Inhibitors of histone deacetylases (HDACs) induce growth arrest, differentiation, and apoptosis of colon cancer cell lines \textit{in vitro} and have demonstrated anti-cancer efficacy in clinical trials. Whereas a role for HDAC1 and -2 in mediating components of the HDAC inhibitor response has been reported, the role of HDAC3 is unknown. Here we demonstrate increased protein expression of HDAC3 in human colon tumors and in duodenal adenomas from \textit{Acipenser} species mice. HDAC3 was also maximally expressed in proliferating crypt cells in normal intestine. Silencing of HDAC3 expression in colon cancer cell lines resulted in growth inhibition, a decrease in cell survival, and increased apoptosis. Similar effects were observed for HDAC2 and, to a lesser extent, for HDAC1. HDAC3 silencing also selectively induced expression of alkaline phosphatase, a marker of colon cell maturation. Concurrent with its effect on cell growth, overexpression of HDAC3 and other Class I HDACs inhibited basal and butyrate-induced p21 transcription in a Sp1/Sp3-dependent manner, whereas silencing of HDAC3 stimulated p21 promoter activity and expression. However, the magnitude of the effects elicited by silencing of individual Class I HDACs was significantly less than that induced by HDAC inhibitors. These findings identify HDAC3 as a gene deregulated in human colon cancer and as a novel regulator of colon cell maturation and p21 expression. These findings also demonstrate that multiple Class I HDACs are involved in repressing p21 and suggest that the growth-inhibitory and apoptotic effects induced by HDAC inhibitors are probably mediated through the inhibition of multiple HDACs.

Acetylation of DNA-bound core histones and sequence-specific transcription factors is a fundamental mechanism of transcriptional regulation. Histone acetylation is typically associated with increased transcription (1) and is regulated by two opposing classes of enzymes: histone acetyltransferases, which add acetyl groups to specific amino acids of the histone protein, and histone deacetylases (HDACs), which catalyze their removal. A second mechanism by which HDACs may regulate gene transcription is by regulating acetylation of DNA sequence-specific transcription factors. Examples include p53, E2F, and Sp3, where deacetylation has been linked to reduced DNA binding or transcriptional activity (2–4).

Through these mechanisms, HDACs are emerging as critical regulators of cell growth, differentiation, and apoptotic programs. We and others have demonstrated that inhibitors of HDACs, such as sodium butyrate, trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), and valproic acid, induce cell cycle arrest, differentiation, and apoptosis in colon cancer cell lines \textit{in vitro} (5–10). These observations suggest a physiological role for transcriptional repression mediated by HDACs in maintaining cell proliferation and survival and inhibiting differentiation. Correspondingly, the deregulation of HDAC-mediated transcriptional repression has been linked to tumorigenesis. The up-regulated expression of HDAC1 and -2 has been demonstrated in human cancers (11, 12), and their abnormal recruitment by mutant transcription factors formed by chromosomal translocation occurs in various leukemias (13). The potential of HDAC inhibitors as anti-cancer agents has been recognized for some time (13), and several, such as depsipeptide, SAHA, MS-275, and valproic acid, are currently undergoing clinical trials for the treatment of hemopoietic and solid tumors (14).

Eighteen mammalian histone deacetylases have been identified to date and have been classified based upon their homology to a prototypical HDAC found in yeast. Class I HDACs (HDAC1, -2, -3, and -8) show homology to the yeast HDAC, Rap1; class II HDACs have a high degree of homology to Hda-1 (15); and class III HDACs are homologous to the yeast Sir2 HDAC (16). The HDAC inhibitors that have been utilized in the \textit{in vivo} and \textit{in vitro} studies described above are, in general, nonspecific inhibitors of Class I and II HDACs.

Consistent with the ability of HDAC inhibitors to induce intestinal cell maturation in colon cancer cell lines, the class I HDACs, HDAC1 and HDAC2, are up-regulated in expression in colon tumors (11), repress p21 expression, promote cell proliferation and survival \textit{in vitro} (11, 12, 17, 18), and are important mediators of intestinal development and differentiation \textit{in vivo} (19). The overlapping functions of HDAC1 and -2 may be a consequence of their co-existence within the same co-repressor complexes (Sin3A, NuRD). The role of other class I and II HDACs in regulating colon cell proliferation is unknown.

Although structurally related to HDAC1 and -2, HDAC3 is a component of the NCoR-SMRT co-repressor complex, which is distinct from co-repressor complexes that typically contain HDAC1 and -2. Whether inhibition of HDAC3 is a component of the HDAC inhibitor response and whether it plays a role in regulating intestinal cell proliferation and differentiation, and apoptosis of colon cancer cell lines is \textit{in vitro} and have demonstrated anti-cancer efficacy in clinical trials. Whereas a role for HDAC1 and -2 in mediating components of the HDAC inhibitor response has been reported, the role of HDAC3 is unknown. Here we demonstrate increased protein expression of HDAC3 in human colon tumors and in duodenal adenomas from \textit{Acipenser} species mice. HDAC3 was also maximally expressed in proliferating crypt cells in normal intestine. Silencing of HDAC3 expression in colon cancer cell lines resulted in growth inhibition, a decrease in cell survival, and increased apoptosis. Similar effects were observed for HDAC2 and, to a lesser extent, for HDAC1. HDAC3 silencing also selectively induced expression of alkaline phosphatase, a marker of colon cell maturation. Concurrent with its effect on cell growth, overexpression of HDAC3 and other Class I HDACs inhibited basal and butyrate-induced p21 transcription in a Sp1/Sp3-dependent manner, whereas silencing of HDAC3 stimulated p21 promoter activity and expression. However, the magnitude of the effects elicited by silencing of individual Class I HDACs was significantly less than that induced by HDAC inhibitors. These findings identify HDAC3 as a gene deregulated in human colon cancer and as a novel regulator of colon cell maturation and p21 expression. These findings also demonstrate that multiple Class I HDACs are involved in repressing p21 and suggest that the growth-inhibitory and apoptotic effects induced by HDAC inhibitors are probably mediated through the inhibition of multiple HDACs.
maturation is presently unknown. Here we show that, like for HDAC1 and -2, expression of HDAC3 is up-regulated in colon tumors and down-regulated during normal epithelial cell maturation along the small intestinal crypt-villus and colonic crypt axes in vivo. Silencing of HDAC3 expression by RNA interference induced a G2/M growth arrest and stimulated p21 promoter activity and protein expression. Silencing of HDAC1 and HDAC2 also induced p21. The stimulatory effects on p21 expression and suppression of cell growth elicited by silencing of individual class I HDACs, however, was significantly less than that induced by HDAC inhibitors. These studies therefore identify a novel role for HDAC3 in repressing p21 and demonstrate that HDAC inhibitors probably exert their effects on intestinal cell proliferation by inhibition of multiple HDACs.

