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DACT3 Is an Epigenetic Regulator of Wnt/β-Catenin Signaling in Colorectal Cancer and Is a Therapeutic Target of Histone Modifications

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SUMMARY

Genetic and epigenetic defects in Wnt/β-catenin signaling play important roles in colorectal cancer progression. Here we identify *DACT3*, a member of the DACT (Dpr/Frodo) gene family, as a negative regulator of Wnt/β-catenin signaling that is transcriptionally repressed in colorectal cancer. Unlike other Wnt signaling inhibitors that are silenced by DNA methylation, *DACT3* repression is associated with bivalent histone modifications. Remarkably, *DACT3* expression can be robustly derepressed by a pharmacological combination that simultaneously targets both histone methylation and deacetylation, leading to strong inhibition of Dishevelled (Dvl)-mediated Wnt/β-catenin signaling and massive apoptosis of colorectal cancer cells. Our study identifies *DACT3* as an important regulator of Wnt/β-catenin signaling in colorectal cancer and suggests a potential strategy for therapeutic control of Wnt/β-catenin signaling in colorectal cancer.

INTRODUCTION

Aberrant activation of Wnt/β-catenin signaling is a major driving force in colon cancer (Kinzler and Vogelstein, 1996; Su et al., 1992; van de Wetering et al., 2002). Mutations in Wnt/β-catenin pathway components including APC, Axin, and β-catenin itself are well-established causes of aberrant signaling activation leading to cancer (Lammi et al., 2004; Liu et al., 2000; Morin et al., 1997; Su et al., 1992). These genetic defects share in common that they result in the accumulation of β-catenin in the nucleus. Nuclear β-catenin interacts with members of the TCF/LEF transcription cofactor family to activate downstream target genes such as Cyclin D1 and Myc that can lead to cell transformation (He et al., 1998; Morin et al., 1997; Tetsu and McCormick, 1999; van de Wetering et al., 2002). The fact that blockade of Wnt/β-catenin signaling in colon cancer cells induces apoptosis or growth inhibition both in vitro and in vivo (Fujii et al., 2007; He et al., 2005; Kwong et al., 2002) has propelled intensive efforts to develop therapeutic strategies that target this pathway (Barker and Clevers, 2006; Lepourcelet et al., 2004; Li et al., 2002).

In addition to genetic mutations in Wnt/β-catenin pathway components, epigenetic events can also contribute to abnormal activation of this signaling pathway in cancer cells. For example, promoter methylation leading to transcriptional silencing of extracellular Wnt inhibitors, such as Secreted Frizzled-Related Proteins (SFRPs), Wnt Inhibitory Factor-1 (WIF-1), and DICKKOPF-1 (DKK-1), have been reported in human colorectal cancer cells (Aguilera et al., 2006; He et al., 2005; Morin et al., 1997; Suzuki

SIGNIFICANCE

Wnt/β-catenin signaling is one of the most frequently deregulated pathways in colorectal cancer and is thus an important therapeutic target. Our study identifies *DACT3* as a negative regulator of Wnt/β-catenin signaling that is epigenetically repressed in colorectal cancer. Repression of *DACT3* in colon cancer does not require DNA methylation but instead involves bivalent histone modifications. Of potential clinical importance, we identify a pharmacological approach that interferes with this epigenetic process and robustly induces *DACT3* expression, leading to inhibition of Wnt/β-catenin signaling and dramatic apoptosis of colon cancer cells. This work identifies an epigenetic component of Wnt/β-catenin regulation in colorectal cancer and thereby opens an important avenue for pharmacological perturbation of this important cancer pathway.

et al., 2002). Conversely, restoration of Wnt inhibitor expression such as SFRP1/2 results in inhibition of Wnt/β-catenin signaling and apoptosis of colorectal cancer cells even in the presence of downstream APC or β-catenin mutations (Baylin and Ohm, 2006; Suzuki et al., 2004). These findings suggest that epigenetic silencing of upstream Wnt inhibitor genes contributes to transformation through the amplification of aberrant signaling that may be initiated by genetic mutations (Baylin and Ohm, 2006; Suzuki et al., 2004). Consequently, epigenetic regulation of the Wnt/β-catenin pathway has emerged as a potential therapeutic target in human cancer, though previously published efforts in this area have mainly focused on direct interference with TCF/β-catenin-mediated transcriptional activation in cancer cells (Barker and Clevers, 2006; Lepourcelet et al., 2004).

The identification of epigenetic regulators of Wnt/β-catenin signaling may pave the way to developing innovative therapeutic strategies. Members of the DACT (Dpr/Frodo) gene family have been shown to modulate Wnt/β-catenin signaling by interacting with Dishevelled (Dvl) (Cheyette et al., 2002), a central component of Wnt signaling (Bilic et al., 2007; Logan and Nusse, 2004). DACT1 and DACT2 antagonize Wnt signaling in some biological contexts, activate it in others, and may also play roles in TGF-β/Nodal signaling (Gloy et al., 2002; Hikasa and Sokol, 2004; Su et al., 2007; Zhang et al., 2004, 2006). A third DACT family member, DACT3, has been described in both mouse and human (Fisher et al., 2006). However, the signaling function of DACT3 and its relevance to oncogenesis are unclear.

RESULTS

DACT3 Expression Is Repressed in Colorectal Cancer Independently of Promoter Methylation

To characterize epigenetic effectors of Wnt/β-catenin signaling in colorectal cancer, we initially focused on 14 representative Wnt signaling inhibitors, including members of the *SFRP*, *WIF1*, *DKK*, and *DACT* gene families, some of which have previously been shown to be transcriptionally inactivated or repressed in various human cancers (Aguilera et al., 2006; He et al., 2005; Suzuki et al., 2002, 2004). We determined gene expression in 24 human colorectal tumors versus matched normal mucosa, using the Illumina Human Ref-8_V2 Sentrix BeadChip (Figure 1A and Table S1 available online). We found that expression of *SFRP* family members was significantly reduced in nearly all human colorectal tumor samples when compared to normal controls ($p < 0.001$), a finding consistent with a previous report (Suzuki et al., 2004). In contrast, expression of *WIF-1* and *DKKs* in tumor samples did not significantly differ from controls, despite previously reported *WIF-1* and *DKK1* silencing in established colorectal cancer cell lines (Aguilera et al., 2006; He et al., 2005). Interestingly, expression of *DACT3* was reduced in all 24 tumor samples ($p < 0.001$), whereas expression levels of *DACT1* and *DACT2* did not show significant differences between tumor and control tissues ($p = 0.24$ and $p = 0.64$, respectively). Consistent with reduced expression of Wnt inhibitors leading to enhanced baseline Wnt/β-catenin signal activation, these tumors exhibited increased expression of established β-catenin/TCF target genes, including *MYC* (He et al., 1998), *CCND1* (Tetsu and McCormick, 1999), *LEF1* (Filali et al., 2002), and *CD44* (Wielenga et al., 1999), compared to control tissues. RT-PCR analysis of eight pairs of randomly selected patient samples confirmed repression of *SFRP1* and *DACT3*, but not of *DACT1* in colorectal cancer compared to controls (Figure 1B). Notably, we also found reductions in *DACT2* expression in three out of eight tumor samples. Thus, beyond previously identified reductions in expression of Wnt inhibitors such as the *SFRP*s, we have found that the expression of *DACT3* is also consistently reduced in colorectal cancer. Additionally, we have found evidence that, in at least some colorectal tumors, *DACT2* expression is also reduced.

*SFRP*s have been shown to be transcriptionally inactivated in cancer cells through promoter methylation (Suzuki et al., 2004). To determine the methylation status of the *DACT3* promoter, we performed methylation-specific PCR (MSP) analysis in the eight colorectal tumor samples around the transcription start site as determined by 5′RACE (Figure S1). The *DACT3* promoter contains a CpG island (Figure 1C). MSP analysis covering the entire CpG island indicates a lack of DNA methylation at the *DACT3* promoter in colorectal tumor samples (Figure 1C). As a positive control, using the same technique we confirmed that the promoter region of *SFRP1* is methylated in these same tumor samples (Figure 1C). This finding suggests that, although *DACT3* expression is reduced in colorectal cancer, this occurs independently of the DNA methylation previously implicated in gene silencing in these cells.

To determine whether the above observations are recapitulated in colorectal cancer cell lines, we performed RT-PCR analysis of the *DACT* genes and of *SFRP1* in seven such lines (Figure 1D). Unlike *SFRP1*, which is consistently silenced in colon cancer, *DACT3* shows a basal level of expression that varies in different cell lines. Notably, expression of the other two DACT family members, *DACT1* and *DACT2*, was also lost in several of these cell lines, though this was not true in the primary tumor tissues examined above. We accordingly examined the methylation status of all three *DACT* genes in these colorectal cancer cell lines. As in the primary tumor samples, the *DACT3* promoter is unmethylated in all the cell lines tested, whereas *DACT1* and *DACT2* promoters were found to be partially and fully methylated, respectively (Figure 1E). Bisulfite genomic sequencing (BGS) in the RKO and HT29 cancer cell lines confirmed the results of this MSP analysis, showing partially and nearly completely methylated CpGs in the *DACT1* and *DACT2* promoters, respectively, but almost no methylated cytosines in the *DACT3* promoter (Figure 1F).

