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The Activation of Beta-Catenin by Wnt Signaling Mediates the Effects of Histone Deacetylase Inhibitors

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Abstract

Most colorectal carcinomas (CRCs) exhibit constitutively active Wnt signaling. We have reported that (a) the histone deacetylase inhibitor (HDACi)² sodium butyrate (NaB) modulates the canonical Wnt transcriptional activity of CRC cells *in vitro* and (b) a linear relationship exists between the increase in Wnt transcriptional activity and the levels of apoptosis in ten CRC cell lines treated with NaB. Herein we report that structurally different HDACis modulate Wnt signaling in CRC cells and a mechanism involved in this action is an increase in beta-catenin that is dephosphorylated at Ser-37 and Thr-41 residues. The increase of active (Ser-37 and Thr-41 dephosphorylated) beta-catenin in CRC cells treated with HDACis is initiated at the ligand level and the inhibition of this increase suppresses Wnt signaling and lowers the levels of apoptosis. CRC cells that develop resistance to the apoptotic effects of HDACis exhibit lower levels of active beta-catenin compared to apoptosis-sensitive parental cells and this resistance is reversed by increasing the levels of active beta-catenin. Results from comparative studies between HDACi-resistant and HDACi-sensitive cells suggest that non-histone targets of HDACis mediate the effects on Wnt signaling and apoptosis.

Keywords

Wnt signaling; histone deacetylase inhibitors; apoptosis; colorectal carcinomas; butyrate

Introduction

Inhibitors of histone deacetylases (HDACis) are promising anticancer agents that preferentially induce growth arrest, differentiation, and apoptosis in malignant, but not normal, cells [reviewed in 1-5]. Several HDACis are currently in clinical trials, and, recently, the U.S. Food and Drug Administration gave approval for the HDACi vorinostat

²Abbreviations used: APC, adenomatous polyposis coli; CRC, colorectal carcinoma; Dkk1, Dickkopf-1; EGFP, enhanced green fluorescent protein; GSK-3beta, glycogen synthase-kinase 3beta; BCT, beta-catenin-Tcf; HDACi, histone deacetylase inhibitors; LRP, low-density lipoprotein receptor-related protein; NaB, sodium butyrate; OA, okadaic acid; PBS, phosphate buffered saline; sFRP, secreted Frizzled related protein; SAHA, suberoylanilide hydroxamic acid, Tcf/Lef, T-cell factor/lymphocyte enhancer factor; TOP/FOP, ratio of luciferase reporter activity of pTOPFLASH to that of pFOPFLASH; TSA, trichostatin A.

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(SAHA) to be used in the treatment of cutaneous T-cell lymphoma. Thus, knowledge of how these agents express their antineoplastic properties is important. The major activity of HDACis is believed to involve inhibition of histone deacetylases, resulting in modified chromatin assembly and altered gene expression [1-5]; however, an increasing body of evidence suggests that non-histone proteins are essential mediators of HDACi function [6].

We have established that HDACis such as sodium butyrate (NaB) and trichostatin A (TSA) modulate Wnt transcriptional activity in human colorectal carcinoma (CRC) cells [7,8]. Canonical Wnt transcriptional activity is induced by the binding of Wnt ligands to cell surface receptors, resulting in inhibition of glycogen synthase kinase-3 beta (GSK-3 beta) activity [9-12]. When active, GSK-3 beta, in complex with adenomatous polyposis coli (APC) and Axin, promotes the phosphorylation and degradation of beta-catenin [13-15]; however, when GSK-3 beta activity is inhibited, dephosphorylated beta-catenin accumulates and interacts with Tcf/Lef DNA binding proteins [16-20]. Beta-catenin-Tcf (BCT) transcriptional complexes are detected by their ability to drive transcription from Tcf/Lef site-containing promoter constructs [19,20].

The constitutive activation of canonical Wnt signaling due to mutations in *APC* [21-23] and *beta-catenin* [20] is believed to promote cell proliferation and tumorigenesis in the colon. However, we and several other research groups have reported that relatively high levels of Wnt signaling result in apoptosis [24-29]. Our findings indicate that hyper-activation of canonical Wnt transcriptional activity induces apoptosis since (a) there is a linear relationship between the fold induction of Wnt transcriptional activity and the degree of apoptosis in ten human CRC cell lines exposed to NaB, (b) cells with suppressed induction of Wnt activity exhibit a decrease in apoptosis in the presence of NaB, and (c) cell fractions with high Wnt activity have a higher ratio of apoptotic to live cells than cell fractions with low levels of Wnt activity [29]. We have also established that the increase in canonical Wnt activity precedes the apoptotic event since (a) the inhibition of apoptosis by a general caspase inhibitor does not abrogate the increase in Wnt activity (unpublished data), and (b) flow cytometry-sorted cells with high Wnt activity exhibit high levels of both live and apoptotic cells; however, if apoptosis were a prerequisite for induction of Wnt activity, all cells with high Wnt activity should have been apoptotic [29]. Based upon our results and the findings of others [24-29], we hypothesize that the relative levels of Wnt signaling determine whether cells proliferate or commit to undergo apoptosis. These observations and the findings that Wnt signaling is modulated by HDACis suggest that the reason HDACis induce reversible growth arrest or apoptosis in different cell types is at least partially determined by the levels of induced Wnt signaling. Thus, HDACis influence the physiology of cells that do not carry Wnt activating mutations to a lesser extent; however, in cells with a deregulated Wnt pathway, HDACis induce higher levels of Wnt which lead to apoptosis.

In the present investigation, we have primarily focused on the effects of NaB in CRC cells, since butyrate is a natural fermentation product of dietary fiber in the colon [30] and the preventive role of dietary fiber against CRCs has been convincingly demonstrated in the most recent completed clinical studies [31,32]. We have, however, also evaluated the effects of other HDACis, two of which are in clinical trial, on the modulation of Wnt activity and apoptosis in CRC cells. We report herein that structurally different HDACis also modulate Wnt signaling in CRC cells and a mechanism involved in this effect is an increase in Ser-37/Thr-41-dephosphorylated beta-catenin initiated at the ligand level. Inhibition of the increase in active beta-catenin levels suppresses the induction of Wnt signaling and the induction of apoptosis by these HDACis. In addition, CRC cells resistant to the apoptotic effects of HDACis exhibit lower levels of Ser-37/Thr-41 dephosphorylated beta-catenin compared to apoptosis-sensitive parental cells; this resistance can be reversed by increasing the levels of

active beta-catenin. The findings suggest that non-histone targets of HDACs likely mediate the effects of these agents on Wnt signaling and apoptosis.