**EXPERIMENTAL PROCEDURES**

*Cell Culture*—The HCT116 and Caco-2 colon cancer cell lines and the FHs normal human small intestinal cell line were obtained from the American Type Culture Collection (Manassas, VA). All were maintained by serial passage at 37 °C, 5% CO2, in minimal essential medium supplemented with 10% fetal bovine serum, 1× antibiotic/antimycotic (100 units/ml streptomycin sulfate, 100 units/ml penicillin G sulfate, and 0.25 μg/ml amphotericin B), 100 μM non-essential amino acids, and 10 mM HEPES buffer solution (all from Invitrogen). The following HDAC inhibitors were utilized. The sodium salt of the short-chain fatty acid, butyrate, and TSA were purchased from Sigma, and SAHA was from Biovision Inc. (Mountain View, CA).

*Microarrays*—All cDNA microarrays used in these studies were generated by the microarray facility at the Albert Einstein College of Medicine, and experiments were performed as previously described (20).

*Paired Human Tumor and Normal Colon Samples*—Ten human colorectal tumors with paired adjacent normal tissue were collected following surgical resection at Montefiore Medical Center through an institutional review board-approved protocol and immediately snap frozen in liquid nitrogen.

*Changes in Gene Expression along the Intestinal Crypt-Villus Axis*—Enterocytes were sequentially isolated along the mouse small intestinal crypt-villus axis as previously described (20–23). The experiment was performed on four independent occasions, each time using a single adult male C57BL/6 mouse, 13 weeks of age. Ten fractions of cells were collected, with fraction 1 highly enriched for cells from the villus tip and fraction 10 enriched for cells from the crypts. Total RNA was extracted from enterocytes isolated from each fraction using the RNeasy RNA isolation kit from Qiagen (Qiagen, Valencia, CA). Linear amplification of 5 μg of total RNA was then performed using T7 bacteriophage RNA polymerase-driven *in vitro* transcription (KIT 0201; Arcturus), and 5 μg of the amplified product was used for probe labeling for microarray analysis. For the microarray experiments, gene expression changes in 6 of the 10 fractions were determined (Factions 1, 2, 4, 6, 8, and 10). In each case, 5 μg of poly(U) RNA from fraction 10 (crypt cells) served as the control and was labeled with Cy3 dUTP. 5 μg of RNA from Fractions 1, 2, 4, 6, 8, and 10, respectively, were labeled with Cy5 dUTP and served as the experimental condition. Labeled probes were combined and hybridized to 27,000 element mouse cDNA arrays. Methods utilized for data normalization and identifying differentially expressed sequences have been previously described (20).

*Immunohistochemistry*—A detailed protocol has been previously described (24). Briefly, duodenal and distal colonic tissue from C57BL/6 mice and duodenal adenomas from Apc1638N/+ mice were formalin-fixed and then paraffin-embedded and stored at 4 °C. The sections were deparaffinized and rehydrated, quenched with 1.5% H2O2 and then blocked with 10% normal goat serum. The following primary antibodies were added to the tissue sections for an overnight exposure at 4 °C: rabbit anti-HDAC1 (1:25 dilution) from Upstate Biotechnology, Inc. (Lake Placid, NY), rabbit anti-HDAC2 (1:50) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), rabbit anti-HDAC3 (1:25) from Novus Biologicals (Littleton, CO), mouse anti-PCNA from Santa Cruz Biotechnology (1:100 dilution), and mouse anti-villin from Novocastra Laboratories (1:50; Newcastle upon Tyne, UK). To detect HDAC1 and HDAC3, an additional epitope retrieval step was added; slides were immersed in antigen unmasking solution (Vector Laboratories, Burlingame, CA) and boiled at 95 °C for 10 min and then washed in distilled water before proceeding with quenching. Detection of primary signal was with either the anti-rabbit or anti-mouse ImmPRESS reagent (Vector Laboratories), followed by incubation with 3’,5’-diaminobenzidine substrate (Vector Laboratories) and hematoxylin counterstaining.