The DNMT inhibitor 5-aza-2′deoxycytidine (5-AzaC) was used to pharmacologically interfere with promoter methylation in the HCT116 colorectal cancer cell line. 5-AzaC treatment decreased methylation at the *DACT1* and *DACT2* promoters (Figure 1G), leading to the increased expression of *SFRP1*, *DACT1*, and *DACT2*, but not *DACT3* (Figure 1H). Similarly, a HCT116 cell line in which the DNA methyltransferase genes *DNMT1* and *DNMT3B* were genetically disrupted (Rhee et al., 2002) showed decreased promoter methylation (Figure 1G; DKO) and increased expression (Figure 1H) of *DACT1*, *DACT2*, and *SFRP1* but no obvious change in *DACT3* (Figures 1G and 1H). A similar result was also obtained in DLD1 cells (Figure 1H). These results support the conclusion that, unlike *SFRP1* and the *DACT1* and *DACT2* genes, promoter methylation does not contribute to the epigenetic repression of *DACT3* in colorectal cancer cells. Furthermore, we found that in patient tumor samples the *DACT1*

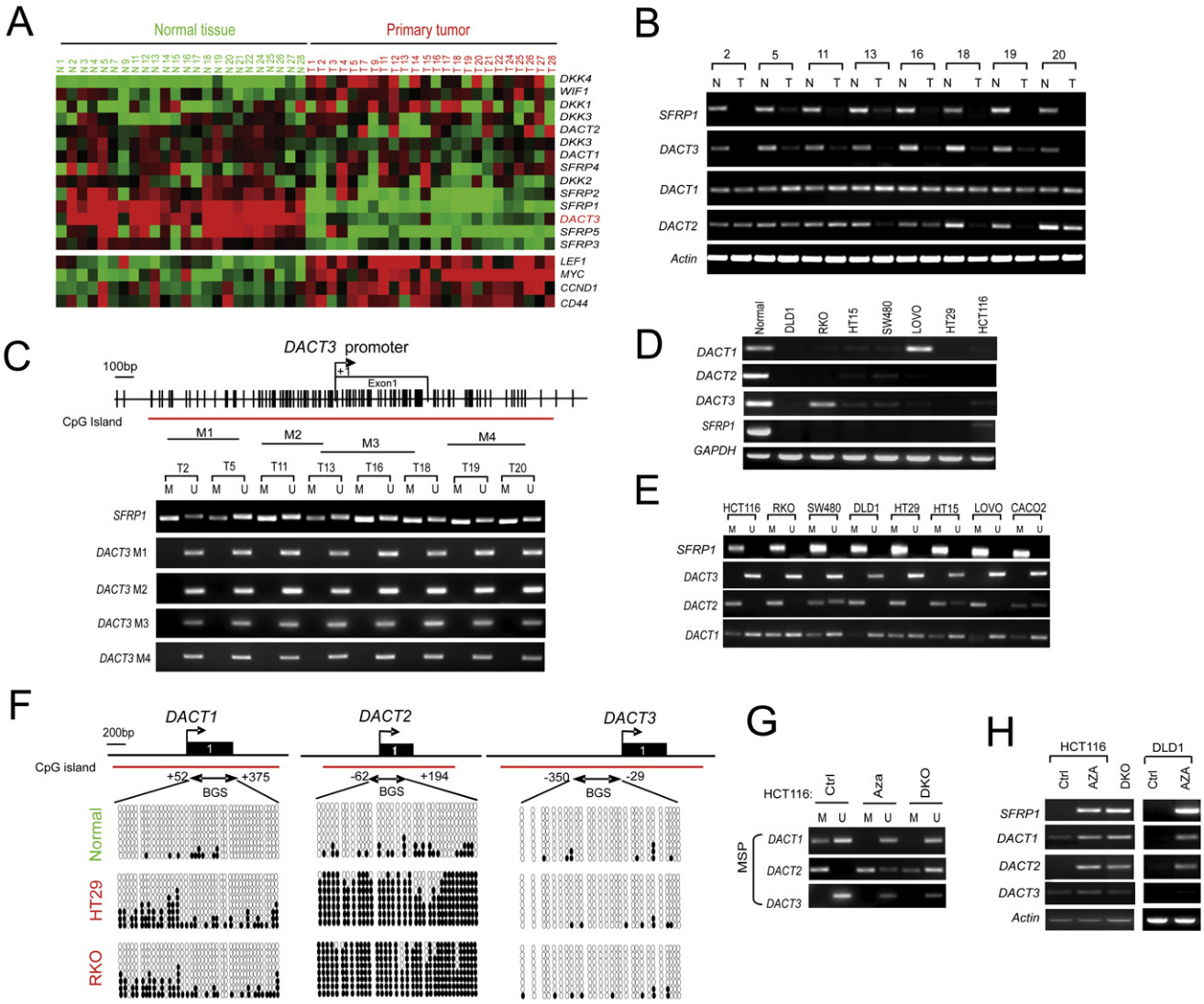

Figure 1. Loss of DACT3 Expression in Colon Cancer Independently of DNA Methylation

(A) Hierarchical clustering of Wnt inhibitors (upper) and Wnt/β-catenin target genes (lower) in human colorectal tumors (T) and matched normal mucosa (N).

(B) RT-PCR analysis of *SFRP1* and *DACT1*, *-2*, and *-3* from eight randomly selected pairs of human colorectal tumor and matched mucosa.

(C) Methylation status of *SFRP1* and *DACT3* in eight tumors determined by methylation-specific PCR (MSP) assay. M1, M2, M3, and M4 represent the examined PCR regions covering the entire CpG island of *DACT3* promoter.

(D) RT-PCR analysis of *SFRP1* and *DACT1*, *-2*, and *-3* in a panel of colorectal cancer cell lines compared to the normal tissue.

(E) Methylation status of *SFRP1* and *DACT1*, *-2*, and *-3* in colorectal cancer cell lines.

(F) Determination of methylation status of CpG sites by sequencing of bisulfite-modified DNA from normal and colorectal cancer cell lines. Arrows indicate the transcription start sites. Open cycles represent unmethylated CpGs; closed cycles denote methylated CpGs.

(G) MSP analysis of *DACT1*, *-2*, and *-3* promoters in HCT116 cells untreated or treated with 5-AzaC and in HCT116-*DNMT1*/*DNMT3b*−/− (DKO) cells.

(H) RT-PCR analysis of *SFRP1* and *DACT1*, *-2*, and *-3* in HCT116 and DLD1 cells treated with 5-AzaC (5 μM) for 3 days and in DKO cells.

promoter was not methylated, and *DACT2* promoter methylation is only detected in several tumor samples with reduced *DACT2* expression (data not shown). Thus, the lack of methylation of *DACT1/2* in the clinical tumor samples we examined may explain why they are not downregulated in these tumors in general.

Epigenetic Repression of *DACT3* Is Associated with Bivalent Histone Modifications

The above data suggest that epigenetic repression of *DACT3* in colon cancer might involve an alternative mechanism, such as histone modification. To determine if chromatin status is associated with *DACT3* repression, we used a chromatin immunoprecipitation (ChIP) assay coupled with quantitative PCR to characterize potentially involved chromatin marks. To this end, we designed a panel of eight to ten primer pairs covering a >7 kb region close to the transcription start site (TSS) of each of the three *DACT* genes and searched for the presence of histone marks, including the repressive marks H3K27me3, H3K9me3, H3K9me2, and H4K20me3, as well as the activating marks H3K4me3 and H3K9/14ac (Figure 2A). Abundant enrichment of the repressive

