

# Boswellic acid induces epigenetic alterations by modulating DNA methylation in colorectal cancer cells

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Accumulating evidence suggests that chemopreventive effects of some dietary polyphenols in part may be mediated by their ability to influence epigenetic mechanisms in cancer cells. Boswellic acids, derived from the plant *Boswellia serrata*, have long been used for the treatment of various inflammatory diseases due to their potent anti-inflammatory activities. Recent preclinical studies have also suggested that this compound has anticancer potential against various malignancies. However, the precise molecular mechanisms underlying their anticancer effects remain elusive. Herein, we report that boswellic acids modulate the DNA methylation status of several tumor suppressor genes in colorectal cancer (CRC) cells. We treated RKO, SW48 and SW480 CRC cell lines with the active principle present in boswellic acids, acetyl-keto- $\beta$ -boswellic acid (AKBA). Using genome-wide DNA methylation and gene expression microarray analyses, we discovered that AKBA induced a modest genome-wide demethylation that permitted simultaneous re-activation of the corresponding tumor suppressor genes. Quantitative methylation-specific PCR and RT-PCR validated the gene demethylation and re-expression of several putative tumor suppressor genes including *SAMD14* and *SMPD3*. Furthermore, AKBA inhibited DNMT activity in CRC cells. Taken together, these results lend further support to the growing notion that the anticancer effect of boswellic acids in part may be due to its ability to demethylate and reactivate methylation-silenced tumor suppressor genes. These results suggest that boswellic acid might be a promising epigenetic modulator in the chemoprevention and treatment of CRC, and also provide a rationale for future investigations on the usefulness of such botanicals for epigenetic therapy in other human malignancies.

## Introduction

*Boswellia* extracts, derived from the plant *Boswellia serrata*, have been known for their anti-inflammatory effects in the treatment of arthritis, ulcerative colitis and Crohn disease.<sup>1-5</sup> Acetyl-11-keto- $\beta$ -boswellic acid (AKBA), one of the major active boswellic acids present in boswellia extracts, is a pentacyclic terpenoid, which has been shown to have antitumor effects in different types of tumor cells including colon,<sup>6,7</sup> prostate,<sup>8,9</sup> leukocytes,<sup>10</sup> liver<sup>11</sup> and brain.<sup>12</sup> Several earlier studies have reported inhibitory effects of AKBA on the nuclear factor kappa B (NF $\kappa$ B),<sup>13</sup> and the signal transducer and activator of transcription-3 (STAT-3)-related pathways,<sup>14</sup> which potentiate apoptosis and inhibit angiogenesis in neoplastic cells. However, the precise molecular mechanisms underlying the antitumor effects of AKBA on colorectal cancer (CRC) still remain elusive.

DNA hypermethylation of CpG islands located within the promoter regions of tumor suppressor genes is a common epigenetic alteration involved in most human cancers. Unlike genomic epigenetic alterations, including aberrant methylation genes is potentially reversible with therapeutic agents. A DNA methyltransferase (DNMT) inhibitor, 5-aza-2'-deoxycytidine (DAC), which reverses DNA hypermethylation, has recently been used clinically for treating human malignancies such as myelodysplastic syndromes, leading to an improvement in the overall clinical outcome of patients with these diseases.<sup>15,16</sup> Given that epigenetic alterations are often present in the earliest stages of cancer, even in the normal-appearing mucosa, there is a growing enthusiasm that demethylating agents could be more beneficial for cancer prevention instead for their use in chemotherapy for advanced cancers.<sup>17,18</sup> Indeed, DAC and zebularine, an oral DNA methylation inhibitor, have been shown to prevent tumor formation in the *Apc*<sup>Min/+</sup> mouse model.<sup>19,20</sup> In spite of its promise, DAC

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treatment is often associated with several side effects including myelosuppression, nausea, vomiting and diarrhea,<sup>15,16</sup> which limits its clinical usefulness, especially for cancer chemoprevention. Another shortcoming of DAC is the manifestation of non-specific demethylation of non-tumor suppressor genes, and possible re-activation of various methylation-silenced proto-oncogenes in humans.<sup>21</sup> In view of this, there is a growing desire to identify demethylating agents that lack adverse side effects, and preferentially demethylate tumor suppressor genes to enhance cancer treatment, and particularly, modulate genes involved in cancer prevention.