*Western Blotting*—Protein isolation, Western blotting, and signal detection were performed as previously described (25). Rabbit anti-HDAC1 (1:500) was from Imgenex (San Diego, CA), rabbit anti-HDAC2 (1:2000) was from Santa Cruz, and rabbit anti-HDAC3 (1:2000) was from Novus Biologicals. A 3-h incubation was used for all HDAC primary antibodies. Additional antibodies used were mouse anti-PCNA (1:500 dilution), mouse anti-β-actin (1:4000; Sigma), rabbit anti-p21 (1:200; Santa Cruz Biotechnology), mouse anti-villin (1:2000, Novocastra Laboratories), and rabbit anti-alkaline phosphatase (1:500; Novus Biologicals). Peroxidase-linked goat anti-mouse IgG (Roche Applied Science) or donkey anti-rabbit IgG (Amersham Biosciences) was used as secondary antibody (1:2000 dilution).

*Transient Transfection Assays*—HCT116 and Caco-2 cells were seeded in 24-well plates at a density of 3 × 104 and 5 × 104 cells/well, respectively. The following day, cells were transiently transfected with a series of luciferase-linked p21 promoter reporter constructs (see supplemental Fig. 3A) using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. These constructs, along with the Sp1-Luc, mSp1-Luc, GAL4, GAL4-Sp3, and GAL-DNSp3 constructs also utilized, have been previously described (26, 27). Also utilized were expression vectors for HDAC1 (pBJ5-FLAG-HDAC1) and a mutant lacking histone deacetylase activity (pBJ5-FLAG-HDAC1–141A) (28), HDAC2 (pME18S-FLAG-HDAC2) (29), and HDAC3 (pCMV-FLAG-HDAC3) (30). In experiments involving butyrate, untreated and treated cells were transfected identically and compared by relative fold induction by the drug after correction for total cellular protein. Luciferase activity was determined in cell lysates using the luciferase or dual luciferase assay kits (Promega, Madison, WI). Transfection efficiency was controlled by co-transfection with TK-Renilla.

*silRNA Experiments*—Expression of HDAC1, HDAC2, and HDAC3 was selectively silenced by utilizing a pool of four predesigned siRNAs (Dharmacon, Lafayette, CO). Two different nontargeting control siRNAs were used, NT1 and NT2. HCT116 or Caco-2 cells were transiently transfected with 100 nM siRNA for 24–120 h, using the Perfecta (calcium phosphate) transient transfection system (Promega, Madison WI). Validation of silencing of protein expression was performed by Western blot.

The sense strands of the individual HDAC siRNA sequences utilized were as follows: HDAC1, CUAUUGAGCUUUCUCCAUACAUUGAAAGCUCGUUACUAUUGGACAUCGCGUGAUAGUUGGU; HDAC2, CCAUUGAUGUGCCCAAUUAUUAUGAAAGUUGGGCUCUAUCUAUGUAGCAGAU-CAUAAUUUUGCAAGAAGCUAGAAUGGUU; HDAC3, GGA-
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FIGURE 1. HDAC3 protein expression in normal and neoplastic colonic epithelium. A, total protein was extracted from 10 human colon cancer specimens and paired normal tissue collected at surgical resection and levels of HDAC3 and carcinoembryonic acid (CEA) quantified by Western blot. Blots were reprobed for actin to ensure equal loading. B, signal intensity was quantified by densitometry and was expressed relative to actin expression. Values are mean ± S.E. of the 10 tumor and normal tissue samples. *, p < 0.01, paired t test. C, levels of HDAC3 in protein lysates prepared from 10 colon cancer cell lines, the normal small intestinal epithelial Fh cell line, and three normal colon samples were quantified by Western blot. Blots were reprobed for actin to ensure equal loading.

AGCGAUGUGGAUUAUAAAGCGAUGUGGAGAUUAUUGC-AUUGAUGACCAGAGUUAUUGGAAUGCGGUUGAAUAGUUAUU.

Flow Cytometry—For cell cycle analyses, HCT116 cells were seeded in triplicate in 6-well plates at a density of 250,000 cells/well. After overnight culture, cells were transfected with NT1 or Class I HDAC siRNA for 24 h. After this time, medium was changed, and the cells were cultured for 24, 48, 72, or 96 h, with medium replaced every 24 h. In parallel, cells treated with the HDAC inhibitors butyrate (2 mM) and SAHA (2 μM) were also cultured. Original cell density was chosen such that the control (NT1) cells reached ~80% confluence at the 96 h time point. Cells were trypsinized, counted for other assays (see below), and resuspended in 50 mg/ml propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100. Cells were stained overnight and analyzed for DNA content utilizing a BD Biosciences FACScan flow cytometer. The percentages of cells in G0/G1, S, and G2/M phases were quantified using ModFit LT for Win32 software (Verity Software House, Topsham, ME).

Cell Viability Assays—Viability of HCT116 cells following a 24–96-h treatment with Class I HDAC siRNAs or HDAC inhibitors, as described above, was assessed in three ways. First, adherent cell number was counted in a Coulter Counter following trypsinization. Second, a clonogenic assay was employed. Briefly, after a 72-h treatment with Class I HDAC or NT1 siRNA, cells were trypsinized and reseeded in triplicate into 6-well plates at a density of 1000 cells/well. Following culture for 7 days, individual colonies were scored after 1% crystal violet staining utilizing Total Lab 1.1 software (Nonlinear Dynamics, Durham, NC), as previously described (31). Fourth, apoptosis was measured as subdiploid DNA content following propidium iodide staining, as previously described (31).

In addition, in separate experiments performed in 96-well plates, cell viability following 24–96-h treatment with Class I HDAC siRNAs or HDAC inhibitors was measured by incorporation of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide (MTT) (Sigma), as previously described (6). Validation of silencing of protein expression was performed by Western blot.