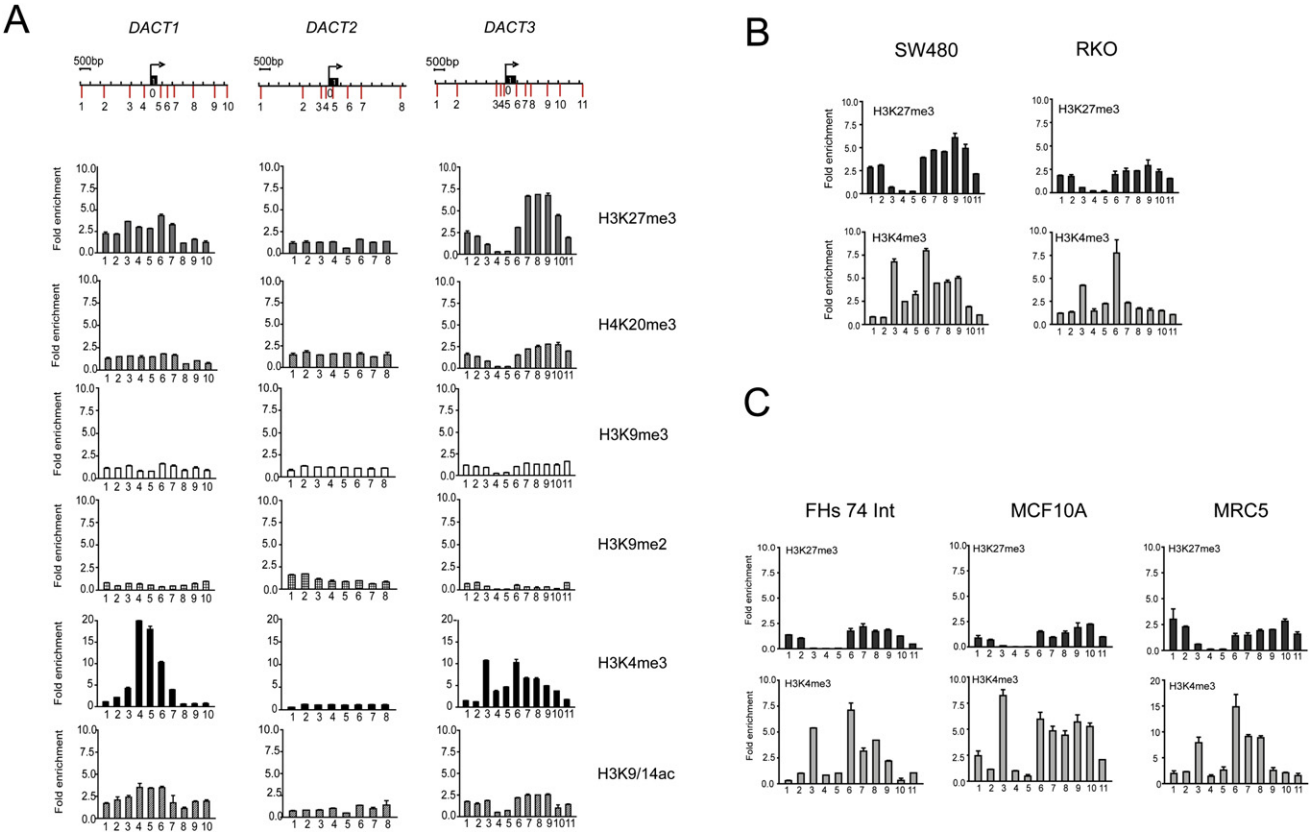

Figure 2. Histone Modifications at *DACT1*, *-2*, and *-3* in Colon Cancer Cells

ChIP assays were performed using antibodies against the indicated histone modifications and analyzed by quantitative PCR. Genomic DNA fragments covering the −3.5 kb to +3.5 kb region with respect to the transcription start site of *DACT1*, *-2*, and *-3* for PCR analysis were indicated with numbers. The relative enrichments (bound/input) (mean ± SD of triplicate measurement) encompassing the indicated regions are shown for each histone mark at each gene locus.

(A) Histone marks at the *DACT1–3* loci in HT-29 cells.

(B) Enrichments of H3K27me3 and H3K4me3 at the *DACT3* locus in SW480 and RKO cells.

(C) Enrichments of H3K27me3 and H3K4me3 at the *DACT3* locus in normal human intestinal epithelial cells (FHs 74 Int), noncancerous breast epithelial MCF10A cells, and lung fibroblast MRC5 cells.

H3K27me3, and to a lesser extent repressive H4K20me3, was detected 500 bp downstream of the TSS of *DACT3* in HT29 cells. In addition, a weaker H3K27me3 was also detected in the more upstream promoter region of *DACT3*. This finding is in agreement with several recent genome-wide studies showing that the majority of H3K27me3 is detected in the proximal downstream region of the TSS in both cancer and embryonic stem cells (Pan et al., 2007; Yu et al., 2007; Zhao et al., 2007). In contrast, no repressive H3K9 methylation marks were detected in the *DACT3* promoter region in HT29 cells.

The activating mark H3K4me3 was also detected at high levels near the *DACT3* TSS in HT29 cells, suggesting that the *DACT3* promoter is simultaneously modified by both repressive and activating (bivalent) histone methylation events in these cells. Such bivalent histone states have previously been correlated with genes transcribed at low levels (Azuara et al., 2006; Bernstein et al., 2006; Mikkelsen et al., 2007; Pan et al., 2007; Zhao et al., 2007). If this is also true for the *DACT3* locus, we would predict that the presence of repressive H3K27me3 should inversely correlate with *DACT3* expression levels. Consistent with this hypothesis, a high level of H3K27me3 was also detected at *DACT3* in SW480 cells that express low levels of *DACT3*; H3K27me3 was detected to a lesser extent in RKO cells that have modest *DACT3* expression (Figure 2B). Moreover, only a high level of only H3K4me3 (but not H3K27me3) was detected at *DACT3* in normal human intestinal epithelial cells (FHs 74 Int) and two other noncancerous cell lines, breast epithelial MCF10A and lung fibroblast MRC5 (Figure 2C), suggesting that this bivalent modification at *DACT3* is cancer specific. To further confirm that the *DACT3* promoter is in a bivalent histone state in cells where its expression is repressed, we have conducted whole-genome mapping of major histone marks in SW480 cells using ChIP-Seq Solexa technology. This high-resolution mapping of histone modifications clearly demonstrates the comodification of H3K27me3 and H3K4me3 at *DACT3* (Figure S2).

Enrichments of H3K4me3, and to a lesser degree H3K27me3, were also detected at *DACT1*, but no enrichment peaks of these histone marks, including H3K4me3, were detected at the *DACT2* locus (Figure 2A). In general for the DACT gene family, our results suggest that the level of H3K4me3 is inversely correlated with the

DNA methylation state: H3K4me3 is detected at the *DACT1* promoter (which is partially methylated), and at the *DACT3* promoter (which is not methylated), but not at the *DACT2* promoter (which is fully methylated) (compare Figures 1F and 2). This finding agrees with recent reports that DNMT preferentially binds to unmethylated H3K4 (Ooi et al., 2007) and that methylation of H3K4 tends to protect surrounding nucleotides from methylation (Weber et al., 2007). Along with the earlier data showing that expression of *DACT3* is insensitive to CpG island methylation, our data are consistent with the hypothesis that, in colon cancer cells, *DACT3* is repressed primarily through histone modifications, and specifically that the *DACT3* locus in such cells exists in a bivalent chromatin state simultaneously containing both repressive and activating histone modifications.

Robust Derepression of *DACT3* by a Pharmacological Approach Inhibiting Both Histone Methylation and Deacetylation

We have recently reported that the S-adenosylhomocysteine hydrolase inhibitor 3-Deazaneplanocin A (DZNep) depletes PRC2 components and inhibits histone methylation, including repressive H3K27me3 and H4K20me3 (Tan et al., 2007). We set out to test whether DZNep, alone or in combination with the histone deacetylase inhibitor Trichostatin A (TSA), the DNMT inhibitor 5-AzaC, or both, could restore *DACT3* expression in colorectal cancer cells. To most accurately measure changes in gene expression, we used the Illumina Beadarray, which allows for accurate measurement of transcript levels without intermediate nucleic acid amplification steps. We found that when used as single agents these drugs minimally induce expression of *DACT3* in DLD1 cells (Figure 3A). However, combined treatment with DZNep and TSA strongly induces *DACT3* expression, whereas other combinations such as DZNep/Aza or TSA/Aza fail to do so (Figure 3A). By contrast, DZNep/TSA combination only induced a modest increase in *DACT1* expression (Figure 3B). Expression of *DACT2*, on the other hand, was only induced by treatments containing 5-AzaC (Figures 1H and 3B), consistent with its epigenetic silencing by more typical promoter methylation.

To determine the specificity of DZNep/TSA combination treatment for *DACT3* derepression versus changes in expression of other known Wnt/β-catenin pathway inhibitors, we again analyzed Illumina gene expression data using RNA from two colon cancer cell lines (DLD1 and HT-29) that were untreated or treated with DZNep, TSA, or both. This analysis revealed that *DACT3* is the only Wnt/β-catenin pathway inhibitor strongly induced by DZNep/TSA treatment and that this occurred in both colon cancer cell lines (Figure 3B and Table S2). Together, our findings show that *DACT3* is distinguished from other Wnt pathway inhibitors in that its repression in colon cancer cells seems to be associated with the bivalent histone modifications but not DNA methylation, and accordingly it can be robustly derepressed by a pharmacologic approach that exclusively targets histone modifications.

In order to better understand the mechanism whereby the DZNep/TSA drug combination reactivates *DACT3* expression, we examined histone modification profiles in treated and untreated cells by western blot (Figure 3C). As previously reported, DZNep treatment alone results in strong reduction of H3K27me3 and H4K20me3, while having little effect on H3K9me3 (Tan et al., 2007). Although the combination treatment resulted in some mild inhibition of H3K9me3, the most notable synergistic change compared to treatment with DZNep or TSA alone was robust induction of H3K9/14 acetylation (Figure 3C). Interestingly, H3K4me3 is also induced by this combination treatment, irrespective of a slight decrease with DZNep treatment alone. Thus, the pharmacologic combination of DZNep with TSA causes an intriguing global shift in histone modifications: it reduces certain repressive histone marks (H3K27me3, H4K20me3, and H3K9me3) while dramatically increasing some activating histone marks (H3K9/14ac and H3K4me3). Mechanistically, this suggests the existence of crosstalk between these chromatin marks, such that inhibition of repressive histone methylation by DZNep creates a favorable chromatin environment for histone acetylation induced by TSA. The magnitude of the effects observed on general histone profiles in these treated cells further suggests that this phenomenon is widespread throughout the genome. This in turn leads to increased expression of specific target genes, prominently including *DACT3*.