Materials and Methods

Cells, plasmids, transfections, luciferase assays, and clonal growth assays

Human CRC cell lines and human transformed embryonic kidney 293 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in alpha-MEM with 10% fetal bovine serum. Transfections were performed with Lipofectamine 2000 (Life Technologies, Rockville, MD) or GenePorter (Gene Therapy Systems, San Diego, CA) as reported previously [8,29]. The vector pRSV-TK (Promega Corp., Madison, WI) was used for normalization of transfection efficiency. The following vectors were provided by various researchers: mouse Dickkopf1 (Dkk1) and LRP5 (Dr. D. Wu, Univ. of Connecticut Health Center, Farmington), secreted Frizzled-related proteins (sFRP) 1, 2, 4, and 5 (Dr. H. Suzuki, Sapporo Medical University, Japan), pTOPFLASH (TOP) and pFOPFLASH (FOP), Tcf1, the Lef1-fusion constructs to VP16 and beta-catenin (Dr. P. K. Vogt, Scripps Research Institute, La Jolla, California), small T antigen (Dr. E. Sontag, University of Texas Southwestern Medical Center, Dallas, Texas). Tcf4 expression vector was from Upstate Biotechnology (Lake Placid, NY). Luciferase assays were performed using a Turner Luminometer and a Dual Luciferase kit (Promega, Madison, WI). Treatment with NaB (Sigma, St. Louis, MO) was performed at 5 mM, with Trichostatin A (Alexis Biochemicals, Carlsbad, CA) at 1 μ M, with SAHA (BioVision Research Products, CA) at 10 μ M, with MS-275 (Alexis Biochemicals) at 10 μ M, and with LiCl (Sigma) at 20 mM. Okadaic acid (Sigma) was used at 20 nM final concentration and was added to cells 15 min prior to exposure to NaB.

Transfections with EGFP-TOP and EGFP-FOP were performed with cells plated at 2×10^6 per well in 12-well dishes 24 hr before transfection with 2 μ g of DNA and Lipofectamine 2000. At 5 hr, cells from each well were washed, trypsinized and aliquoted into 6 wells of 24-well dishes. At 24 hr after transfection, cells were treated with NaB, okadaic acid (OA), or the combination of these two agents. In cotreatment experiments, cells were preincubated with OA for 15 min before the addition of NaB. Cells were harvested 24 hr later and subjected to flow cytometry as described [29]. Transfections with inhibitors of Wnt activity were carried out with GenePorter in 24-well dishes with 0.4 μ g or 1 μ g of Dkk1, sFRP, dnLRP5, or empty expression construct and 0.1 μ g or 0.4 μ g of luciferase reporter construct (TOP or FOP). Conditioned medium from 293 cells, transfected with 5 μ g of pCINeo or Dkk1 expression vector and Lipofectamine 2000 in 6-well dishes, was obtained at 32 hr post-transfection. At this time, HCT-116 cells plated in 6-well dishes were incubated with the conditioned medium in the absence or presence of 5 mM NaB for a total of 17 hr. Transfections with pre-designed Tcf1 siRNA (cat. # 16708, ID: 215766, Ambion, Austin, TX) or negative control siRNA (cat. # 4611, Ambion) were performed with Lipofectamine 2000 according to the protocol of the manufacturer.

Clonal growth assays were performed as indicated previously [29]. For these assays, HCT-116 cells were transiently transfected with 1.2 μ g of empty vector or Dkk1 expression vector. Treatment with 5 mM NaB was initiated at 26 hr post-transfection and continued for 16 hr. Equal numbers of cells from each treatment and transfection were plated in triplicate in 6-well dishes; at 10 days, colonies were stained with crystal violet solution and their numbers determined.

Apoptotic assays

Apoptotic analyses were performed using the Vybrant Apoptosis Assay Kit #2 (Molecular Probes, Eugene, OR) or the Annexin V-PE Apoptosis Detection Kit I (BD Pharmingen) as previously described [29]. Attached and floating cells were collected from both treated and control cells. The percentage of apoptotic cells represents the ratio of the number of apoptotic cells to that of the total analyzed cells, multiplied by 100. The fold increase in apoptotic cells is the ratio of the percentage of apoptotic cells in treated samples to that of mock treated samples. Statistical analyses of this group of samples were performed using WinMDI 2.8 software (provided by Dr. Joseph Trotter, Scripps Clinic, San Diego, CA).

Western blot analysis

Nuclei isolated using a Nuclei EZ kit (Sigma) or intact cells were lysed as described previously [33] and equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and immunostained with antibodies against total beta-catenin (#C-19220, BD Biosciences, San Diego, CA), active beta-catenin (#05-665, Upstate Biotechnology, Charlottesville, VA), Tcf1 and Tcf4 (Upstate Biotechnology), or actin (Sigma). For detection of acetylated histones H3 and H4, nuclei were isolated from cells with the Nuclei EZ kit (Sigma) and proteins were extracted with N-myc buffer [33]; sulfuric acid was added to a final concentration of 0.2 M. Samples were incubated on ice for 20 min and supernatants were precipitated with three volumes of ethanol. The pellets were washed with 70% and 100% ethanol, dissolved in water, and the protein level quantified by the method of Bradford. Equal amounts of protein were resolved on 15% SDS-polyacrylamide gels. Acetylated Histone H3 (Lys 9), acetylated Histone H4 (Lys 5), and dimethyl-Histone 3 (Lys 9) were detected with antibodies from Upstate Biotechnology (cat # 06-942, 06-759, and 07-212, respectively). Western blots were visualized with an anti-mouse-horseradish peroxidase antibody (Sigma) and chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA). Quantitative analysis was performed on a Molecular Dynamics Densitometer (Sunnyvale, CA).

RNase protection analyses

To prepare the Tcf1 probe, Tcf1 cDNA-containing vector was cut with SmaI and PstI restriction endonucleases to obtain a fragment of 358 nt encompassing parts of exons Ib and II. This probe protected 358 nt of the full length Tcf1 transcript (when the upstream Tcf1 promoter is used) and 254 nt when a shorter Tcf1 message is synthesized from the downstream Tcf1 promoter [34]. The Tcf1 fragment was cloned in the antisense orientation from the T7 promoter of pGEM4z (Promega).

The Tcf4 probe was prepared by excising a 227 nt DNA fragment from the Tcf4 expression construct (Upstate Biotechnology) with BspHI, followed by blunt-ending, and SacI treatment. This fragment was inserted in the antisense orientation to the T7 promoter in the pGEM4z vector pre-cut with SacI and SmaI. The full length Tcf4 probe is 283 nt when linearized with EcoRI. Probes were generated by transcription of the linearized templates for Tcf1 and Tcf4 with the MAXI script *in vitro* transcription kit of Ambion and biotin-16-uridine-5'-triphosphate from Roche. Non-radioactive RNase protection analyses were performed with the SuperSignal RPA III Chemiluminescent Kit (Pierce, IL).

Co-immunoprecipitation and NoShift assay

Nuclei were isolated from mock and NaB treated CRC cells using the Nuclei EZ Prep kit (Sigma) and lysed as described previously [33]. For each sample, 100 µg of nuclear protein were diluted to 1 µg/µl with phosphate buffered saline, mixed with 100 µl of a 50% protein A agarose bead slurry (Upstate Biotechnology) and 1 µg of antibody, and incubated with

rotation for 2 hr at 4° C. The beads were collected by centrifugation, washed 4 times with 300 µl of RIPA buffer and resuspended in 60 µl of 2× Laemmli buffer.

