficacy for cancer treatment was recently underscored by the
approval of SAHA for the treatment of cutaneous T-cell lymphoma. Whereas the downstream effectors of HDACi-induced apoptosis in tumor cells have been studied in detail (3, 17), the molecular events that initiate HDACi-induced apoptosis are unknown. Possibilities include transcription-dependent effects, such as altered expression of proapoptotic and antiapoptotic molecules, or transcription-independent effects, such as induction of aberrant mitosis, ROS production, or altered acetylation of prosurvival molecules, such as HSP90.

In the present study, the importance of de novo transcription in HDACi-induced apoptosis in colon cancer cell lines was established. Through gene expression profiling of multiple colon cancer cell lines sensitive and resistant to HDACi, we identified a reproducible transcriptional response preferentially induced in response to HDACi treatment in sensitive cell lines. Importantly, this transcriptional response was observed in response to multiple, structurally distinct HDACi and across a range of colon cancer cell lines with extensive molecular and mutational heterogeneity including differences in microsatellite instability status, ploidy, and the mutation and methylation status of multiple tumor suppressor genes and oncogenes (28).

A striking feature of the HDACi-induced transcriptional response is that a number of the induced genes, particularly the IE and SR genes, are coordinately induced in response to several other stimuli. This includes the coordinate induction of IE gene expression in response to serum stimulation, phorbol ester, and growth factor treatment (39, 40) and the coordinate induction of the metallothionein gene family, clustered within the q13 region of chromosome 16, in response to metal induction and oxidative stress (41).
coordinate induction of these genes in response to HDACi treatment suggested they likely reflected the modulation of a specific transcription factor or factors that coordinately regulate their expression.

Several findings collectively indicated a key role for Sp1/Sp3 transcription factors in driving this transcriptional response. First, the promoters of HDACi-induced genes had a high GC content and were significantly enriched for Sp1/Sp3.
binding sites. Second, localization of Sp1 and Sp3 at the promoters of several of these genes was detectable by ChIP analysis. Third, induction of Sp1/Sp3 reporter activity was preferentially induced by HDACi in sensitive cell lines. Finally, disruption of Sp1/Sp3 DNA binding with mithramycin or siRNA-mediated downregulation of Sp1 or Sp3 attenuated induction of a number of HDACi targets. Importantly, both mithramycin and Sp1/Sp3 siRNA attenuated HDACi-induced apoptosis, establishing a direct link between Sp1/Sp3-mediated transcriptional response and initiation of HDACi-induced apoptosis. It is important to note, however, that expression of a subset of the genes induced by HDACi, including FOS and IGFBP3, were not modulated by Sp1 and/or Sp3 silencing, indicating additional transcription factors also likely participate in HDACi-induced transcriptional changes.

Whereas the precise mechanism by which initiation of this transcriptional response triggers apoptosis remains to be determined, it is noteworthy that several genes comprising the HDACi transcriptional response have previously been linked with HDACi-induced apoptosis through over and underexpression studies. These include IGFBP3 (42), EGR1 (43), NRR4A1 (TR3/Nur77; ref. 14), and GADD45B (44). However, in each case, induction of these genes only partially accounts for the overall apoptotic response, suggesting that their collective induction, which may sufficiently alter the cellular transcriptional equilibrium in favor of apoptosis, may be the critical determinant of HDACi-induced apoptosis. Lending support to this concept is the finding that a number of these transcripts are basally repressed in colon tumors compared with normal colon tissue. In the context of tumorigenesis therefore, selective repression of these transcripts may alter the transcriptional balance in favor of cell survival.

Whereas HDACi treatment uniformly induces expression of antiproliferative genes, such as p21 in both sensitive and resistant cell lines, it is notable that several of the genes preferentially induced by HDACi in sensitive cells, including the IE genes FOS, JUN, EGR1, and EGR3, are typically linked to the promotion of cell proliferation, such as in response to serum or growth factor treatment (34). HDACi treatment therefore clearly induces a conflicting transcriptional response comprising both proproliferative and antiproliferative signals, which is magnified in HDACi sensitive cells. HDACi induction of IE gene expression is also sustained for over

Figure 6. Expression of multiple HDACi-induced genes is reduced in primary colon tumors relative to adjacent normal mucosa. The percentage of (A) HDACi-induced and (B) uninduced (control) sequences significantly overexpressed or underexpressed in colon tumors. C, identity of the nine genes induced by HDACi and significantly repressed in colon tumors relative to normal colonic epithelium (*, P < 0.05). D, immunohistochemical validation of reduced expression of ATF3 and metallothionein in normal colon and colon tumors. Images downloaded from the publicly available protein atlas database (32, 33) as per the image use policy of protein atlas.
24 hours, whereas growth factor induction is typically maximal at 30 minutes to 1 hour after stimulation and rapidly returns to baseline (38). Whether these conflicting transcriptional signals or the sustained induction of IE gene expression are perceived by the cells as a SR and whether this is ultimately the stimulus for apoptosis induction is worthy of further investigation. In support of this hypothesis, it is notable that cotreatment of colon cancer cells with agents such as phorbol 12-myristate 13-acetate, which independently induce IE gene expression, potentiates HDACi-induced apoptosis (45).

In conclusion, this study shows that HDACi-induced apoptosis in colon cancer cells is linked to a consistent transcriptional response involving induction of a transcriptional network enriched for IE and SR genes, which are regulated, to a large extent, by the Sp1/Sp3 transcription factors. Importantly, several components of this transcriptional response are basally repressed in colon tumors suggesting HDACi-induced apoptosis may be initiated through induction of a transcriptional network whose basal repression is linked to tumor cell survival.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Apoptotic Sensitivity of Colon Cancer Cells to Histone Deacetylase Inhibitors Is Mediated by an Sp1/Sp3-Activated Transcriptional Program Involving Immediate-Early Gene Induction

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