We used ChIP to further assess changes in histone modifications specifically at the *DACT3* locus in response to DZNep/TSA combination treatment. Consistent with the global changes in modified histone levels found by western blot analysis, cells treated with DZNep/TSA had a decrease in H3K27me3 and H4K20me3, and a concomitant increase in H3K4me3 and H3K9/14ac at the *DACT3* locus (Figure 3D). Such effects were much weaker at the *DACT1* locus and thus correlate well with the relative effects of this pharmacologic treatment on *DACT3* and *DACT1* gene expression, respectively. The striking changes in histone methylation and acetylation marks at the *DACT3* locus are consistent with and help to explain the equally striking changes in *DACT3* expression levels following DZNep/TSA treatment.

To summarize, our biochemical, pharmacologic, and expression data all support a model in which repression of *DACT3* expression occurs via a bivalent histone domain. Furthermore, this repression can be effectively reversed by a combined pharmacological approach that simultaneously inhibits both histone methylation and deacetylation, resulting in a major change in chromatic structure at a subset of such bivalently modified genes, prominently including the *DACT3* locus.

Derepression of *DACT3* Is Associated with Inhibition of Wnt/β-Catenin Signaling and Massive Apoptosis in Colorectal Cancer Cells

Based on a previously published study (Zhang et al., 2006), one effect of increased DACT protein levels might be degradation of the Dishevelled (DVL) protein, which is central to Wnt signal transduction (Bilic et al., 2007; Logan and Nusse, 2004). Activation of the Wnt/β-catenin pathway in cancer cells and in other contexts is molecularly observable as accumulation of unphosphorylated non-membrane-associated β-catenin in the nucleus and cytoplasm (Peifer and Polakis, 2000; Polakis, 2007). Accordingly, we examined the effects of our drug treatment on DVL2 and β-catenin levels. DVL2 was arbitrarily chosen as a representative of the DVL gene family, as it is one of three functionally redundant, conserved DVL family members expressed in all or most cell types (Hamblet et al., 2002). Consistent with observed

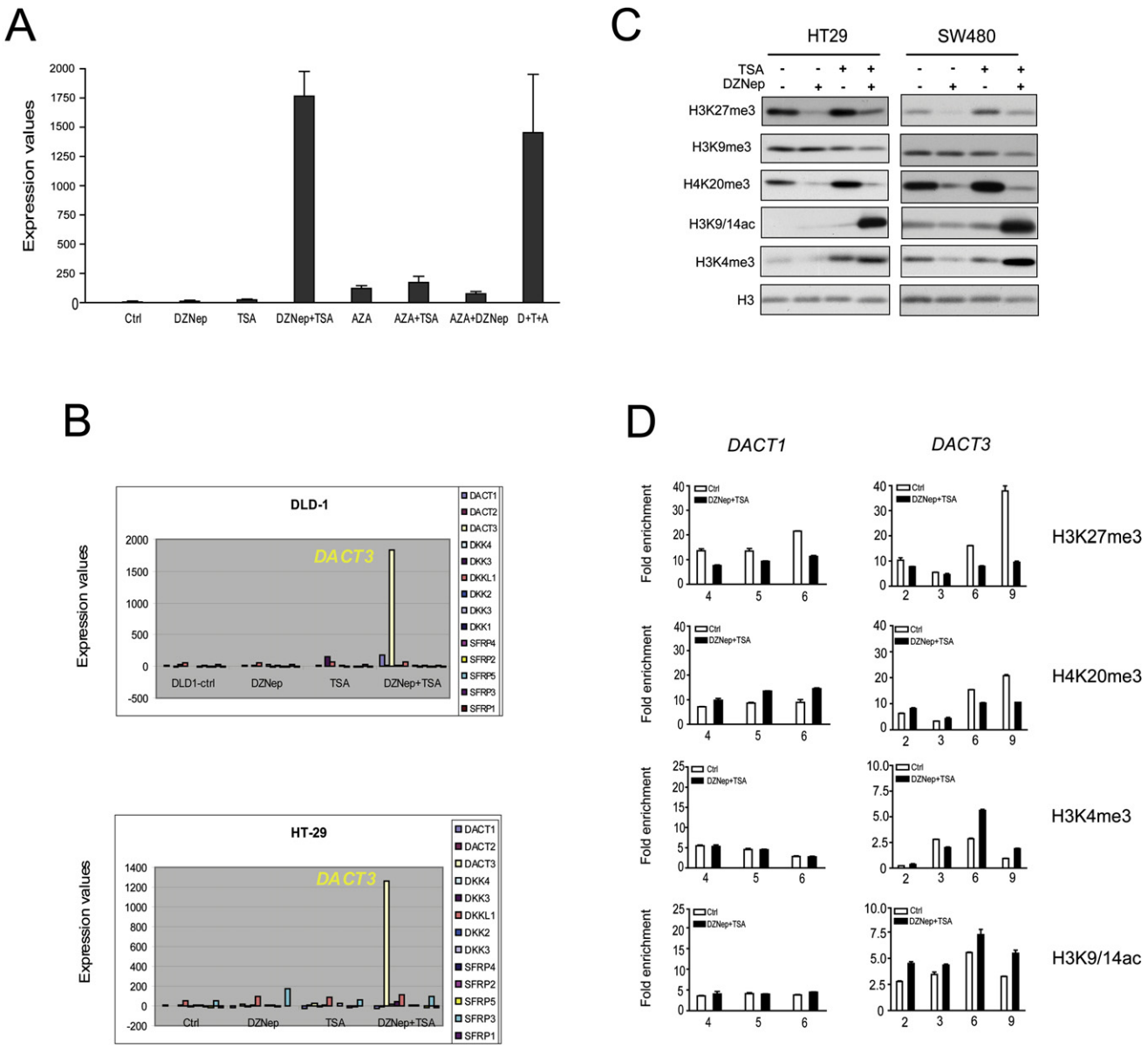

Figure 3. Effects of Combination of DZNep and TSA on *DACT3* and Histone Modifications

(A) *DACT3* mRNA expression analysis using Illumina Beadarray in DLD1 cells treated with 5 μM DZNep, 5 μM 5-AzaC, 200 nM TSA, or their combinations. The data shown represent mean ± SD of three independent experiments.

(B) Indicated colorectal cancer cell lines were treated with DZNep, TSA, or both. RNA was harvested and subjected to array analysis. Changes of expression of known Wnt/β-catenin antagonists are shown.

(C) Immunoblotting analysis of bulk histone modifications induced by DZNep, TSA, or both in HT29 cells and SW480 cells.

(D) ChIP analysis indicated the changes of histone marks at the *DACT1* and *DACT3* loci in HT29 cells untreated or treated with DZNep/TSA. The values represent the normalized enrichments against the changes of a low background region before and after the drug treatment. The data represent mean ± SD of three independent experiments.

changes in transcript levels, DACT3 protein levels were markedly increased (in both SW480 and DLD1 cells) upon DZNep and TSA combination treatment as determined by western blot analysis (Figure 4A and Figure S3). As predicted, the combination treatment resulted in a decrease in DVL2 levels. In contrast, treatment with either DZNep or TSA alone did not cause such changes (Figure 4A). Concomitantly, levels of activated (nonphosphorylated) β-catenin dropped in DZNep/TSA-treated cells by western blot (Figure 4A). This was confirmed by immunocytochemistry showing diminished nuclear β-catenin staining upon treatment with DZNep/TSA (Figure 4B). Finally, the expression of TCF/β-catenin target genes, including *MYC*, *LEF1*, *CCND1*, and *CD44*, was markedly decreased in DZNep/TSA-treated cells, but not in cells treated with either DZNep or TSA alone (Figure 4C and Table S3). These findings uniformly indicate that Wnt/β-catenin signaling is inhibited by DZNep/TSA combination treatment.