For NoShift assays, nuclear extracts were isolated from untreated and NaB treated HCT-116 and SW620 CRC cells using a Nucbuster kit (Novagen, Madison, WI) or as described above [33]; the NoShift kit was used according to the manufacturer's instructions. Twenty-five µg of nuclear protein was used per sample, with a 1:100 dilution of beta-catenin antibody (BD Transduction Laboratories, San Diego, CA). Oligonucleotides, with 3' end biotin labels, containing sequences for Tcf site binding (TOP) or mutant sequences (FOP) were constructed and the complementary TOP or FOP oligonucleotides were annealed and diluted to a concentration of 10 pmol/µl. Each sample was incubated with either TOP or FOP annealed oligonucleotides. The TOP oligonucleotides used were: 5' GGGTAAGATCAAAGGGGGTAA 3'; 5' TTACCCCTTTGATCTTACCC 3'. The FOP oligonucleotides were: 5' GGGTAAGG CCAAAGGGGGTAA 3'; 5' TTACCCCTTTGGCCTTACCC 3'. The background readings of no extract controls were subtracted from readings obtained from the TOP and FOP oligonucleotides.

Statistics

All P values indicated were calculated using the Student's t-test; statistical significance was set at $P < 0.05$.

Results

NaB increases the steady-state levels of Ser-37/Thr-41 dephosphorylated (active) beta-catenin in human CRC cell lines

We have reported that NaB does not increase the levels of nuclear localized total beta-catenin in CRC cells [8]. Subsequently, Clevers and colleagues reported that Wnt signaling controls the phosphorylation status of Ser-37 and Thr-41 of beta-catenin and this transcriptionally active beta-catenin form has a physiological function [35,36]. Therefore, we examined the effects of NaB on the levels of transcriptionally active beta-catenin, utilizing a monoclonal antibody which recognizes beta-catenin with non-phosphorylated Ser-37 and Thr-41. The dephosphorylated status of Ser-37 and Thr-41 is indicative of active Wnt signaling *in vitro* and *in vivo* [35,36]. We found that in eight out of ten CRC cell lines (SW48, SW620, LS174T, HCT-116, DLD-1, COLO201, HT29, LoVo) NaB increased the levels of beta-catenin that is non-phosphorylated at Ser-37 and Thr-41 (Fig. 1A). The only exceptions to these findings were RKO cells, which have undetectable levels of active beta-catenin, and SW480 cells, in which exposure to NaB for 24 hr or less produced decreased levels of active beta-catenin in the total cellular lysates and unchanged levels of nuclear active beta-catenin (Fig. 1). In most cell lines exposure to NaB was performed for 24 hr; in COLO201, SW48, and SW620 cells the increase in active beta-catenin was detected earlier, at 7 to 17 hr of treatment with 5 mM NaB. The increase in dephosphorylated beta-catenin levels in nuclei was confirmed in SW620 and HCT-116 CRC cell lines treated with 5 mM NaB for 24 hr (Fig. 1B).

To ascertain the role of increased levels of Ser-37/Thr-41 dephosphorylated beta-catenin in the upregulation of Wnt activity in NaB treated cells, we utilized okadaic acid (OA), which inhibits the activity of serine-threonine protein phosphatases and modulates Wnt signaling by changing the phosphorylation status of its components [37-39]. Treatment of HCT-116 and SW620 cells with both NaB and OA resulted in levels of active beta-catenin comparable to those in mock treated cells (Fig. 1C). Next, we measured the canonical Wnt transcriptional activity in cells in which the increase in active beta-catenin was suppressed by OA. Wnt transcriptional activity was measured with the TOP/FOP luciferase reporter

system that has wild-type (TOP) or mutant (FOP) Tcf binding sites upstream of a minimal c-fos promoter [19]. The ratio of luciferase expression driven by wild-type to that driven by mutant promoter sequences specifically assays the contribution of Wnt activity to the expression of luciferase [7,19]. Measuring Wnt transcriptional activity by assaying the expression levels of endogenous Wnt-targeted genes such as *c-myc* is not appropriate since (a) HDACs influence gene expression independent of Wnt signaling; thus, NaB both stimulates transcriptional initiation [40] and blocks transcriptional elongation of the *c-myc* gene [40,41], (b) it has been reported that whereas low levels of Wnt signaling activate particular endogenous promoters, higher levels of Wnt signaling suppress the same promoters due to additional sequences that control gene expression [42], and (c) different Wnt-sensitive genes are activated depending upon whether Wnt signaling is induced at the ligand level or at subsequent intracellular steps [43]. The use of the TOP/FOP reporters overcomes the above mentioned problems. Therefore, we transfected SW620 and HCT-116 CRC cells with the TOP or FOP reporters and treated the cells with NaB and/or OA (Fig. 1D). Exposure of the SW620 cells to NaB resulted in a 6-fold increase in the TOP/FOP ratio; whereas, cotreatment with NaB and OA resulted in only a 1.1-fold increase in this ratio, producing a 5.5-fold decline in the TOP/FOP ratio ($P < 0.02$). In HCT-116 cells, NaB enhanced Wnt activity by 27-fold; while, cotreatment with NaB and OA partially suppressed the increase in Wnt activity produced by NaB alone to 9-fold ($P < 0.001$).

We have established that only a fraction of the cells in a CRC population exhibit Wnt activity, and that NaB increases the number of Wnt positive cells to different extents in each CRC cell line [29]. Thus, we next determined whether OA suppresses the increase in the number of Wnt positive cells. The number of Wnt positive and Wnt negative cells was determined by utilizing vectors expressing the green fluorescent protein under the control of the TOP or FOP promoter, as previously described [29]. Consistent with our previous findings [29], a mock treated population of HCT-116 cells contained $10.7 \pm 2.2\%$ Wnt positive cells; exposure to NaB increased this percentage nearly 4-fold to $39 \pm 6.2\%$ ($P < 0.003$). Exposure to OA alone resulted in $14.9 \pm 2.8\%$ of the cells exhibiting high levels of Wnt activity, a value statistically equivalent to that of the mock treated cells. Cotreatment with NaB and OA resulted in a 2-fold lower percentage of Wnt positive cells ($20.9 \pm 2.2\%$, $P < 0.01$) relative to cells exposed to NaB alone (Fig. 1E). In mock treated SW620 cells, $13.4 \pm 1.7\%$ of the cellular population exhibited high levels of Wnt activity. NaB treatment alone increased this number more than 2-fold to $30.8 \pm 1.5\%$ ($P < 0.001$), and treatment with OA alone resulted in $20.7 \pm 3.7\%$ of the cells expressing high levels of Wnt activity, a relatively small increase ($P < 0.05$) compared to that of the mock treated sample. Cotreatment with NaB and OA resulted in $14.1 \pm 1.1\%$ Wnt-positive cells, a result statistically equivalent to that of the mock treated cells (Fig. 1E).