In this regard, a growing body of literature supports that natural dietary compounds generally have multiple molecular targets within cancer cells, and are ordinarily considered quite safe. Consequently, there is renewed interest to understand the molecular mechanisms underlying the anticancer effects of several promising dietary botanicals, with the hope that some of these may eventually be used for cancer chemoprevention and treatment in future. Although limited, recent evidence indicates that the chemopreventive potential of several dietary polyphenols in part may be mediated by their ability to reactivate methylation-silenced tumor suppressor genes in cancer cells.<sup>22</sup>

In the present study, we hypothesized that AKBA might exert its antitumor effects in CRC cells partly by modulating the methylation status of various tumor suppressor genes. Using both array-based and candidate-gene approaches for the determination of methylation and gene expression status, herein, we provide novel evidence that in addition to its inhibitory effects on cellular signaling pathways, the anticancer effects of AKBA in part are due to its ability to induce demethylation and subsequent reactivation of putative tumor suppressor genes in CRC cells.

## Results

**AKBA inhibits cell viability and proliferation, and induces apoptosis in CRC cells.** To elucidate whether AKBA (the molecular structure; Fig. 1A) has any growth inhibitory effects in CRC cells, we performed MTT assays to determine cell viability in RKO, SW48 and SW480 cell lines treated with boswellic acid. We found that AKBA treatment for 72 h resulted in a dose-dependent growth inhibition in all three CRC cell lines: 1–16% with 5  $\mu$ M doses, 10–18% with 10  $\mu$ M, 35–46% with 20  $\mu$ M and 89–98% with 40  $\mu$ M AKBA [in all CRC cell lines vs. control cell lines (Fig. 1B)]. To further confirm the inhibitory effects of AKBA on cell proliferation, we performed BrdU assays and observed a similar dose-dependent effect on cell proliferation in all cell lines. There was little effect of AKBA in RKO and SW480 cells treated with 5 or 10  $\mu$ M concentrations, but we observed a 12–53% inhibition in cell proliferation with the 20  $\mu$ M doses and a 91–99% inhibition with 40  $\mu$ M AKBA in all CRC cell lines after 96 h of treatment (Fig. 1C).

Furthermore, to determine whether the growth inhibition observed following AKBA treatment in CRC cell lines might be due to the induction of apoptosis, we next performed an immunofluorescence assay using an anti-active caspase-3 antibody in RKO, SW48 and SW480 cell lines. As shown in Figure 1D–F,