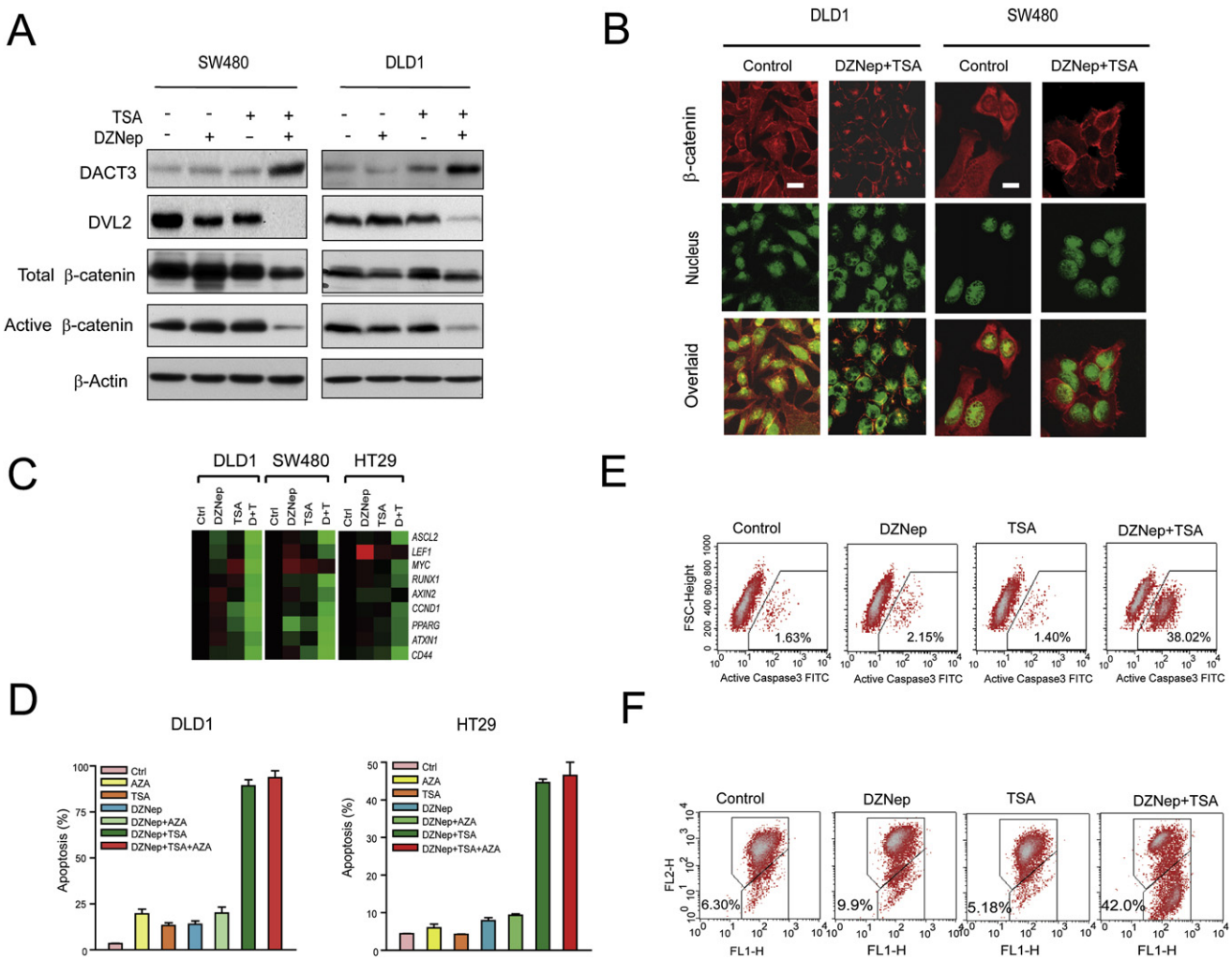

Figure 4. Combination of DZNep and TSA Results in Blockade of Wnt/β-Catenin Signaling and Massive Apoptosis in Colorectal Cancer Cells

(A) Immunoblot analysis of DACT3, DVL2, total β-catenin, and nonphosphorylated β-catenin (Active-β-catenin) in SW480 and DLD1 cells treated with DZNep (5 μM), TSA (100 nM), or both.

(B) Immunofluorescent images of DLD1 and SW480 cells treated with DZNep/TSA. β-catenin staining is red, and nuclear staining is green (DRAQ5). Scale bars represent 10 μm.

(C) Indicated colorectal cancer cell lines were treated as above; RNA was harvested and subjected to array analysis. Changes of expression of known Wnt/β-catenin target genes were shown using the gene clustering program. Green represents downregulated genes.

(D) DLD1 and HT29 cells were treated as in (A), and cell death was determined by PI staining and FACS analysis. The data represent mean ± SD of three independent experiments.

(E) HT29 cells were treated as in (A), and Caspase-3 activity was measured by FACS analysis.

(F) HT29 cells were treated as in (A), followed by JC-1 staining and FACS analysis.

Furthermore, we found that the decrease of β-catenin upon DZNep/TSA can be effectively rescued by treatment of cells with a small-molecule inhibitor of GSK-3, while the downregulation of DVL2 remained unaffected (Figure S4). This suggests that DACT3-DVL2 routes through GSK-3 to regulate β-catenin stability. Among 81 genes whose expression was increased by DZNep/TSA treatment in all the three cancer cell lines, *DACT3* is by far the most heavily induced (Table S4). To our knowledge no other gene in this list is directly relevant to Wnt/β-catenin signaling. Taken together, our results support that DZNep/TSA combination treatment decreases Wnt/β-catenin signal transduction in colorectal cancer cell lines by increasing *DACT3* gene expression and protein levels, secondarily destabilizing endogenous DVL proteins necessary for efficient Wnt signal transduction. Conversely, they suggest that repression of *DACT3* is a key epigenetic event in colorectal cancer formation.

Inhibition of Wnt/β-catenin signaling is expected to block the prosurvival pathway and induce apoptosis in cancer cells that are addicted to this pathway (Fujii et al., 2007; He et al., 2005). Indeed, we found that inhibition of Wnt/β-catenin signal transduction by DZNep/TSA was accompanied by a strong synergistic induction of cell death in DLD1 and HT29 cells, as assessed by propidium iodide (PI) and fluorescence-activated cell sorting (FACS) (Figure 4D). Similar results were obtained using other

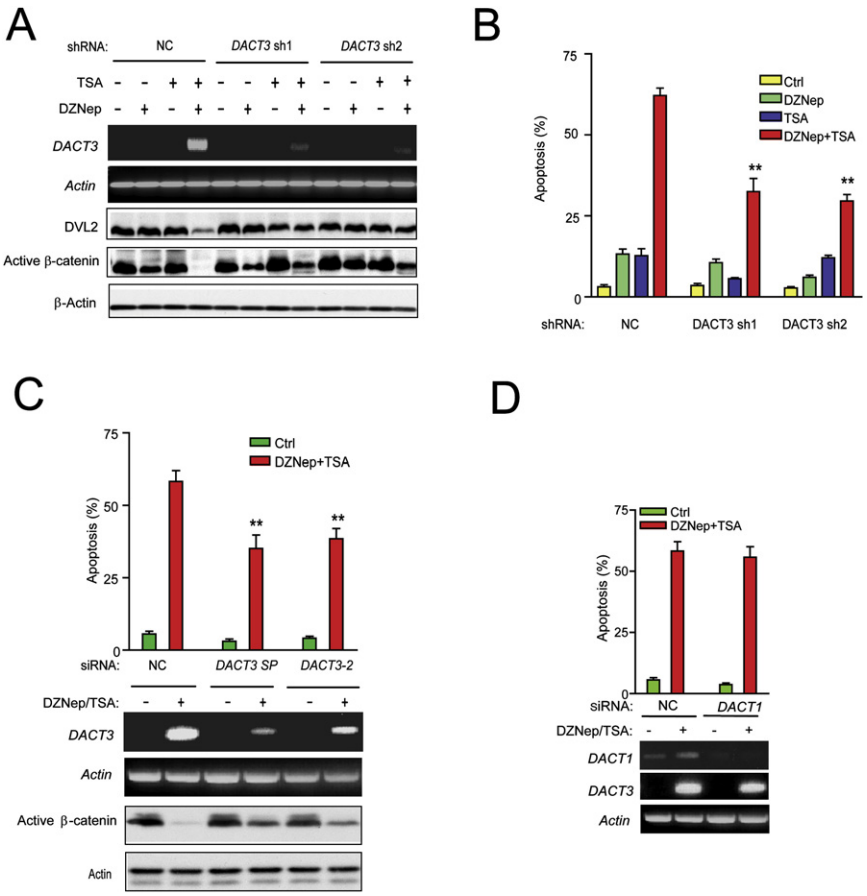

Figure 5. *DACT3* Plays a Role in Inhibition of Wnt/β-Catenin Signaling and Apoptosis Induction by DZNep/TSA

(A) Two DLD1 stable clones expressing *DACT3* shRNA (*DACT3*-sh1 and *DACT3*-sh2) or DLD1 cells expressing a nontargeting control shRNA (NC) were treated with DZNep, TSA, or both. *DACT3* and *actin* mRNA levels were determined by RT-PCR analysis; DVL2, active β-catenin, and total β-actin protein levels were assessed by immunoblotting.

(B) NC, *DACT3*-sh1, and *DACT3*-sh2 cells were treated as above, and apoptosis was determined by PI staining and FACS analysis. The data represent mean ± SD of three independent experiments. $^{**}p < 0.01$.