We also determined whether a genetic modulator of protein phosphatase activity would also inhibit the ability of NaB to induce Wnt activity; thus, the effects of OA on Wnt activity levels were mimicked by overexpression of SV40 small T-antigen, which, like OA, is a known inhibitor of protein phosphatase PP2A [44] (Fig. 1F).

While these data strongly suggest that an increase in the levels of active beta-catenin is necessary for the induction of Wnt activity observed in CRC cells treated with NaB, there is no linear correlation between these two phenomena. For example, in HCT-116 cells, quantitative analyses of Western blot data show that treatment with NaB increases the levels of active beta-catenin by 2.2-fold; whereas, the induction of Wnt activity is 21.8-fold [29]. In SW620 cells treated with NaB, the induction of the levels of active beta-catenin is 1.9-fold, and the induction of Wnt activity is 5.8-fold [29].

To ascertain whether the increased levels of active nuclear beta-catenin in NaB treated CRC cells corresponded to higher levels of Tcf-associated beta-catenin, we measured the levels of beta-catenin immunoprecipitated by Tcf4 and Tcf1 antibodies (Fig. 2). For these experiments we utilized HCT-116 cells that exhibit high levels of induction of both Wnt activity and apoptosis after exposure to NaB and SW620 cells that produce a weaker response to NaB. No increase occurred in the complex formed between Tcf4 and beta-catenin in SW620 cells treated with NaB and, in addition, no Tcf1-beta-catenin complexes were detected in this cell line (Fig. 2A). In contrast, an increase in the levels of beta-catenin associated with Tcf4, and the induction of a lesser degree of association between beta-catenin and Tcf1, were observed in HCT-116 cells treated with NaB (Fig. 2B).

Western blot analyses of nuclear lysates used for immunoprecipitation experiments demonstrated that NaB did not significantly influence the steady-state levels of Tcf4 in SW620 cells; however, it decreased these levels in HCT-116 cells (Fig. 2C). The increased association of beta-catenin with Tcf4 in NaB treated HCT-116 cells was confirmed by transcription factor binding assays with nuclear protein extracts from mock or NaB treated HCT-116 cells and oligonucleotides containing either wild-type (TOP) or mutant (FOP) Tcf-Lef binding sites (NoShift assays, see Materials and Methods). No increase in association of beta-catenin with Tcf4 was observed with similar assays performed with SW620 cells (data not shown).

Butyrate modulates canonical Wnt signaling at the ligand level in CRC cells

Since the phosphorylation status of the Ser-37 and Thr-41 residues of beta-catenin can be regulated by the binding of Wnt ligands to their receptors at the plasma membrane [35,36], we tested whether NaB influences Wnt activity at the ligand level. This was accomplished by cotransfecting CRC cells with TOP or FOP luciferase reporters and vectors encoding Wnt antagonists, such as Dkk1, sFRPs, and dominant negative (dn) LRP5. Dkk1 is a secreted molecule that prevents the binding of Wnt ligands to co-receptors LRP5/6 [45-47]; sFRPs are proteins that exhibit sequence similarity to the extracellular domain of the Frizzled receptor [48-50]; and dnLRP5 consists of the extracellular domain of the receptor. The dnLRP5 and sFRPs may sequester Wnt ligands and/or form nonfunctional complexes with Frizzled receptors.

Expression of Dkk1 reduced the NaB upregulation of the TOP/FOP ratio which measures Wnt activity from 22.4 ± 2.1 -fold to 7.7 ± 0.14 -fold in HCT-116 cells and from 1.67 ± 0.6 -fold to 0.95 ± 1.7 -fold in SW48 cells (Fig. 3A). sFRP2 also decreased the induction of Wnt activity by NaB in HCT-116 cells from 38.5-fold to 12.2-fold ($P < 0.005$, Fig. 3B). Transfection with dnLRP5 decreased the TOP/FOP ratio in both mock ($P < 0.002$) and NaB treated ($P < 0.001$) HCT-116 cells; therefore, dnLRP5 did not produce a statistically significant change in the fold induction of TOP/FOP in these cells (Fig. 3B). In SW620 cells, exogenous Dkk1 and dnLRP5 had no significant effect on the upregulation of the TOP/FOP ratio (Fig. 3C and data not shown); whereas, sFRP2 inhibited the TOP/FOP ratio in a statistically significant manner in both mock and NaB treated SW620 cells (Fig. 3C).

The expression of the two Wnt antagonists that most effectively inhibited the induction of Wnt activity by NaB in HCT-116 cells (i.e., sFRP2 and Dkk1) also affected the levels of active beta-catenin. Thus, an inhibition of the increase in active beta-catenin by NaB in HCT-116 cells cultured with conditioned medium from Dkk1 transfected 293 cells was observed (Fig. 3D), as well as in HCT-116 cells transiently transfected with Dkk1 (Fig. 3E). Suppressed induction of active beta-catenin by NaB was less obvious in sFRP2 transfected HCT-116 cells (data not shown), most likely due to a different mechanism of action and/or the degree of secretion of the Wnt antagonist.

We have previously demonstrated the existence of a linear relationship between the level of Wnt activity and the degree of apoptosis occurring in ten CRC cell lines treated with NaB [29]. Therefore, it was of interest to ascertain whether Wnt activity induced at the cell surface in HCT-116 cells contributed to the sensitivity of these cells to the apoptotic effects of NaB. To evaluate this possibility, we compared the ability of Dkk1 transfected and empty vector transfected HCT-116 cells to form colonies after exposure to NaB (Fig. 3F). NaB treated cells, transfected with empty vector, formed $47.1 \pm 3.2\%$ colonies compared to those produced by mock treated, empty vector-transfected cells; whereas, $68.7 \pm 3.0\%$ of NaB treated, Dkk-1 transfected cells formed colonies compared to mock treated, Dkk-1 transfected cells (Fig. 3F). The difference in colony formation between control and Dkk-1 transfected cells was statistically significant ($P < 0.002$).

Structurally distinct HDACis mimic the effect of NaB on CRC cells

We have previously shown that trichostatin A (TSA) mimics the effects of NaB on Wnt activity in CRC cells [7,8]. To determine whether the ability to increase Wnt transcriptional activity in CRC cells is a general characteristic of HDACis, we measured the effects of other HDACi, i.e., TSA and SAHA (both of which are hydroxamic acids) and MS275 (a benzamide derivative), that are structurally different from NaB. The concentrations of these agents were selected based upon the optimal induction of the TOP/FOP ratio in HCT-116 cells (data not shown). We initially established that TSA ($1 \mu\text{M}$), SAHA ($10 \mu\text{M}$), and MS275 ($10 \mu\text{M}$) induced apoptosis in HCT-116 cells of a magnitude comparable to that produced by 5 mM NaB (Fig. 4A), a level of butyrate normally found in the colonic lumen [30,51]. In our studies, the fold increase in apoptosis is the ratio of the percentage of apoptosis in treated samples to that of mock treated samples; the percentage of apoptosis represents the ratio of the number of apoptotic cells to that of all analyzed cells, multiplied by 100. At the indicated concentrations, TSA, SAHA, and MS275 induced the TOP/FOP ratio approximately 20-fold (Fig. 4B) and increased the levels of active beta-catenin as measured by Western blot analyses (Fig. 4C). Furthermore, the exogenous expression of Dkk-1, an inhibitor of Wnt activity at the ligand level, suppressed the induction of Wnt transcriptional activity by each HDACi (Fig. 4D).