RESULTS

HDAC3 Expression Is Up-regulated in Human Colon Tumors—We first examined Class I HDAC expression during colorectal tumorigenesis, utilizing 10 paired colon tumor/normal samples obtained from patients who had undergone surgical resection of a colon tumor. Western blot analysis demonstrated that protein expression of HDAC3 was increased by 2.8-fold in the tumor samples compared with adjacent normal tissue (Fig. 1, A and B, p = 0.003). The tumor content of the samples was confirmed by probing for the known colon tumor marker, carcinoembryonic antigen, which was up-regulated in the tumor in 9 of the 10 tissue pairs. Similar to HDAC3, and consistent with the findings of two reports published during the course of these studies (11, 12), HDAC1 and HDAC2 expression was also significantly up-regulated in human colon tumors compared with adjacent normal mucosa (supplemental Fig. 1). HDAC3 was also robustly expressed by each cell line of a panel of 10 established colon cancer cell lines, markedly higher than the expression of HDAC3 observed in a normal small intestinal cell line and in three normal samples from the tissue pairs described above (Fig. 1C).

HDAC3 Expression Is Restricted to the Proliferative Compartment of Normal Mouse Small Intestine and Colon and Is Robustly Expressed in Mouse Intestinal Adenomas—We next examined HDAC3 expression by immunohistochemistry in normal mouse intestine and in adenomas derived from Apc1638N/+ mice. These mice harbor an inactivating mutation of APC and develop multiple adenomas in the small intestine (33). As shown in Fig. 2, A and B, HDAC3 expression was nuclear and localized to the proliferative compartment of duodenal crypts (arrows). Duodenal or jejunal tumors from five Apc1638N/+ mice were examined in parallel for HDAC3 expression. Staining was also exclusively nuclear in the epithelial cells from the tumor samples (Fig. 2C). However, ~90% of cells in each of the tumors examined were positive for HDAC3, markedly higher than the percentage of cells positive in normal duodenal...
tissue. Maximal expression of HDAC3 in the proliferative crypt compartment in normal small intestine was further confirmed at the mRNA and protein level. Examination of HDAC3 expression along the mouse small intestinal crypt-villus axis, utilizing a cDNA microarray database previously generated in this laboratory (20), demonstrated that steady state mRNA levels of HDAC3 were maximal in crypt cells and significantly reduced in cells at the villus tip (Fig. 2D). A similar down-regulation of HDAC3 protein level was observed (Fig. 2E). Similarly to HDAC3, HDAC2 was also robustly down-regulated in villus cells at both the mRNA and protein level, whereas less pronounced changes were observed for HDAC1 (Fig. 2, D–E). Validating the fractionation procedure, mRNA and protein expression of the marker of cell proliferation, PCNA, was maximal in crypt cells, whereas expression of the differentiation marker, villin, was maximal in villus cells (Fig. 2E and supplemental Fig. 2A).

Finally, we examined expression of HDAC3 and other Class I HDACs in normal mouse distal colon by immunohistochemistry (Fig. 2, F–J). Similar to our findings in the small intestine, HDAC3 staining was predominantly nuclear, with the highest expression observed at the base of colonic crypts (Fig. 2F). We also extended these findings to examine expression of HDAC1 and HDAC2. Like HDAC3, expression of HDAC2 was markedly decreased in the differentiated cells of the upper third of the crypt axis (Fig. 2H), although a less pronounced change was observed for HDAC1 (Fig. 2G). We also performed immunohistochemical staining for cells positive for PCNA to validate our results. As expected, PCNA-positive cells were largely confined to the crypt base (Fig. 2J). These changes were confirmed by quantifying cells positive for Class I HDAC or PCNA staining in the lower, middle, and upper third of the crypt (supplemental Fig. 2B).

Role of Class I HDACs in Colon Cell Growth, Apoptosis, and Differentiation in Vitro—The confinement of expression of Class I HDACs to the proliferative compartment of normal small intestinal and colonic epithelium suggests that a physiological function of class I HDACs may be to maintain cell proliferation. Consistent with this role, pharmacological inhibition of HDACs in colon cancer cells results in cell cycle arrest and up-regulation of expression of a key regulator of the cell cycle, the Cdk inhibitor p21 (34).

We determined whether these effects of HDAC inhibitors could be mimicked by silencing of individual Class I HDACs to directly test the role of HDAC1 to -3. We first confirmed selective and robust silencing of HDAC1, HDAC2, and HDAC3 expression, as shown in Fig. 3A. A
72-h treatment with 100 nM siRNA duplexes down-regulated protein levels of the target HDAC by ~90% in HCT116 cells, whereas two non-targeting control duplexes, NT1 and NT2, had no effect. Similar selective down-regulation of HDAC1, HDAC2, and HDAC3 mRNA following treatment with the relevant siRNA was confirmed by quantitative reverse transcription-PCR (data not shown).

**Cell Growth**—Representative histograms showing the effects of Class I HDAC silencing on cell cycle indices in asynchronous HCT116 cells following fluorescence-activated cell sorting analysis are presented in Fig. 3B. Silencing of HDAC2 and HDAC3 both induced a significant reduction of the percentage of cells in S phase, whereas HDAC1 induced a more modest reduction. A time course of the effects of Class I HDAC...
silencing on S phase is shown in Fig. 3C. These data indicate proliferative roles for these Class I HDACs. The mechanism of growth arrest appeared to differ between HDAC2 and HDAC3 silencing, since HDAC2 silencing induced both a modest increase in the G0/G1 (data not shown) and G2/M cell populations, whereas HDAC3 silencing exclusively induced a G2/M arrest (Fig. 3C). These changes in cell cycle indices were consistent with those induced by HDAC inhibitors (Fig. 3B), but were of a considerably lesser magnitude.