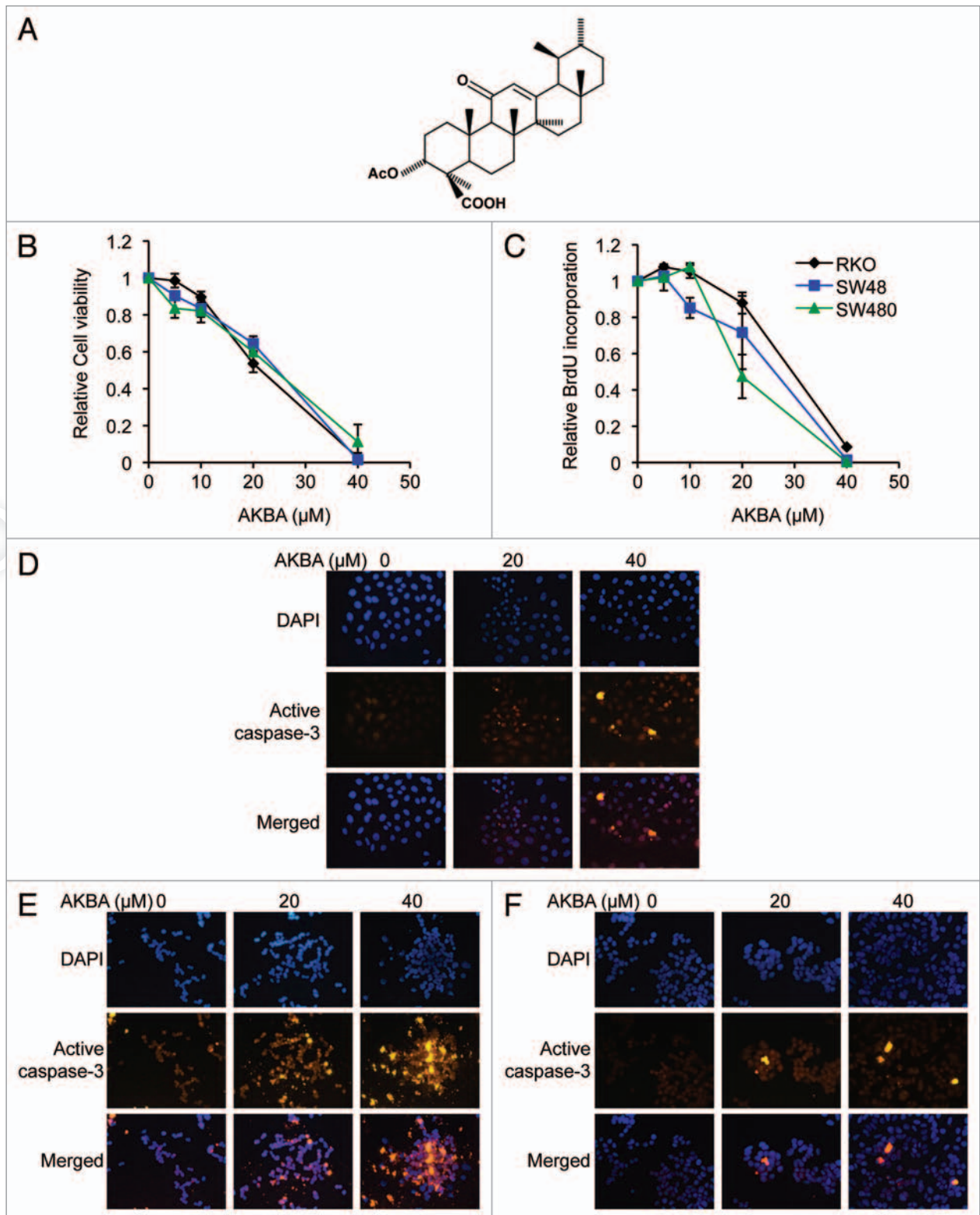
AKBA treatment increased the number of active caspase-3 positive cells, as evidenced by a greater number of bright yellow cells, in all three cell lines, in a dose-dependent manner. These results suggest that AKBA's antitumor effects are mediated both by the induction of apoptosis as well as through inhibition of cell proliferation in CRC cells.

**AKBA induces demethylation of several CpG loci in CRC cells.** To study the effects of AKBA treatment on the DNA methylation status in CRC, we next analyzed genome-wide DNA methylation patterns in control and AKBA-treated cell lines using Illumina's Infinium HumanMethylation27 microarrays. For these experiments, we chose the SW48 CRC cell line for the initial genome-wide methylation analysis, because this cell line possesses a CpG island methylator phenotype (CIMP), which is characterized by aberrant hypermethylation of numerous CpG islands in the promoter regions of many tumor suppressor genes, including the *MLH1* gene.<sup>23</sup> Methylation patterns of SW48 cells treated with DMSO alone (controls) were compared with cells treated with 2.5  $\mu$ M DAC or 20  $\mu$ M AKBA for 6 d. The scatter plots and the histograms illustrate that a large number of CpG sites had reduced methylation levels after both DAC and AKBA treatments compared with control cell lines (Fig. 2A). The mean  $\beta$ -values of all CpG loci in SW48 cells treated with AKBA were significantly lower than those of control cells (0.366 for AKBA vs. 0.401 for controls,  $p < 0.0001$  by two-tailed Student's *t*-test). These results indicate that AKBA induced demethylation of a large number of CpG sites, although the demethylating activity of AKBA was markedly lower than the global demethylation induced by DAC (0.286; Fig. 2A).