Mechanism(s) by which resistant malignant cells evade the apoptotic effects of HDACis

NaB resistant HCT-R cells were derived from HCT-116 cells by continuous exposure to increasing concentrations of NaB. These cells grow in the presence of 5 mM NaB, a concentration that results in high levels of apoptosis in wild-type parental cells [29]. HCT-R cells exhibit a markedly lower induction of Wnt transcriptional activity compared to parental HCT-116 cells in the presence of NaB and other HDACis (Table I). Furthermore, HCT-R cells were relatively resistant to the apoptotic effects produced by all of the HDACis compared to parental cells (Fig. 5A). Thus, HCT-R cells exhibited 1.3-, 1.4-, 1.7-, and 3.3-fold increases in the number of apoptotic cells; whereas, the wild-type parental HCT-116 cells exhibited 4.3-, 5.6, 8.5-, and 11.1-fold increases in apoptotic cells when exposed to $10 \mu\text{M}$ of MS275, 5 mM of NaB, $10 \mu\text{M}$ of SAHA, or $1 \mu\text{M}$ of TSA, respectively, for 24 hrs.

Since HCT-R cells developed by exposure to NaB were cross-resistant to the apoptotic effects of other HDACis, we ascertained whether HDACis were able to induce net histone acetylation in these cells. NaB and TSA increased acetylation of histones H3 and H4 in HCT-R cells, albeit the level of histone acetylation was lower in HCT-R cells than in HCT-116 cells after 24 hr of exposure to TSA (Fig. 5B). However, after 7 hr of treatment with TSA, the acetylation status of histones H3 and H4 did not differ significantly between HCT-R and HCT-116 cells (Fig. 5B).

The steady-state levels of the major Wnt signaling components were also analyzed in HCT-R cells. Western blot analyses demonstrated that treatment with NaB did not upregulate the levels of Ser-37/Thr-41-dephosphorylated beta-catenin in HCT-R cells to the same extent as in parental HCT-116 cells (Fig. 5C). Quantitative analyses of Western Blots data show an increase in the levels of active beta-catenin of 1.1-fold in HCT-R cells in presence of NaB; whereas, in parental HCT-116 cells exposure to NaB results in a 2.2-fold increase of dephosphorylated beta-catenin. The expression of Tcf proteins Tcf4 and Tcf1, were also determined, in particular the possible expression of their repressive, dominant negative forms [34, 52] was addressed. RNase protection analyses of *Tcf1* and *Tcf4* mRNAs were performed with probes designed to distinguish between full-length and dominant negative forms of the Tcf proteins. No mRNAs encoding dominant negative forms of the Tcf proteins were detected in HCT-R and HCT-116 cells, and no differences in the steady-state levels of the *Tcf4* mRNA were observed between these cell lines (Fig. 6A). However, HCT-R cells exhibited an increased expression of *Tcf1* mRNA compared to HCT-116 cells both in the presence and absence of NaB (Fig. 6A). At the protein level, Tcf4 levels were lower in HCT-R cells; whereas, Tcf1 protein levels in HCT-R cells corresponded to the high levels of *Tcf1* message (Fig. 6B). To test the possibility that excess Tcf1 protein in HCT-R cells repressed Wnt activity in the presence of HDACis, HCT-116 cells were cotransfected with TOP/FOP reporter vectors and the expression vectors for Tcf1 or Tcf4. Cells transfected with Tcf1 or Tcf4 exhibited suppressed induction of Wnt activity in the presence of NaB (Fig. 7A); whereas control (empty vector) transfected cells exhibited a 13.2-fold induction of Wnt activity after exposure to NaB. Transfection with Tcf1 reduced this level of induction to 2.3-fold ($P < 0.002$) and transfection with Tcf4 reduced the level of induction of Wnt activity to 2.1-fold ($P < 0.002$). The expression of Tcf1, but not that of Tcf4, suppressed the fold induction of apoptosis by 40% in HCT-116 cells treated with NaB (Fig. 7B). To further analyze the role of Tcf1 in the HDACi-resistant cell phenotype, the effects of a decrease in Tcf1 levels were examined. Transient transfection of *Tcf1* siRNA decreased endogenous Tcf1 levels in HCT-116 cells and resulted in a 33% suppression in the fold-induction of Wnt activity and a 38% suppression in the fold-induction of apoptosis in the presence of NaB (Fig. 7C,D). The effect of reduced Tcf1 levels in HCT-R cells were not assessed due to the ineffective suppression of the high endogenous *Tcf1* levels by siRNA. Since we established that in the presence of 5 mM NaB, HCT-R cells exhibit lower levels of dephosphorylated beta-catenin than the parental HCT-116 cells (Fig. 5), we hypothesized that in the HDACi-resistant cells the excess Tcf1 not bound to beta-catenin has a repressive role on Wnt activity. To test this hypothesis, we increased the levels of dephosphorylated beta-catenin through inactivation of GSK-3 beta by lithium chloride treatment [35]. Cotreatment of HCT-R cells with LiCl and NaB resulted in a detectable upregulation of Ser-37/Thr-41 dephosphorylated beta-catenin (Fig. 8A), an increase in Wnt transcriptional activity (Fig. 8B), and higher levels of apoptosis (Fig. 8C).

Discussion

Different levels of Wnt activity have been proposed to result in different cell fates. Thus, based upon analyses of *APC* mutations in patients with familial adenomatous polyposis, Wong et al. [28] proposed that cells with high levels of Wnt activity undergo apoptosis; whereas, cells with moderate levels of Wnt activity maintain a proliferative state and cells with low levels of Wnt activity undergo differentiation. Numerous other reports also support a relationship between high levels of Wnt activity and apoptosis [24-28,53-55]. Consistent with these reports are our observations of a causative relationship between the levels of Wnt activity and the degree of apoptosis in ten CRC cell lines treated with NaB [29]. We reported that expression of a dominant negative form of Tcf4 (dnTcf4) in HCT-116 and DLD-1 CRC cells, which respond to butyrate with high induction of Wnt activity and apoptosis, suppresses both the increase in Wnt activity and apoptosis [29]. The ability of

Wnt signaling to direct cells to various outcomes may be explained by the activation of distinct sets of genes due to: (a) various levels of Wnt signaling [42,56], (b) the participation of different Lef/Tcf factors in the BCT complexes [57], and/or (c) the activation of the Wnt pathway at the cell surface or at subsequent intracellular steps [43].