(C) SW480 cells transfected with *DACT3* SmartPool siRNA (*DACT3*-SP) and an independent *DACT3* siRNA (*DACT3*-2) were treated as in (A). The data represent mean ± SD of three independent experiments. $^{**}p < 0.05$. *DACT3* mRNA levels, DVL2, active β-catenin protein levels, and apoptosis were determined as previously.

(D) SW480 cells transfected with *DACT1* siRNA were untreated or treated with DZNep/TSA and harvested for mRNA and apoptosis analysis as above. The data represent mean ± SD of three independent experiments.

colon cancer cell lines (data not shown). In contrast, cells treated with DZNep/Aza, a treatment that does not efficiently derepress *DACT3* expression, did not undergo comparable levels of cell death. Similarly, addition of 5-AzaC to the DZNep/TSA combination, which does not produce further increases in *DACT3* gene expression, also does not produce additional increases in cell death. This suggests that modulation of DNA methylation by 5-AzaC and resultant effects on other potential gene targets does not contribute to further effects on Wnt/β-catenin signaling, and that induction of *DACT3* alone by DZNep/TSA is associated with maximal cell death. The cell death induced by DZNep/TSA was further determined to be apoptotic. As shown in Figure 4E, DZNep/TSA combination treatment, but not single-agent treatment, resulted in a dramatic activation of Caspase-3 in HT29 cells (Figure 4E). Moreover, the combination treatment induced a sharp drop in mitochondrial transmembrane potential (MTP) ($\Delta\Psi m$), indicative of mitochondrial dysfunction that is characteristic of apoptosis (Figure 4F).

The specific effects of DZNep/TSA treatment on histone modifications, *DACT3* gene expression, activated β-catenin protein levels, and apoptosis were also seen in treatments combining DZNep with other HDAC inhibitors such as PXD101 and suberoylanilide hydroxamic acid (SAHA). This shows that the effects of this pharmacological strategy that we have defined depend on the combined action of DZNep with an HDAC inhibitor and are not idiosyncratic consequences of TSA administered in combination with DZNep (Figure S5).

Functional Validation of *DACT3* as a Critical Regulator of Wnt/β-Catenin Signaling

To assess whether *DACT3* transcriptional derepression is required to decrease Wnt/β-catenin signaling in treated colorectal cancer cells, we generated cell lines derived from DLD1 that stably express a short-hairpin RNA targeting *DACT3*. Levels of the *DACT3* mRNA following DZNep/TSA treatment were greatly diminished in two DLD1 clones expressing the *DACT3* shRNA compared to control shRNA cells (Figure 5A). Western blot analysis indicated that DZNep/TSA-induced effects on both DVL2 and unphosphorylated β-catenin levels were diminished in *DACT3* shRNA cells (Figure 5A). Moreover, when apoptosis was assayed, the induction of apoptosis by DZNep/TSA was markedly reduced in cell lines expressing *DACT3* shRNA (Figure 5B). These *DACT3* knockdown effects are unlikely to be caused by off-target effects of the siRNA, because they were also observed in SW480 cells transiently transfected with two other independent *DACT3* siRNAs (Figure 5C). By contrast, knockdown of *DACT1*, which shows only a slight increase upon DZNep/TSA treatment, had no inhibitory effect on apoptosis (Figure 5D). In summary, our results indicate that transcriptional derepression of *DACT3* contributes to inhibition of Wnt/β-catenin signal transduction and to apoptosis following DZNep/TSA treatment of colorectal cancer cells.

We next set out to directly test the function of DACT3 in regulating Wnt/β-catenin signal transduction. DACT family members in various species have previously been shown to interact with

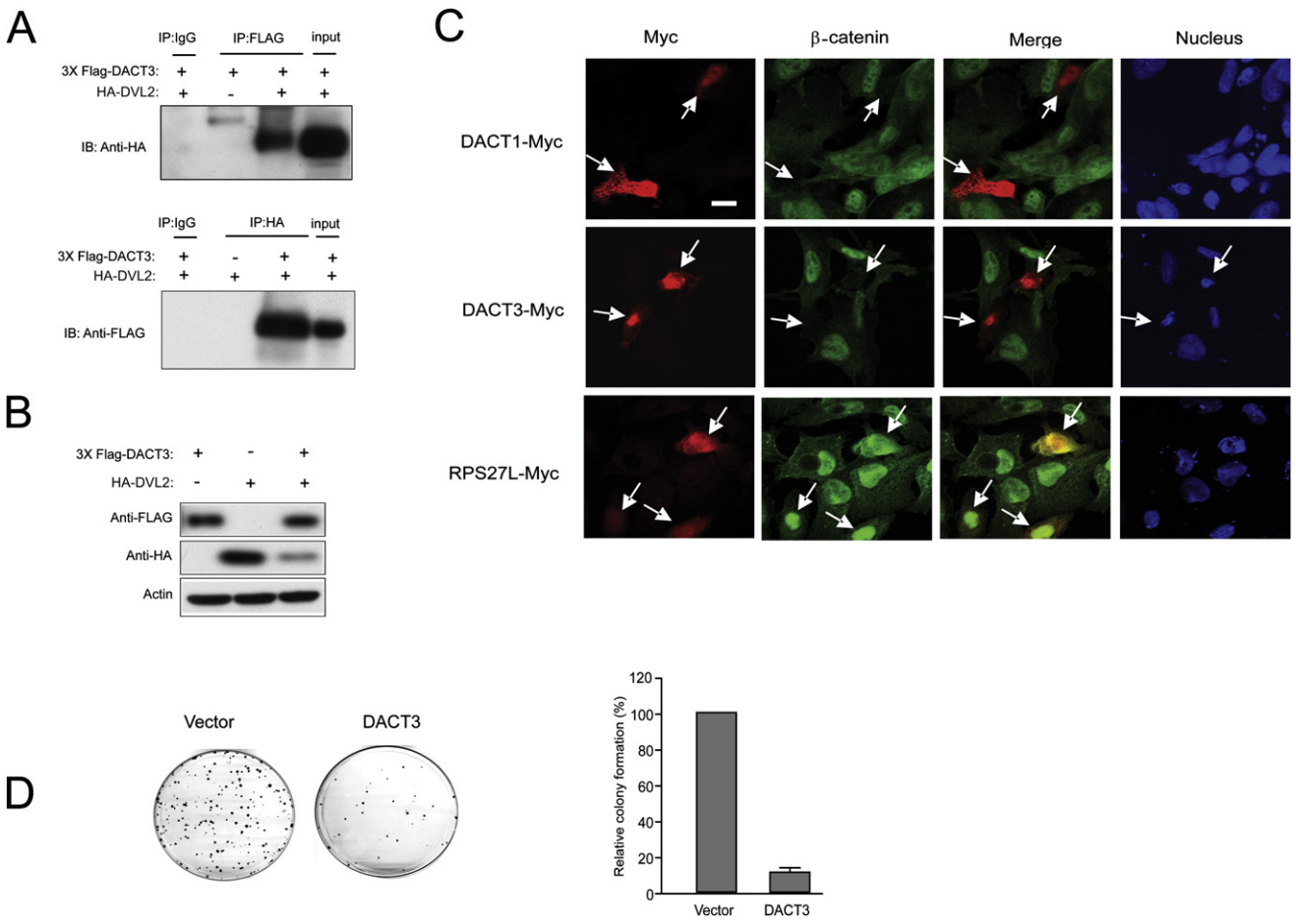

Figure 6. Effects of DACT3 Overexpression on DVL2 and β-Catenin in Colorectal Cancer Cells

(A) Exogenously expressed DACT3 associates with DVL2. SW480 cells were transiently transfected with the indicated Flag- or HA-tagged expression constructs. DACT3 was immunoprecipitated with anti-Flag, and the immunoprecipitates were probed with anti-HA antibody. Reciprocal coimmunoprecipitation between DACT3 and DVL2 is shown on the lower panel.

(B) Western blot analysis of HA-tagged DVL2 protein levels in SW480 cells cotransfected with Flag-DACT3 or an empty vector.

(C) Immunofluorescent images of SW480 cells transfected with Myc-tagged DACT1 or DACT3 expression construct for 72 hr. Myc-tagged DACT1 or DACT3 and β-catenin levels were detected by staining with anti-myc (red) and anti-β-catenin (green). Nuclei were stained by DRAQ5 (blue). Arrows indicate transfected cells expressing DACT3-myc. Scale bar represents 10 μm.

(D) Colony formation assay showing ectopic expression of DACT3 suppresses colon cell growth. DLD1 cells were transfected with a DACT3 expression vector or the empty vector and selected for 12 days with Zeocin. The number of colonies relative to the control in triplicates is shown on the right (error bar: mean ± SD).