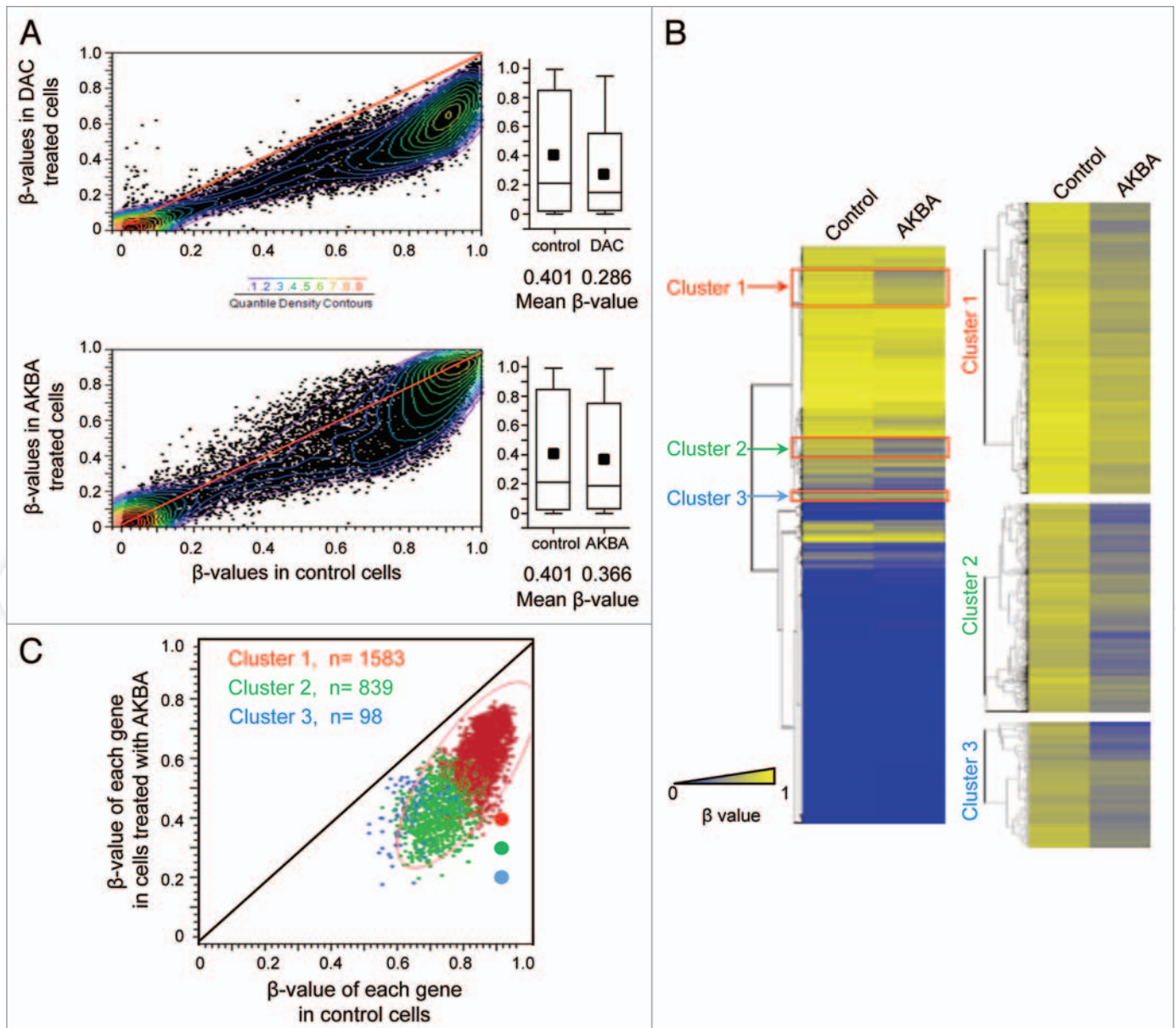
We were interested in elucidating whether a specific subset of genes existed that was preferentially demethylated following AKBA treatment in CRC cell lines. Hierarchical clustering analysis successfully identified three clusters of genes, "cluster 1" ( $n = 1,583$ ), "cluster 2" ( $n = 839$ ) and "cluster 3" ( $n = 98$ ), which were most prominently demethylated in AKBA treated CRC cells (Fig. 2B and C). Our observations of localized demethylation changes in a significant proportion of genes suggests that perhaps certain subsets of genes are more susceptible to DNA demethylating activity in AKBA treated cells.

**A subset of genes is demethylated and upregulated by AKBA treatment.** To better understand the biological relevance of AKBA-induced demethylation, and to identify the subsets of genes in which demethylation correlated with their reactivation, we performed genome-wide gene expression analysis using the Illumina's HumanHT-12 v4 Expression BeadChip microarrays in SW48 cells treated with AKBA. The data output of gene expression arrays was then compared with the  $\beta$ -values from the global methylation arrays by scatter-plot analysis (Fig. 3). When we combined gene methylation and expression array data, 76% of the genes in cluster 1, 78% in cluster 2 and 73% in cluster 3 exhibited a simultaneous decrease in methylation and a corresponding increase in gene expression. These data suggest that the expression of the majority of genes in these three clusters were actually upregulated when demethylated.