We demonstrate that a mechanism by which HDACis upregulate Wnt activity in CRC cells occurs by increasing the levels of transcriptionally active beta-catenin. Beta-catenin is stabilized by its N-terminal dephosphorylation. Four N-terminal amino acid residues of beta-catenin have been implicated as targets for phosphorylation: Ser-33, Ser-37, Thr-41, and Ser-45 (<http://www.stanford.edu/~rnusse/arm/bcatmut.html>). Of these four, the monoclonal antibody used in our studies recognizes non-phosphorylated Ser-37 and Thr-41 [35,36]. This antibody was developed by Clevers and his colleagues and has been proven to be capable of visualizing the generation of active beta-catenin triggered by the canonical Wnt pathway *in vitro* and *in vivo* [35,36]. Our analyses of the phosphorylation status of Ser-37 and Thr-41 (Fig. 1 and Fig. 4) reveals that HDACi treatment contributes to the dephosphorylation of these residues in eight out of ten human CRC cell lines. In addition, we have established that the induction of canonical Wnt activity [29] is concomitant with and dependent upon the upregulation of this form of active beta-catenin. Thus, induction of Wnt transcriptional activity by HDACis is suppressed when the upregulation of Ser-37/Thr-41-dephosphorylated beta-catenin is blocked by cotreatment of CRC cells with NaB and OA (Fig. 1C,D). In addition, expression of small T antigen, a genetic inhibitor of protein phosphatase 2A [44] also inhibits the upregulation of Wnt activity by NaB (Fig. 1F). The ability of Ser-37/Thr-41-dephosphorylated beta-catenin to hyperinduce Wnt activity in CRC cells that already express deregulated Wnt signaling due to *APC* and/or *beta-catenin* mutations is not surprising. The *APC* mutations in cells such as SW480, SW620, DLD-1, HT-29, and LoVo, are most likely not fully penetrant, since selection against *APC* mutations that hyper-activate canonical Wnt signaling has been demonstrated [55]. In contrast, CRC cells with mutations in *beta-catenin* (i.e., HCT-116, SW48, COLO201, and LS174T) have one wild *beta-catenin* allele, the product of which should be sensitive to stabilization via dephosphorylation of Ser-37 and Thr-41. In addition, the mutated *beta-catenin* allele in these four CRC cell lines carries changes in codons other than those encoding Ser-37 and Thr-41; therefore, dephosphorylation at these two residues may further stabilize beta-catenin.

The relevance of increased levels of nuclear active beta-catenin was confirmed by immunoprecipitation demonstrating that the levels of Tcf4-associated beta-catenin were also increased by NaB in HCT-116 cells (Fig. 2B), even though the steady-state levels of Tcf4 were decreased in these cells (Fig. 2C). Gel-shift analyses have shown that HCT-116 cells are characterized by relatively low levels of Tcf-beta-catenin complexes, resulting from a small pool of free beta-catenin and an excess of Tcf [58]. Therefore, active beta-catenin might well be the limiting factor for efficient complex formation in HCT-116 cells, a finding which explains the marked upregulation of Wnt activity in these cells when active beta-catenin is induced by HDACis. In contrast, enhanced levels of Tcf4-beta-catenin complexes were not observed in SW620 cells treated with NaB; however, OA treatment decreased the induction of Ser-37/Thr-41-dephosphorylated beta-catenin and Wnt activity in NaB treated SW620 cells (Fig. 1C,D). Unlike HCT-116 cells, SW620 cells are characterized by relatively high levels of BCT complexes [58]; thus, the increase in active beta-catenin in HDACi treated SW620 cells likely results in small changes in BCT complex formation that are difficult to detect by immunoprecipitation (Fig. 2A). The results from immunoprecipitation analyses also correspond to our observation of a greater induction of Wnt transcriptional activity following exposure to NaB in HCT-116 cells (20-fold) compared to SW620 cells (6-fold) [29]. The fact that there is no linear correlation between the induction of the levels of active beta-catenin and of Wnt activity in CRC cells exposed to

HDACis suggests that additional mechanisms account for the increase of canonical Wnt activity. For example, we have observed that exposure of CRC cells to NaB enhances binding between BCT complexes and target DNA sequences [8 and unpublished data].

The increased percentage of Wnt positive cells in NaB treated CRC cell populations ([29] and Fig. 1E) indicates that exposure to NaB induces Wnt activity in cells with undetectable Wnt signaling. OA inhibited the upregulation of active beta-catenin, as well as the increase in the number of Wnt positive cells following exposure to NaB (Fig. 1E). These findings led us to hypothesize that NaB induces and/or amplifies Wnt signaling at the ligand level and that this signal functions through autocrine and paracrine modes of action. Autocrine Wnt signaling in HCT-116 and SW480 CRC cells have also been reported by others [59, 60]. Our results, however, differed from the reported inhibitory effects of sFRPs on Wnt signaling in HCT-116 cells [60]; the differences may be due to the luciferase reporter systems used to measure Wnt activity. We have found that the Wnt antagonists Dkk-1 and sFRP2 do not have effects on the TOP/FOP ratio in mock treated HCT-116 and SW48 cells (these cells have *beta-catenin* mutations in Ser-45 and Ser-33, respectively); however, these Wnt antagonists inhibited the induction of Wnt transcriptional activity by NaB (Fig. 3). The *APC* mutant SW620 cells were sensitive to the inhibitory effects of sFRP2 (Fig. 3C) and other sFRPs (data not shown), both in the absence and presence of NaB, presumably because these cells have two wild-type *beta-catenin* alleles and an *APC* mutation that is not fully penetrant. The different sensitivities of the CRC cell lines to the inhibitors of Wnt activity may be due to either the expression of different Wnt ligands and receptors [48, 59-62] or to cell-specific differences in the expression and/or processing of the transfected Wnt antagonists.

The induction/augmentation of Wnt signaling at the plasma membrane by HDACis (Fig. 4) may be due to: (a) increased expression of Wnt ligands and/or their receptors; (b) decreased expression of Wnt signaling inhibitors that function at the ligand level; (c) modifications of Wnt ligands and/or their receptors; and/or (d) increased cellular secretion of Wnt ligands or increased cell surface presentation of their receptors. The first two of these possibilities should be detectable by microarray analyses that compare the expression profiles of mock and HDACi treated cells, such as HCT-116, LS174T, or COLO201, which exhibit high induction of Wnt activity and apoptosis after exposure to HDACis. However, modification of Wnt ligands and receptors, increased secretion of Wnt ligands, and/or receptor presentation on the cell surface may also result in upregulated Wnt activity at the ligand level. Future work will elucidate which of these events contributes to the increased dephosphorylation at Ser-37 and Thr-41 of beta-catenin in HDACi treated CRC cells.