DVL proteins through a highly conserved C-terminal motif and to negatively regulate β-catenin function (Cheyette et al., 2002; Zhang et al., 2006). To confirm that DACT3 also interacts with DVL proteins, we performed coimmunoprecipitation experiments by transfecting SW480 cells with expression vectors for Flag-tagged DACT3 and HA-tagged DVL2. Under these conditions using either tag antibody, DVL2 efficiently immunoprecipitates with DACT3, and conversely DACT3 efficiently immunoprecipitates with DVL2 (Figure 6A). Furthermore, cotransfection of DVL2-HA with DACT3-Flag led to a marked decrease in DVL2-HA protein expression compared to DVL2-HA-expressing cells cotransfected with an empty vector (Figure 6B). These results demonstrate that, as predicted from homology with other DACT family members, DACT3 can indeed interact with DVL family members as exemplified by DVL2. They further show that this interaction can reduce DVL protein stability, at least under these cotransfection conditions.

We similarly investigated the effect of ectopic *DACT3* expression on β-catenin levels in SW480 cells using confocal immunochemistry. SW480 cells expressing Myc-tagged DACT3 or DACT1 had nearly undetectable levels of nuclear β-catenin, whereas untransfected cells or cells transfected with a nonrelevant gene product, Myc-tagged RPS27L, retained high levels of nuclear β-catenin (Figure 6C). Moreover, SW480 cells ectopically expressing DACT3 displayed condensed nuclei typical of apoptosis. These results demonstrate that ectopically expressed *DACT3*, like other Dact family members (Cheyette et al., 2002; Hikasa and Sokol, 2004; Zhang et al., 2006), can negatively regulate Wnt/β-catenin signaling, including in colorectal cancer cells.

To determine if DACT3 suppresses cell growth as a result of inhibition of oncogenic Wnt/β-catenin signaling, we performed the colony formation assay with DLD1 cells transfected with a *DACT3*-expressing plasmid or the control empty vector. Cells transfected with *DACT3* show a dramatic decrease in colony numbers compared to control cells (Figure 6D). This result further demonstrates that DACT3 functions as a potential tumor suppressor in colon cancer.

DISCUSSION

The present study uncovers an epigenetic event that contributes to the constitutive activation of Wnt/β-catenin signaling in human colorectal cancer. We show that transcriptional repression of the *DACT3* gene occurs frequently both in colorectal cancer cell lines and in patient-derived tumors. Our analysis indicates that *DACT3*, together with *SFRPs*, might be a key epigenetic regulator of the Wnt/β-catenin signaling pathway in this disease process. This study thereby establishes *DACT3* as a potentially important target for cancer therapies aimed at controlling aberrant Wnt/β-catenin signaling.

Unlike the *SFRP* genes whose expression is often completely silenced by promoter DNA methylation, *DACT3* appears to be expressed at low levels in colon cancer cell lines. We provide evidence to show that this epigenetic event occurs through a bivalent histone modification that contain both repressive (H3K27me3) and activating (H3K4me3) histone marks at the *DACT3* locus and does not involve or require methylation of the promoter DNA. As a result of this epigenetic regulation, levels of *DACT3* can be strongly induced by a pharmacological treatment that targets histone modifications: specifically, combination treatment that simultaneously interferes with both histone methylation and deacetylation. By contrast, this treatment does not reactivate genes silenced predominately by DNA methylation, such as *SFRP1* or *DACT2*, and only modestly induces *DACT1*, whose repression in colon cancer cells is linked to both DNA methylation and histone modifications. These data suggest that this combination treatment of DZNep/TSA preferentially reactivates genes predominately repressed by bivalent histone modifications with minimum DNA methylation.

The bivalent chromatin state has been previously described in both embryonic stem (ES) cells and some differentiated cells, where it is in general associated with genes expressed at low levels (Azuara et al., 2006; Barski et al., 2007; Bernstein et al., 2006; Mikkelsen et al., 2007; Pan et al., 2007; Zhao et al., 2007). Some tumor suppressor genes with bivalent histone marks in ES cells lose the H3K4me3 mark during oncogenesis, but instead become fully silenced through DNA methylation (Ohm et al., 2007; Schlesinger et al., 2007; Widschwendter et al., 2007). Our data suggest that a bivalent chromatin state occurring in cancer cells might also contribute to cancerous transformation. Importantly, this epigenetic modification at *DACT3* locus appeared to be cancer specific, as we did not observe the same modifications at the *DACT3* locus in normal intestinal epithelial cells. Genes found in such a bivalent chromatin state in cancer cells (such as *DACT3*) therefore represent unique therapeutic targets. Unlike tumor suppressors that are silenced by DNA methylation, genes carrying a bivalent chromatin such as *DACT3* may be more subject to manipulation by treatments such as DZNep/TSA that target both histone methylation and deacetylation. Identifying these alternatively regulated tumor suppressor genes is also important because they are likely to be insensitive to therapies that only target DNA methylation, such as Aza.

Although highly speculative, it is possible that the chromatin pattern we have observed at the *DACT3* locus in our colorectal cancer cells is part of the molecular signature of stem cell-like cancer cells. There is evidence that Wnt/β-catenin signaling plays a central role in the maintenance of epithelial stem cells and of early progenitors (de Lau et al., 2007; Fodde and Brabletz, 2007). There is also data suggesting that the initial epigenetic event(s) that upregulate Wnt/β-catenin signaling may occur in colorectal adenomas before they acquire fully transforming APC mutations (Siu et al., 1999; Suzuki et al., 2004). Bivalent histone modification at *DACT3* could be one such epigenetic event. The combined pharmacologic approach we have described, which targets an epigenetic signature characteristic of colorectal cancer cells and abrogates Wnt/β-catenin signaling, may have the potential to target cancer stem cells that rely on this mechanism of gene silencing and that require high levels of Wnt/β-catenin signaling activity for their self-renewal and survival.

Our study illustrates the diversity and complexity of epigenetic mechanisms involved in gene repression in cancer cells. Use of DZNep as a histone methylation inhibitor in combination with other chromatin remodeling compounds may make it possible to discover other cancer genes regulated through various epigenetic mechanisms. The combined effects of DZNep and the HDAC inhibitor TSA on overall histone modification profiles is provocative, because together this drug combination appears to switch a repressive chromatin state into an active one by simultaneously reducing repressive histone marks (H3K27me3) and increasing activating marks (H3K4me3 and H3K9/14ac). This pharmacologic intervention, together with a DNA methylation inhibitor, might therefore have the potential to revert a globally "malignant" chromatin state found in some cancer cells into a more normal "benign" one. Although it remains to be biochemically determined how DZNep/TSA treatment generates such strong synergy in histone acetylation, we propose that this reflects direct crosstalk between these two types of histone modification. We further predict that such pharmacologic epigenetic "reprogramming" of histone modification profiles across the genome can lead to profound changes in gene expression, affecting multiple signaling pathways simultaneously. Indeed, our microarray data shows that *DACT3* is not the only gene regulated in this manner; the expression of at least 81 other genes was significantly affected by combined DZNep/TSA treatment. It is possible that inhibition of Wnt/β-catenin signaling cooperates with other signaling effects to yield the maximal apoptotic response observed upon this combination treatment.

We show that increasing the levels of DACT3 protein in colorectal cancer cells, either through pharmacologic derepression or via ectopic expression, results in robust degradation of DVL2 and to decreases in activated β-catenin. This DACT3-DVL2 signaling interaction might route through GSK-3 to regulate β-catenin stability; in support of this we have found that a small-molecule inhibitor of GSK-3 can rescue the decreases in activated β-catenin caused by DZNep/TSA treatment. As reported here we have also found that decreases in activated β-catenin caused

by increasing DACT3 can occur even in the presence of APC mutations. This finding is consistent with numerous reports suggesting that β-catenin phosphorylation and degradation still takes place in colorectal cancer cells carrying APC mutations (Calviello et al., 2007; Rice et al., 2003; Suzuki et al., 2004; Yang et al., 2006). In particular, it has been shown that restored expression of SFRP1/2 can effectively degrade β-catenin in colorectal cancer cells carrying these downstream mutations (Calviello et al., 2007; Rice et al., 2003; Suzuki et al., 2004). These findings together with ongoing studies suggest that there are alternate molecular mechanisms that contribute to β-catenin regulation independently of APC in colorectal cancer cells.

Our data also suggest that multiple abnormal epigenetic events might contribute to aberrant activation of the Wnt/β-catenin signaling pathway in colon cancer. These multiple events might occur in the same cell, or they may be present in different cells within a tumor. By increasing heterogeneity in a tumor cell population, epigenetic events may contribute to cancer progression and also contribute to treatment resistance and cancer recurrence.

In short, this study demonstrates that epigenetic repression of *DACT3* leads to aberrant Wnt/β-catenin signaling in colorectal cancer cells. Our data represent an important advance toward understanding how this epigenetic event contributes to deregulation of Wnt/β-catenin signaling in colorectal cancer. This work also provides an important proof of principle that such epigenetic events can be specifically targeted by pharmacologic strategies to yield robust effects on Wnt/β-catenin signaling with important consequences for cancer eradication.