We also determined the effects of Class I HDAC silencing on cell number and on cell viability using the MTT assay. As shown in Fig. 4A, silencing of HDAC2 or HDAC3 induced an ~20% reduction in cell number 72–96 h after transfection compared with cells treated with NT1. Silencing of HDAC1 induced a more modest, but nevertheless statistically significant, reduction in cell number. Likewise, silencing of HDAC3 and other class I HDACs resulted in reduced cell viability as assessed by the MTT assay (Fig. 4B).

Apoptosis/Cell Survival—Finally, to determine the effect of HDAC3 and other class I HDACs on colon cell survival, we performed clonogenic assays on cells transfected with the appropriate siRNA for 72 h. As shown in Fig. 5A, silencing of HDAC3 resulted in the formation of ~25% fewer colonies compared with HCT116 cells transfected with a nontargeting siRNA (NT1). A similar effect was observed following HDAC2 silencing, whereas HDAC1 silencing elicited a less pronounced effect (Fig. 5A). Cells treated with HDAC inhibitors for 24 h prior to seeding for clonogenic assays displayed an ~65–75% reduction in the ability to form colonies compared with untreated control cells (Fig. 5A), a markedly more potent effect than individual Class I HDAC silencing. Scans of representative experiments are shown in Fig. 5B. Silencing of HDAC3 and HDAC2, but not HDAC1, also resulted in a modest increase in the percentage of subdiploid, apoptotic cells after 72 h of treatment, as shown in Fig. 5C and in the histograms displayed in Fig. 3B. Importantly, however, the increase in apoptosis induced by silencing of individual HDACs was significantly less than that induced by HDAC inhibitors (Figs. 5C and 3B).

Differentiation—Finally, the effect of Class I HDAC silencing on differentiation of colon cancer cells was determined using the well established marker of colon cell differentiation, ALP. As shown in Fig. 6, A and B, silencing of HDAC3, but not HDAC1 or HDAC2, induced a significant up-regulation of ALP activity and protein levels in Caco-2 cells. This suggests that HDAC3 may, among the Class I HDACs studied, selectively play a role in repressing colon cell differentiation.

Class I HDACs Repress p21 in Colon Cancer Cells In Vitro in a Sp1/Sp3-dependent Manner—We then examined the role that Class I HDACs play in regulating expression of p21. Whereas HDAC1 and -2 have been previously shown to regulate p21 expression in vitro (11, 34), the role of HDAC3 in regulating p21 expression and cell growth in
intestinal epithelial cells is unknown and was therefore examined in HCT116 cells. First, we confirmed that the HDAC inhibitor, butyrate, markedly induced p21 protein levels in HCT116 colon cancer cells (Fig. 7A). Consistent with the known HDAC-inhibitory activity of butyrate, a parallel induction in acetylated histone H3 and H4 levels was observed (Fig. 7A). Silencing of HDAC3 induced a 2-fold increase in p21 expression ($p < 0.05$) (Fig. 7B). Consistent with these previous reports, silencing of HDAC1 and -2 also induced p21, demonstrating that p21 repression is mediated by multiple class I HDACs. This was supported by the observation that the magnitude of p21 induction following silencing of individual class I HDACs was significantly lower than that induced by butyrate (Fig. 7B).

We next examined the mechanism by which HDAC3 and other Class I HDACs repressed p21 in colon cancer cells. Since HDAC inhibitors activate p21 promoter activity in several cell types in an Sp1/Sp3-dependent manner (26, 35), we postulated that HDAC3 may repress p21 by a similar mechanism. Consistent with these findings, transfection of HCT116 or Caco-2 colon cancer cells with a series of p21 promoter constructs (see supplemental Fig. 3A) demonstrated that a proximal p21 promoter fragment, pWP133, which contains six canonical Sp1/Sp3 binding sites was sufficient for maximal HDAC inhibitor-mediated induction of p21 promoter activity (supplemental Fig. 3, B and C). Furthermore, deletion or mutation of specific Sp1/Sp3 sites within the proximal p21 promoter resulted in marked impairment of butyrate induction of p21 promoter activity (supplemental Fig. 3, B and C). The greatest extent of inhibition of butyrate induction was observed when the Sp1/Sp3 site between −42 and −77 relative to the transcription start site was mutated. However, since abrogated butyrate induction was also observed following transfection with pWP-101 mtSp1–4 compared with pWP-101, the Sp1/Sp3 site between −69 and −64 was also partially involved in butyrate-mediated activation. Interestingly, deletion or
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FIGURE 8. Class I HDAC regulation of p21 promoter activity. A, effect of Class I HDAC silencing on p21 promoter activity, as determined by the luciferase assay. HCT116 cells were transfected with pWP-133 and either a nontargeting siRNA or siRNAs targeting HDAC1, HDAC2, or HDAC3 (100 nM) for 72 h. P21 promoter activity in cells treated with and without 2 mM butyrate for 24 h was determined in parallel. Values shown are mean ± S.E. of five independent experiments. *, p < 0.05 relative to relevant control. Student’s t test. B and C, effect of HDAC1, HDAC2, or HDAC3 overexpression on basal (B) and 2 mM butyrate-induced p21 promoter activity (C). HCT116 cells were co-transfected with pWP-133, TK-Renilla, and increasing concentrations of HDAC1, HDAC2, or HDAC3 expression vectors or the empty vector control (0–2 μg). The values are mean ± S.E. and are expressed as a percentage of pWP-133 activity measured in appropriate empty vector controls (the first bar of each set of five bars).

mutation of Sp1/Sp3 sites also markedly reduced basal p21 reporter activity, indicating that Sp1/Sp3 plays a dual role in mediating both activation and repression of p21 transcription.