The finding that cells resistant to the Wnt-modulating and apoptotic effects of HDACis (i.e., HCT-R cells) still exhibit enhanced histone acetylation following exposure to HDACis suggests that non-histone targets are involved in the action of the HDACis. Likely non-histone targets for the activity of HDACis are proteins that interact with the Wnt signaling pathway, including those involved in chromatin remodeling. One such target may be the chromatin remodeling factor Brg1, whose dominant negative form markedly inhibits the ability of NaB to upregulate Wnt activity in HCT-116 cells (unpublished data). The increased endogenous Tcf1 expression in HCT-R cells most likely contributes to their HDACi resistant phenotype, since exogenous overexpression of Tcf1 and Tcf4 in HCT-116 CRC cells decreased the induction of Wnt activity by NaB (Fig. 7A) and the overexpression of Tcf1, but not of Tcf4, inhibited NaB induced apoptosis (Fig. 7B). Interestingly, Tcf1, a transcriptional target for Tcf4-beta-catenin complexes, has been proposed to have a tumor-suppressor role in the intestine [23,63]; however, Tcf1 expression is upregulated in adenomas compared to normal proliferating cells in the crypt [64]. This duality of Tcf1 function *in vivo* could be explained by our findings. Thus, when Tcf1 is expressed at low

levels and is coupled to active beta-catenin to form BCT complexes, the downregulation of Tcf1 results in suppressed upregulation of Wnt transcriptional activity and apoptosis (Fig. 7C,D). However, the overexpression of Tcf1 also suppresses the upregulation of Wnt activity in HCT-116 cells (Fig. 7A), presumably because the excess of Tcf1 that is not complexed with beta-catenin acts as a transcriptional repressor. Thus, HCT-R cells with relatively high levels of Tcf1 (Fig. 6B) and low levels of active beta-catenin (Figs. 5C and 8A) exhibit resistance to the Wnt-modulating and apoptotic effects of HDACis. However, the increase in active beta-catenin levels produced by cotreatment of these cells with LiCl and NaB increased both Wnt transcriptional activity and cellular apoptosis, most likely by providing sufficient beta-catenin to complex with Tcf1 (Fig. 8). The contribution of increased active beta-catenin to the sensitivity of HCT-116 cells to the apoptotic effects of NaB has been demonstrated by clonal growth assays. Thus, Dkk-1 expressing HCT-116 cells, in which the induction of Ser-37/Thr-41-dephosphorylated beta-catenin and Wnt activity by NaB is suppressed (Fig. 3), are less vulnerable to the effects of NaB compared to mock transfected HCT-116 cells (Fig. 3F).

The findings reported herein and our observation of a causative relationship between Wnt signaling and apoptosis [29] suggest that malignancies in which HDACis hyperactivate Wnt activity will respond to HDACi treatment by programmed cell death; whereas, malignancies with relatively high levels of Tcf species and low levels of active beta-catenin will respond to the same treatment by reversible growth arrest. In this second category of malignancies, the downregulation of Wnt signaling may be a more appropriate choice of treatment, since suppression of Wnt activity also leads to apoptosis in some cell types and can be achieved by a variety of methodologies, both genetic and pharmacological [65-68]. Thus, we propose a bidirectional modulation of Wnt activity for therapeutic purposes. This approach applies not only to human colorectal cancer, but to any form of human cancer in which Wnt signaling plays an important role; e.g., cancer of the prostate [69-72]. For example, we have observed that treatment with NaB upregulates Wnt activity (by ~ 5-fold) in LNCap prostate cancer cells and cotreatment with NaB and LiCl leads to a greater induction (~ 20-fold) of Wnt-specific transcription (unpublished data). Therefore, in summary, identification of the relative levels of Wnt signaling components in human tumor tissue may therefore assist in determining whether upregulation or downregulation of Wnt activity is the more efficient therapeutic strategy for use with HDACis.

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Fig. 1. NaB induces Wnt transcriptional activity in human CRC cells in part through the upregulation of Ser-37/Thr-41-dephosphorylated beta-catenin levels

(A) Representative Western blot analyses of total lysates from CRC cells that were mock treated or treated with 5 mM NaB for 16 to 24 hr, except for COLO201 cells which were treated for 7 hr. (B) Representative Western blot analyses of nuclear lysates from CRC cells that were mock treated or treated with 5 mM NaB for 24 hr. (C) Representative Western blot analyses of nuclear lysates from CRC cells that were mock treated or treated with 20 nM OA, 5 mM NaB, or cotreated with NaB and OA for 24 hr. Beta-catenin dephosphorylated at residues Ser-37 and Thr-41 was detected with anti-ABC antibody (Upstate Biotechnology) and actin was detected with anti-beta-actin antibody (Sigma). Equal loading for Western blot

analyses of nuclear proteins was confirmed by Ponceau staining. (D) HCT-116 and SW620 CRC cells were transfected with 0.4 μ g/well of TOP or FOP reporter vectors and were treated with 20 nM OA, 5 mM NaB, or the sequential combination of NaB and OA for 24 hr. Luciferase values were normalized utilizing the activity from cotransfected pRLTK. (E) HCT-116 and SW620 cells were transfected with EGFP-TOP and EGFP-FOP vectors, as described in Materials and Methods, and treated as described in (D). Cells were analyzed by flow cytometry; plots of relative cell number versus fluorescence for EGFP-TOP and EGFP-FOP transfected cells were generated and overlaid to estimate the percentage of cells with Wnt activity, as described previously [29]. (F) Cells were cotransfected with TOP or FOP reporters and the mutant or wild-type small T antigen expression vector and treated with 5 mM NaB or mock treated for 24 hr. The fold upregulation by NaB was calculated as a ratio of the TOP/FOP activity in the presence of NaB to that in the absence of NaB. Data for all transfections are from three independent experiments. *Bars*, SDs.

Fig. 2. Levels of Tcf associated beta-catenin in mock and NaB treated CRC cells
 (A,B) Representative Western blot analyses of beta-catenin co-immunoprecipitated with Tcf4 antibody as described in Materials and Methods. Equal amounts of immunoprecipitated samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunostained with antibody to beta-catenin (Transduction Laboratories). (C) Representative Western blot analyses of the steady-state levels of Ser-37/Thr-41-dephosphorylated beta-catenin and Tcf4 in the nuclear lysates used for the immunoprecipitation experiments. Equal loading was confirmed by staining the unused portion of the membranes with Ponceau's solution.

Fig 3. Wnt antagonists inhibit the upregulation of Wnt transcriptional activity, decrease the levels of Ser-37/Thr-41-dephosphorylated beta-catenin, and sustain the clonogenic growth of human CRC cells treated with NaB

CRC cells were cotransfected with 0.1 μ g/well of TOP or FOP reporter vectors and 1 μ g/well of Dkk1 expression vector or empty pCINeo vector control in (A) or with 0.4 μ g/well of TOP or FOP reporter vectors and 0.4 μ g/well of sFRP2, dnLRP5, or empty vector, pCMV-HA or pcDNA Neo in (B and C). Twenty-four hr post-transfection, cells were treated with 5 mM NaB or were mock treated and harvested 24 hr later. Luciferase values were normalized utilizing the activity from cotransfected pRLTK. Data are from three independent experiments. (D, E) Representative Western blot analyses of Ser-37/Thr-41-

dephosphorylated beta-catenin levels in HCT-116 cells cultured for 17 hr in conditioned medium from pcINeo- or Dkk1-transfected 293 cells in the presence or absence of NaB (D) and Ser-37/Thr-41-dephosphorylated beta-catenin levels in HCT-116 cells transiently transfected with empty vector or Dkk1 expression construct and treated with 5 mM NaB for 24 hr or left untreated (E). Total cell lysates (50 µg of protein) were analyzed as in Figure 1. (F) HCT-116 cells transiently transfected with empty vector control (CTRL) or Dkk-1 expression vector were mock treated or treated with 5 mM NaB for 16 hr and their ability to form colonies was measured as described in Materials and Methods. Triplicate samples were obtained for each transfection and each treatment. The results indicate the percentage of colonies formed by NaB treated cells relative to the number of colonies formed by mock treated cells. Values represent the mean of three independent transfection experiments $\pm$ SD. Bars, SDs.