Next, using the gene expression results from the three clusters, we performed Ingenuity Pathway Analysis (IPA) to determine



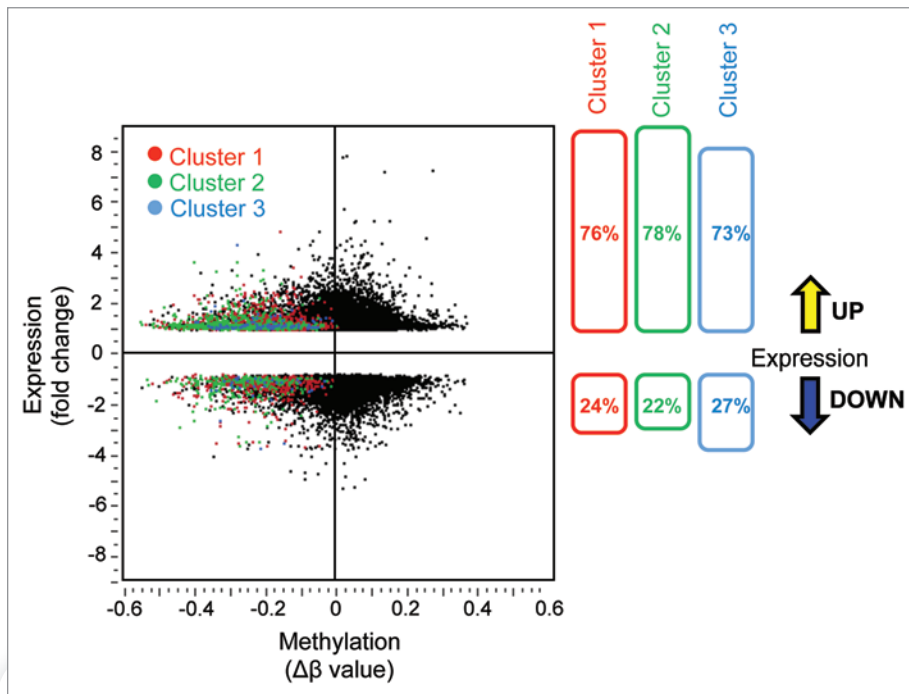
**Figure 1.** AKBA exerts anticancer effects in CRC cell lines. (A) The molecular structure of AKBA. (B) AKBA treatment inhibits cell viability in RKO, SW48 and SW480 cells using a MTT assay. (C) AKBA reduces proliferation of CRC cells in a BrdU assay. AKBA induces apoptosis in RKO (D), SW48 (E) and SW480 (F) cells in an immunofluorescence staining assay that uses an anti-active caspase-3 antibody. Data are represented as mean  $\pm$  standard error of mean (SEM) from three independent experiments.



**Figure 2.** AKBA demethylates CpG islands in CRC cells. (A) A scatter plot comparing the  $\beta$ -values of all CpG loci between control and DAC-treated (2.5  $\mu$ M) SW48 CRC cells (top), and control and AKBA (20  $\mu$ M) treated cells (bottom). Colored dots represent  $\beta$ -value densities for each CpG locus. The histograms represent mean  $\beta$ -value distribution in SW48 cells treated with DAC (top part) and those treated with AKBA for 6 d (bottom part). The horizontal line within the box represents the median values. The upper and lower lines in the box plot represent 75th and 25th percentiles. The horizontal bars above and below the boxes represent the maximum and the minimum values. The small black square within each box plot represents median  $\beta$ -values. The differences in the mean  $\beta$ -values between the control, DAC and AKBA treated SW48 cells were analyzed by a two-tailed Student's t-test. (B) A heat map illustration of the DNA methylation pattern of the control and AKBA treated cells (left). The gradient of blue to yellow represents individual  $\beta$ -values, and the degree of methylation from low (blue) to high (yellow; 0 to 1). The three clusters of genes that demonstrated the most significant DNA demethylation after AKBA treatment are shown with red boxes. A close-up view of the heat maps corresponding to the subset of demethylated genes in the clusters 1, 2 and 3 are represented on the right. (C) The scatter plot of the  $\beta$ -values from the gene clusters 1, 2 and 3 (red, green and blue respectively) in AKBA vs. control cells.