Consistent with the p21 transcriptional activation mediated by HDAC inhibitors, silencing of expression of HDAC3 significantly stimulated p21 promoter activity in HCT116 cells (Fig. 8A). Similarly, silencing of HDAC1 and -2 also induced an increase in p21 promoter activity. However, paralleling the relative magnitude of p21 protein up-regulation, the induction of p21 promoter activity mediated by individual Class I HDAC silencing was significantly less than that induced by butyrate treatment (Fig. 8A). The ability of HDAC3 to repress p21 expression was further confirmed by overexpressing HDAC3 under basal and butyrate-stimulated conditions, with HDAC1 and -2 examined in parallel. As shown in Fig. 8B, HDAC3 overexpression induced a concentration-dependent inhibition of basal p21 promoter activity in HCT116 cells. Similar effects were observed for HDAC1 and -2. Furthermore, overexpression of HDAC1, HDAC2, and HDAC3 each significantly inhibited butyrate’s stimula-
tory effect on pWP-133 transcription (Fig. 8C), collectively indicating that multiple HDACs are involved in repressing p21 promoter activity and in mediating the butyrate response. Overexpression of these Class I HDACs also attenuated butyrate induction of pWP-133 activity in Caco-2 cells (data not shown).

Whether the inhibitory effects of HDAC overexpression were due to deacetylase activity of the constructs was directly tested by comparing the inhibition of butyrate and SAHA-induced p21 promoter activity mediated by a WT-HDAC1 expression vector and an HDAC1 expression vector encoding a mutant protein with markedly reduced deacetylase activity, HDAC1-141A. HDAC1-141A overexpression resulted in minimal inhibition of butyrate and SAHA induction of p21 promoter activity compared with the WT construct (supplemental Fig. 4A). Equal expression of WT-HDAC1 and HDAC1-141A in the cells was confirmed by probing for FLAG by Western blot (supplemental Fig. 4B).

Butyrate-induced activation of Sp1/Sp3 binding elements in colon cancer cells was further confirmed utilizing a luciferase reporter construct containing three consensus Sp1/Sp3 binding sites (Sp1-luc). As shown in Fig. 9A, butyrate induced a 28-fold increase in Sp1/Sp3 reporter activity in HCT116 cells while having minimal effects on a control reporter construct containing three mutant Sp1/Sp3 binding sites (mtSp1-luc). Similar effects were observed in Caco-2 cells (data not shown). We next tested whether silencing of HDAC3 and other Class I HDACs could mimic butyrate-induced activation of the Sp1/Sp3 reporter construct. As shown in Fig. 9B, activity of Sp1-luc was stimulated 3–5-fold following silencing of HDAC1, HDAC2, and HDAC3 expression. Minimal changes on mtSp1-luc activity were observed, confirming the specificity of these effects. We confirmed these findings by Class I HDAC overexpression studies. As shown in Fig. 9C, butyrate induction of Sp1-luc activity in HCT116 cells was inhibited by >50% following overexpression of HDAC1, HDAC2, or HDAC3. Similar results were observed in Caco-2 cells (data not shown).

Finally, to more directly confirm the ability of HDAC3 and other Class I HDACs to repress Sp1/Sp3-dependent transcription, we utilized a one-hybrid system, in which Sp3 was fused to GAL4, and transactivation was measured using a luciferase reporter driven by a minimal promoter linked to five consensus GAL4 DNA binding sites (27). As shown in Fig. 9D and consistent with butyrate induction of Sp1/Sp3 reporter activity, butyrate induced a 35-fold stimulation of GAL4-Sp3 transactivation in HCT116 cells. The specificity of butyrate induction of GAL4-Sp3 was demonstrated by markedly reduced transactivation following transfection with a fusion construct containing the dominant negative form of Sp3, which lacks the transactivation domain (GAL4-DNSp3) (Fig. 9D). We then tested whether silencing of HDAC3 and other class I HDACs could mimic butyrate-induced activation of GAL4-Sp3. As shown in Fig. 9E, HDAC1, -2, and -3 silencing induced a 5–10-fold increase in GAL4-Sp3 transactivation compared with NT1 or NT2. Minimal effects upon activity of the GAL4 or GAL4 DNSp3 constructs were observed (Fig. 9E). These findings were further confirmed by the ability of HDAC1, HDAC2, or HDAC3 overexpression to inhibit butyrate-induced transactivation of GAL4-Sp3 (Fig. 9F).

DISCUSSION

Inhibitors of the HDAC family of transcriptional co-repressors, such as butyrate, induce cell cycle arrest, markers of cell differentiation, and apoptosis in colon cancer cell lines in vitro (5–10). HDAC inhibitors have also been shown to inhibit tumorigenesis in vivo, in both xenograft and mouse models of a variety of tumor types (14). Based on these preclinical findings, a number of HDAC inhibitors are currently under-
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We observed that HDAC3 was maximally expressed in the proliferative compartment in normal mouse small intestine and colon, suggesting that HDAC3 expression may play a role in regulating cell proliferation. Direct evidence for such a role was demonstrated in vitro in HCT116 cells by the reduction in cell number, and accumulation of cells in the G2/M phase of the cell cycle following HDAC3 silencing. A role for HDAC3 in cell survival and apoptosis was also evident by the reduced clonogenicity and increase apoptosis observed in HDAC3-silenced cells.