Fig. 4. Structurally different HDACis induce Wnt signaling at the ligand level and increase the levels of Ser-37/Thr-41-dephosphorylated beta-catenin in HCT-116 cells
 (A) Apoptosis in HCT-116 cells treated for 24 hr with TSA (1 μ M), SAHA (10 μ M), MS275 (10 μ M), or 5 mM NaB was measured with the Vybrant Apoptosis Assay Kit #2 as described in Materials and Methods; controls were treated with the appropriate vehicle. Duplicate samples were measured and representative experimental results are shown. (B) HCT-116 cells were transiently cotransfected with 0.5 μ g/well of TOP or FOP reporter vectors and the pRL-TK luciferase vector and treated as in (A) for a total of 24 hr. Controls included cells treated with vehicle (dimethyl sulfoxide or methanol). (C) HCT-116 cells were treated as in 4A for 24 hr, harvested for total lysates and analyzed by Western blotting with antibodies to the dephosphorylated form of beta-catenin and actin (see Fig. 1). C_M is the methanol vehicle control; C_D represents the dimethyl sulfoxide vehicle control. (D) CRC cells were cotransfected with 0.1 μ g/well of TOP or FOP reporter vectors and 1 μ g/well of Dkk1 expression vector or empty pCINeo vector. Twenty-four hr post-transfection, cells were treated with various HDACi or vehicles as in 4A and harvested 24 hr later. Luciferase values were normalized utilizing the activity from cotransfected pRLTK. All experiments were repeated a minimum of three times. *Bars*, SDs.

Fig. 5. Characterization of the HDACi-resistant phenotype of HCT-R cells

(A) Suppressed apoptosis in HDACi treated HCT-R cells. Apoptotic assays were carried out with BD Pharmingen Annexin V-PE apoptosis detection kit (#559763). HCT-116 cells were treated in 24-well dishes with 1 μ M TSA, 10 μ M SAHA, 10 μ M MS275, 5 mM NaB, or corresponding vehicle, for 24 hr. The percentage of apoptotic cells was determined by flow cytometry. The fold increase in apoptosis represents the fold increase in percentage of apoptotic cells in the treated samples versus vehicle treated control samples. Two experiments with duplicate samples were performed for each HDACi and representative results are shown. (B) Representative Western blot analyses of acetylated histones H3 and H4 in the absence and presence of 5 mM NaB or 1 μ M TSA at 24 and 7 hr of treatment. Six μ g of protein were loaded per sample and equal loading was ascertained by staining the upper portion of each Western blot membrane with Ponceau's solution. (C) Representative Western blot analyses of active beta-catenin levels in total lysates of HCT-R cells treated with various HDACis as in (A). Bars, SDs.

Fig. 6. Expression of Tcf proteins in HCT-116 and HCT-R cells

(A) Representative RNase protection assay performed as described in Materials and Methods for both Tcf1 and Tcf4 cellular transcripts. HCT-116 and HCT-R cells were mock treated (C represents control) or were treated with 5 mM NaB for 24 hr. (B) Representative Western blot analyses of Tcf1 and Tcf4 level, performed on nuclear lysates from HCT-116 and HCT-R cells that were mock treated (C represents control) or treated with 5 mM NaB for 24 hr.

Fig. 7. Tcf1 and Tcf4 expression represses the ability of NaB to induce Wnt activity in CRC cells
 (A) HCT-116 cells were cotransfected with 0.2 μ g of TOP or FOP reporter, 1.0 μ g of Tcf1 or Tcf4 expression vector, and control (CTRL) plasmid pRL-TK per well, and cells were treated as described in Fig. 1. Inset in (A) shows Tcf1 and Tcf4 expression levels in nuclear lysates of transiently transfected cells. Equal loading was confirmed by Ponceau staining.
 (B) HCT-116 cells were cotransfected with 0.2 μ g of green fluorescent protein (GFP) expression vector and 1.0 μ g of Tcf1 or Tcf4 expression vector or control pcDNA-Neo vector. GFP-positive cells from controls and cells treated with 5 mM NaB were analyzed for levels of apoptosis by flow cytometry (Annexin V-PE apoptosis detection kit I, BD Pharmingen).
 (C) HCT-116 cells were co-transfected with 0.2 μ g of TOP or FOP reporter, pRLTK at a 1:50 ratio, and 100 nmol of non-specific siRNA or Tcf1-specific siRNA per well. TOP/FOP activity was assayed as described above. Inset in (C) is a representative Western blot analysis of the Tcf1 levels in HCT-116 cells transfected with 100 nmol of non-

specific control siRNA or Tcf1 siRNA. No changes in Tcf4 and Ran (nuclear antigen) were observed in all treatments. (D) Apoptotic analyses of HCT-116 cells transfected with 100 nmol of non-specific control siRNA or Tcf1 siRNA and treated with NaB for 24 hr. Flow cytometric analyses were performed as in (B). *Bars*, SDs.

Fig. 8. LiCl treatment counteracts the NaB-resistant phenotype of HCT-R cells

(A) Representative Western blot analyses of the levels of Ser-37/Thr-41-dephosphorylated beta-catenin in total cell lysates of HCT-116 and HCT-R cells treated with 5 mM NaB, 20 mM LiCl or a combination of both reagents. (B) TOP/FOP ratio in HCT-R and HCT-116 cells transiently transfected with TOP or FOP as in Fig. 1D and treated as in (A). (C) Percentage of apoptotic cells in HCT-R cells treated for 24 hr as in Fig. 5; apoptotic assays were carried out as in Fig. 5. Bars, SDs.

Table 1
The comparative induction of Wnt activation produced by HDACi inhibitors in HCT-R and parental HCT-116 cells

CELLS	TOP/FOP Ratio ^a , 24 hr treatment					
	CTRL+D ^b	NaB	TSA	CTRL+M ^c	MS275	SAHA
HCT-R	2.1±0.9	7.0±1.2 (3 ×)	13.9±6.1 (7 ×)	5.6±1.5	13.6±2.5 (2.4 ×)	24.8±1.7 (4.4 ×)
HCT-116	3.2±0.8	114.1±20.3 (36 ×)	128.0±11.9 (40 ×)	6.4±1.5	105.7±9.2 (16 ×)	176.6±16.8 (28 ×)

^a TOP/FOP ratio following exposure for 24 hr to HDACis (5 mM NaB, 1 μ M TSA, 10 μ M MS275, 10 μ M SAHA).

^b DMSO vehicle control.

^c Methanol vehicle control.