EXPERIMENTAL PROCEDURES

Samples, Cell Lines, and Drug Treatment

Human tissue samples were obtained from the Singapore Tissue Network using protocols approved by the Institutional Review Board of the National University of Singapore; informed consent was obtained from each individual who provided the tissues. The colorectal cancer cell lines and nontransformed cell lines used in this study were purchased from the American Type Culture Collection (Manassas, VA). HCT116 cells with genetic disruption of DNMT1 and DNMT3B (HCT116 DKO) were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, MD). For drug treatment, cells were seeded the day before the drug treatment. Cells were treated with 5 μM 3-Deazaneplanocin A (DZNep) (obtained from Dr. Victor E. Marquez at National Cancer Institute, USA) or 5 μM 5-AzaC (Sigma) for 72 hr and Trichostatin A (TSA; Cell Signaling) at 100–200 nM for 24 hr. For 5-AzaC treatment, the medium was replaced with freshly added 5-AzaC for every 24 hr. For cotreatment of cells with DZNep and TSA, DZNep was added for 24 hr followed by TSA for an additional 24 hr for gene expression analysis and 48 hr for FACS analysis.

Mapping of *DACT3* Transcription Start Sites and Cloning of Full-Length *DACT3* cDNA

Total RNA (10 μg) was isolated from HEK293 cells with RNeasy Mini Kit (QIAGEN). *DACT3* transcription start sites were mapped by RNA ligation-mediated 5′RACE using FirstChoice RLM-RACE kit (Ambion) according to the manufacturer's protocol with the exception of reverse transcription and PCR steps. Reverse transcription after linker ligation was carried out at 64°C for 1 hr using Thermo-X polymerase (Invitrogen) and a gene-specific oligo: 5′-GACCC AGGCGACCATAGGAGCTGGATC-3′. Nested PCR was carried out using PfuUltraPhusion polymerase (Stratagene) with forward primers provided by the FirstChoice RLM-RACE kit and gene-specific reverse primers: 5′-GCTGG ATCCAGAGAAGCCACTGTCCCCA-3′ and 5′-CACAGAAGGTTGAGGGTGG TGAATCTGGACCT-3′. PCR products were cloned into pCR-BluntII-TOPO vector (Invitrogen) and sequenced with M13 primers. A tagged *DACT3* expression construct containing the longest open reading frame, based on mRNA start site mapping, was generated for overexpression studies. Full-length *DACT3* coding region was amplified by RT-PCR using 5 μg of total mRNA from HEK293 cells and the following primers: 5′-ATTGAATTCAATGATCCGG GCCTTCTCGTTCCCGGT-3′ and 5′-ATTAGATCTTCACACTGTAGTCATGAC CTTGAGAGAACCCGA-3′. The PCR product was cloned into p3xFLAG-CMV10 between EcoRI and BglII sites and sequenced.

RNA Interference

The SMARTpool siRNA targeting *DACT3* and the nontargeting control were purchased from Dharmacon (Lafayett, CO). A separate *DACT3* siRNA was obtained from Sigma-Proligo that targeted the following sequence: 5′-GGUUC UCUCAAGGUCAUGA-3′. To generate *DACT3* shRNA stable cells, a *DACT3* siRNA sequence (GGAGAAUCGCCUGCCUUCA) was cloned into the pSIREN-RetroQ retroviral expression vector. The pSIREN-RetroQ-Neg Vector was used as negative control shRNA (BD Bioscience), and cells were selected and expanded as described (Tan et al., 2007).

Immunoblot Analysis

Immunoblotting was performed as described previously (Tan et al., 2007). The blots were probed with the following antibodies. Anti-H3K27me3 (07-449), anti-H3K9me3 (07-442), anti-H3K9/K14 ac (06-599), anti-H3K4me3 (07-473), and anti-active β-catenin (05-665) were purchased from Upstate. Anti-H4K20me3 (ab9053) was from Abcam. Anti-β-catenin (6B3) and anti-H3 (3H1) were from Cell Signaling, and anti-DVL2 (sc-8026) was from Santa Cruz. The rabbit polyclonal antibody to DACT3 was raised against a 14 amino acid peptide from human DACT3 (LSLESGGLEQESGR) and was purified through affinity column.

Transfection and Immunoprecipitation

SW480 cells were transiently transfected using Fugene 6.0 (Roche). At 48 hr posttransfection, the cells were lysed with 1 ml of lysis buffer (20 mM Tris-HCl [pH 7.4], 2 mM EDTA, 25 mM NaF, 1% Triton X-100) plus protease inhibitors (Roche) for 30 min at 4°C. After 12,000 × g centrifugation for 30 min, the lysates were immunoprecipitated with anti-FLAG M2 agarose affinity gel (Sigma) or anti-HA affinity matrix (Roche) overnight at 4°C. The precipitants were washed three times with washing buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS), and the immune complexes were eluted with sample buffer containing 1% SDS for 5 min at 95°C and analyzed by SDS-PAGE. Immunoblotting was performed with primary antibodies against HA tag (sc-805, Santa Cruz) or FLAG tag (F1804, Sigma).

Apoptosis and Flow Cytometric Analysis

Cells were harvested and fixed in 70% ethanol. Fixed cells were stained with PI (50 μg/ml) after treatment with RNase (100 μg/ml). The stained cells were analyzed for DNA content by FACS in a FACSCalibur (Becton Dickinson Instrument, San Jose, CA). Apoptotic sub-G1 fraction was quantified using the CellQuest software (Becton Dickinson). To measure the mitochondrial transmembrane potential (MTP), cells were stained with JC-1, according to the manufacturer's instructions (BD Bioscience), and cells positive for JC-1 detection were measured using CellQuest software (BD Bioscience). To measure Caspase-3 activity, cells were fixed with Cytofix/Cytoperm solution (BD Biosciences) as instructed and then stained with fluorescein isothiocyanate-conjugated rabbit anti-active Caspase-3 monoclonal antibody (BD Biosciences). Quantification of cells positive for the Caspase-3 detection was performed by flow cytometry.

Microarray Gene Expression Analysis and Semiquantitative RT-PCR

Total RNA was isolated using Trizol (Invitrogen) and purified with the RNeasy Mini Kit (QIAGEN). Reverse transcription was performed using an RNA Amplification kit (Ambion). The microarray hybridization was performed using the Illumina Gene Expression Sentrix BeadChip HumanRef-8_V2, and data analysis was performed using GeneSpring software from Agilent Technologies as described (Tan et al., 2007). For RT-PCR, total RNA was reverse transcribed using oligo(dT)12-18 primer with Superscript II reverse transcriptase (Invitrogen). cDNA (100 ng) was used for PCR, and the primer sequences are shown

in Table S5. The microarray data have been submitted to the GEO public database (accession number GSE10972).

DNA Methylation Analysis

The CpG island DNA methylation status was determined by PCR analysis after bisulfited modification and followed by MSP and BGS (Yoshikawa et al., 2001). All BGS and MSP primers were designed to be close transcription start sites and in the CpG islands of the genes investigated. Primer sequences are shown in Table S5.

ChIP Assays

ChIP assays were performed as described previously (Zhao et al., 2005). The immunoprecipitated DNA was quantitated by real-time quantitative PCR using the PRISM 7900 Sequence Detection System (Applied Biosystems). Primer sets were chosen to amplify approximately 100–150 bp around the indicated region. We used the following antibodies in the ChIP study: anti-H3K27me3 (Upstate), anti-H3K9me3 and anti-H3K9me2 (Abcam), anti-H3K20me3 (Upstate), and anti-H3K9/14ac and anti-H3K4me3 (Upstate). The enrichments of these histone marks at the examined regions were quantitated relative to the input amount. To compare the two pools of DNA materials, from cells untreated and from cells treated, a further normalization of the ΔCt values against a region that shows low background enrichment was performed. The sequences of the PCR primers are shown in Table S5.

Immunofluorescence Staining and Confocal Microscopy

The cells were seeded in 4-well or 8-well culture chamber slides. After treatment or transfection for 72 hr, cells were fixed with 3.7% paraformaldehyde in PBS and permeablized with 0.2% Triton X-100. Cells were sequentially incubated with primary antibodies (anti-Myc or anti-β-catenin) and Alexa Fluor 488- or Alexa Fluor 546-conjugated secondary antibodies (Invitrogen) for 1 hr each and mounted in Fluorsave (Merck) mounting medium. DRAQ5 (Biostats, UK) was diluted in mounting medium for nuclear staining. The stained cells were examined by Zeiss LSM510 confocal microscopy.

Colony Formation Assay

Colony formation assays were performed as described previously to evaluate tumor cell growth in vitro (Yoshikawa et al., 2001). DLD1 cells were plated at 3×10^4 per well using 6-well plates and transfected with either pcDNA4.0-DACT3 or backbone pcDNA4.0 (2.0 μg) using Fugene 6 (Roche) according to the manufacturer's protocol. The cells were replated in triplicates and cultured for 10–15 days in complete DMEM medium containing Zeocin (100 μg/ml). The surviving colonies were stained with Gentian violet after methanol fixation, and visible colonies (×50 cells) were counted.

ACCESSION NUMBERS

The microarray data have been submitted to the GEO public database at http://www.ncbi.nlm.nih.gov/geo/ (accession number GSE10972).

SUPPLEMENTAL DATA

The Supplemental Data include five supplemental figures and five supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/13/6/529/DC1/.

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