In parallel, HDAC3 silencing induced p21 protein levels and promoter activity. Dissection of the mechanism of p21 repression by HDAC3 demonstrated a dependence on the Sp1/Sp3 family of transcription factors. First, overexpression of HDAC3 significantly repressed basal as well as butyrate-induced activity of a proximal p21 reporter construct containing six Sp1/Sp3 binding sites. Mutation analyses of one or more of the Sp1/Sp3 sites within this fragment established that over 95% of the basal activity of this construct was Sp1/Sp3-dependent. Second, reverse transcriptional repression by HDAC3 resulted in transcriptional activation. Finally, HDAC3 silencing resulted in transcriptional repression by HDAC3, whereas conversely, HDAC3 overexpression repressed butyrate-induced transactivation of GAL4-Sp3. Effects similar to those induced by HDAC3 were observed for HDAC1 and -2, with HDAC1 and -2 silencing resulting in reduced...
cell growth, enhanced cell survival, and p21 induction in a Sp1/Sp3-dependent manner. Supporting our results, a recently published study demonstrated that HDAC1 and HDAC3 overexpression represses p21 expression in an Sp1-dependent manner in cells of nonintestinal epithelial origin (36).

An emerging model for regulation of p21 is the recruitment of HDACs by Sp1/Sp3 transcription factors, which in turn repress p21 transcription (reviewed in Ref. 37). The present findings identify HDAC3 as a necessary component for p21 repression and demonstrate that multiple class I HDACs are required for efficient p21 repression. The coordinate repression of p21 by HDAC1 and -2 is consistent with the co-existence of these HDACs in co-repressor complexes, such as Sin3A and NuRD (38). The current demonstration that HDAC3 also represses p21 expression is important given its existence in the NCoR-SMRT co-repressor complex (39–41), which is distinct from repressor complexes containing HDAC1 and HDAC2. The repression of p21 by HDAC3 is also consistent with a previous report that demonstrated that overexpression of N-CoR inhibited p21 promoter induction following serum withdrawal (42). It is noteworthy that the class II HDACs, HDAC4 and HDAC5, also interact with the HDAC3-NCoR-SMRT complex (43), indicating that class I and II HDACs may act in concert to repress gene expression. Collectively, these findings suggest that multiple HDAC-containing repressor complexes may be recruited to the p21 promoter to repress p21 promoter activity and subsequently modulate cell growth.

Whereas a number of the phenotypic effects induced by HDAC3 were paralleled by HDAC1 and -2, silencing of HDAC3 alone induced expression and activity of alkaline phosphatase, a marker of colon cell differentiation and a gene consistently up-regulated in colon cancer cells in response to butyrate treatment. Therefore, whereas certain cellular processes, such as p21, are coordinately regulated by multiple Class I HDACs, others, such as ALP, may be preferentially regulated by specific Class I HDACs. The latter is consistent with a recent study in Drosophila demonstrating that, following Class I HDAC overexpression, ~90% of the genes differentially expressed were unique to a given HDAC, indicating that each has a largely distinct profile of transcriptional targets (44).

Importantly, despite greater than 90% efficiency of silencing of HDAC1, HDAC2, and HDAC3 expression, individual class I HDAC silencing failed to fully recapitulate HDAC inhibitor-mediated p21 induction, growth arrest, or apoptosis. Therefore, it is likely that HDAC inhibitors inhibit multiple class I HDACs in order to exert their phenotypic effects, a finding that may contribute toward the further development of HDAC inhibitors in cancer treatment by suggesting that generic rather than isoform-specific HDAC inhibitors may have greater therapeutic efficiency. Whether the inhibition of HDACs additional to those examined herein, including the class I HDAC9 and the class II HDACs HDAC4, -5, -6, -7, -9, and -10, are also required for the HDAC inhibitor response remains to be determined. We also note that these findings do not eliminate the possibility that HDAC-independent mechanisms may play a role in the cellular response to these agents.

In conclusion, these findings identify HDAC3 as a protein up-regulated in colon tumors and as a regulator of proliferation and differentiation of colon cancer cells in vitro. The localization of HDAC3 and other class I HDACs to the proliferative zone in both small intestine and colon is consistent with a physiological role for these enzymes in maintaining cell proliferation and inhibiting maturation, a function that is borne out by the stimulation of growth arrest, maturation, and apoptosis upon their pharmacological inhibition with HDAC inhibitors in vitro. These studies also demonstrate that multiple class I HDACs are involved in the repression of the cell cycle mediator p21 and in the regulation of cell proliferation and illustrate that HDAC inhibitors probably mediate their response through the inhibition of multiple HDACs.

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REFERENCES

1. Peterson, C. L., and Laniel, M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 10627–10632
2. Gu, W., and Roeder, R. G. (1997) Cell 90, 595–606
3. Marigo, G., Wagener, C., Gutiérrez, M. I., Cartwright, P., Helin, K., and Giacca, M. (2000) J. Biol. Chem. 275, 10887–10892
4. Ammananamachi, S., Freeman, J. W., and Brattain, M. G. (2003) J. Biol. Chem. 278, 35775–35780
5. Mariadason, J. M., Barkla, D. H., and Gibson, P. R. (1997) Am. J. Physiol. 272, G705–G712
6. Mariadason, J. M., Rickard, K. L., Barkla, D. H., Augenlicht, L. H., and Gibson, P. R. (2000) J. Cell. Physiol. 183, 347–354
7. Mariadason, J. M., Velcich, A., Wilson, A. J., Augenlicht, L. H., and Gibson, P. R. (2001) Gastroenterology 120, 889–899
8. Heerdt, B. G., Houston, M. A., and Augenlicht, L. H. (1994) Cancer Res. 54, 3288–3293
9. Gurvich, N., Tygankova, O. M., Meinhold, J. L., and Klein, S. P. (2004) Cancer Res. 64, 1079–1086
10. Litvak, D. A., Evers, B. M., Hwang, K. O., Hellmich, M. R., Ko, T. C., and Townsend, C. M. Jr. (1998) Surgery 124, 161–170
11. Fischle, W., Kiermer, V., Dequiedt, F., and Verdin, E. (2001) Biochem. Cell Biol. 79, 337–348
12. Marks, P. A., Rifkind, R. A., Richon, V. M., Breslow, R., Miller, T., and Kelley, W. K. (2001) Nat. Rev. Cancer 1, 194–202
13. Lagger, G., O’Carroll, D., Rembold, M., Khier, H., Tischler, J., Weitzer, G., Schuetten-gruber, B., Hauser, C., Brunnermeier, R., Juenewein, T., and Seiser, C. (2002) EMBO J. 21, 2672–2681
14. Glaser, K. B., Li, J., Staver, M. J., Wei, R. Q., Albert, D. H., and Davidson, S. K. (2003) Biochem. Biophys. Res. Commun. 310, 529–536
15. Tou, L., Liu, Q., and Shvidarsa, R. A. (2004) Mol. Cell. Biol. 24, 3132–3139
16. Mariadason, J. M., Nicholas, C., L’Italien, K. E., Zhuang, M., Smartt, H. J. M., Heerdt, B. G., Yang, W., Corner, G. A., Wilson, A. J., Klampfer, L., Arango, D., and Augenlicht, L. H. (2005) Gastroenterology 128, 1081–1088
17. Weiner, M. M. (1973) J. Biol. Chem. 248, 2536–2541
18. Weiner, M. M. (1973) J. Biol. Chem. 248, 2542–2548
19. Ferraris, R. P., Villenas, S. A., and Diamond, J. (1992) Am. J. Physiol. 262, G1047–G1059
20. Veil, A., Yang, W., Heyer, J., Fragale, A., Nicholas, C., Vanni, S., Uchel, A., Lipkin, M., Yang, K., and Augenlicht, L. (2002) Science 295, 1726–1729
21. Marzaglia, L., Bordonaro, M., Askham, F., Shi, L., Kurguchi, M., Veil, A., and Augenlicht, L. H. (2001) Cancer Res. 61, 3465–3471
22. Sowa, Y., Orita, T., Minamikawa, S., Nakano, K., Mizuno, T., Nomura, H., and Sakai, T. (1997) Biochem. Biophys. Res. Commun. 241, 142–150
23. Sowa, Y., Orita, T., Minamikawa-Hirane, S., Mizuno, T., Nomura, H., and Sakai, T. (1999) Cancer Res. 59, 4266–4270
24. Hassig, C. A., Tong, J. K., Fleischer, T. C., Owa, T., Grable, P. G., Ayer, D. E., and Schreiber, S. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3519–3524
25. Laherty, C. D., Yang, W. M., Sun, J. M., Davie, J. R., Seto, E., and Eisenman, R. N. (1997) Cell 89, 349–356
26. Yang, W. M., Yao, Y. L., Sun, J. M., Davie, J. R., and Seto, E. (1997) J. Biol. Chem. 272, 28001–28007
27. Mariadason, J. M., Arango, D., Shi, Q., Wilson, A. J., Corner, G. A., Nicholas, C., Aranes, M. J., Lesser, M., Schwartz, E. L., and Augenlicht, L. H. (2003) Cancer Res. 63, 8791–8812
28. Mosmann, T. (1983) J. Immunol. Methods 65, 55–63
29. Yang, W. C., Mathew, J., Veil, A., Edelman, W., Uchel, A., Lipkin, M., Yang, K., and Augenlicht, L. H. (2001) Cancer Res. 61, 565–569
30. Archer, S. Y., Meng, S., Shi, A., and Hedin, R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6791–6796
31. Huang, L., Sowa, Y., Sakai, T., and Pardee, A. B. (2000) Oncogene 19, 5712–5719
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36. Huang, W., Tan, D., Wang, X., Han, S., Tan, I., Zhao, Y., Lu, J., and Huang, B. (2006) Biochem. Biophys. Res. Commun. 339, 165–171
37. Gartel, A. L., and Radhakrishnan, S. K. (2005) Cancer Res. 65, 3980–3985
38. Jepsen, K., and Rosenfeld, M. G. (2002) J. Cell Sci. 115, 689–698
39. Guenther, M. G., Lane, W. S., Fischle, W., Verdin, E., Lazar, M. A., and Shiekhattar, R. (2000) Genes Dev. 14, 1048–1057
40. Li, J., Wang, J., Nawaz, Z., Liu, J. M., Qin, J., and Wong, J. (2000) EMBO J. 19, 4342–4350
41. Codina, A., Love, J. D., Li, Y., Lazar, M. A., Neuhaus, D., and Schwabe, J. W. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 6009–6014
42. Bailey, P., Downes, M., Lau, P., Harris, J., Chen, S. L., Hamamori, Y., Sartorelli, V., and Muscat, G. E. (1999) Mol. Endocrinol. 13, 1155–1168
43. Fischle, W., Dequiedt, F., Hendzel, M. J., Guenther, M. G., Lazar, M. A., Voelter, W., and Verdin, E. (2002) Mol. Cell 9, 45–57
44. Cho, Y., Griswold, A., Campbell, C., and Min, K. T. (2005) Genomics 86, 606–617