whether there were any specific cell-signaling pathways that are preferentially affected by AKBA treatment. IPA analysis revealed that AKBA treatment of CRC cells modulated five different canonical pathways most profoundly. Based upon the categorical configuration within IPA, these five pathways were classified as: (1) molecular mechanisms of cancer, (2) axonal

guidance signaling, (3) G-protein coupled receptor signaling, (4) role of osteoblasts, osteoclasts and chondrocytes in rheumatoid arthritis, and (5) CRC metastasis signaling (Fig. 4A). Most of the genes modulated by AKBA treatment were listed as part of the CRC metastasis signaling pathway. These genes were related to NF $\kappa$ B and tumor necrosis factor, both of which have



**Figure 3.** Correlation between DNA demethylation and re-expression of the corresponding genes in AKBA treated CRC cells. The scatter plot illustration of DNA methylation ( $\beta$ -values) data obtained from genome-wide methylation arrays and the changes in gene expression analyzed by microarray in SW48 cells treated with AKBA. The methylation status after AKBA treatment is shown for each gene from clusters 1, 2 or 3 (colored red, green or blue, respectively, left part). As shown on the right, the majority of demethylated genes within each of the three clusters were upregulated after AKBA treatment (76, 78 and 73% respectively).

previously been shown to be targets of AKBA in other human cancers (Fig. 4B).

In order to narrow down the list of AKBA-targeted genes in CRC, we focused our attention on those genes that have a confirmed or putative tumor suppressor role in cancer, contain CpG islands in their promoter region, and demonstrated at least a 2-fold increase in gene expression following AKBA treatment. Accordingly, we selected nine putative tumor suppressor genes that met these criteria: *cyclin G<sub>2</sub>* (*CCNG2*), *DNA damage-inducible transcript 4* (*DDIT4*), *death effector domain-containing protein* (*DEDD*), *fragile histidine triad gene* (*FHIT*), *PR domain containing 13* (*PRDM13*), *protein-tyrosine phosphatase gamma* (*PTPRG*), *sterile  $\alpha$  motif domain containing 14* (*SAMD14*), *sphingomyelin phosphodiesterase 3* (*SMPD3*), *tumor protein p53-binding protein 2* (*TP53BP2*). To confirm and validate the demethylation and upregulated gene expression of these nine putative tumor suppressor genes, we analyzed methylation levels by quantitative methylation-specific PCR (MSP) and gene expression levels by real-time RT-PCR in SW48, RKO and SW480 cell lines treated with AKBA. These experiments revealed that six of the nine genes were indeed upregulated and demethylated in two of the three AKBA-treated CRC cell lines (Fig. 5). Among all nine genes, *SAMD14* and *SMPD3*, both of which were included in cluster 1 in the methylation microarray analysis (Fig. 2B and C), were shown to be demethylated and upregulated in SW48 cells by the validation analysis (Fig. 5). *SAMD14* is downregulated

in various human lung adenocarcinoma cell lines and its promoter region is frequently hypermethylated in early invasive pulmonary adenocarcinoma.<sup>24</sup> Similarly, *SMPD3* is one of the genes whose methylation and expression levels were found to be modulated in the CRC metastasis signaling pathway identified by IPA analysis (Fig. 4). Overexpression of *SMPD3* leads to an inhibition of cell growth in the human breast cancer cell line MCF7,<sup>25</sup> and the promoter region of *SMPD3* has been reported to be hypermethylated in human breast cancers.<sup>26</sup> The demethylation and upregulation of putative tumor suppressor genes could help explain the anticancer effects of AKBA.

**AKBA inhibits DNMT activity.** Since DNMT catalyzes the process of methylation, and to further clarify the mechanisms underlying the demethylating activity of AKBA, we aimed to elucidate whether AKBA could inhibit DNMT activity in CRC cells. Use of the DNMT inhibition assay demonstrated that AKBA markedly inhibited DNMT activities in SW48 and SW480 cell lines at 40  $\mu$ M concentrations, while these effects were modest at 20  $\mu$ M concentrations (Fig. 6). We also observed that treatment with the classical DNMT inhibitor, DAC (5  $\mu$ M), resulted in an inhibition (~25%) of DNMT activity in these cell lines (data not shown). Taken together, the results of our experiments show that AKBA seems to have a rather modest inhibitory effect on DNMT activity, which is consistent with previous observations for other botanical compounds.<sup>22</sup>

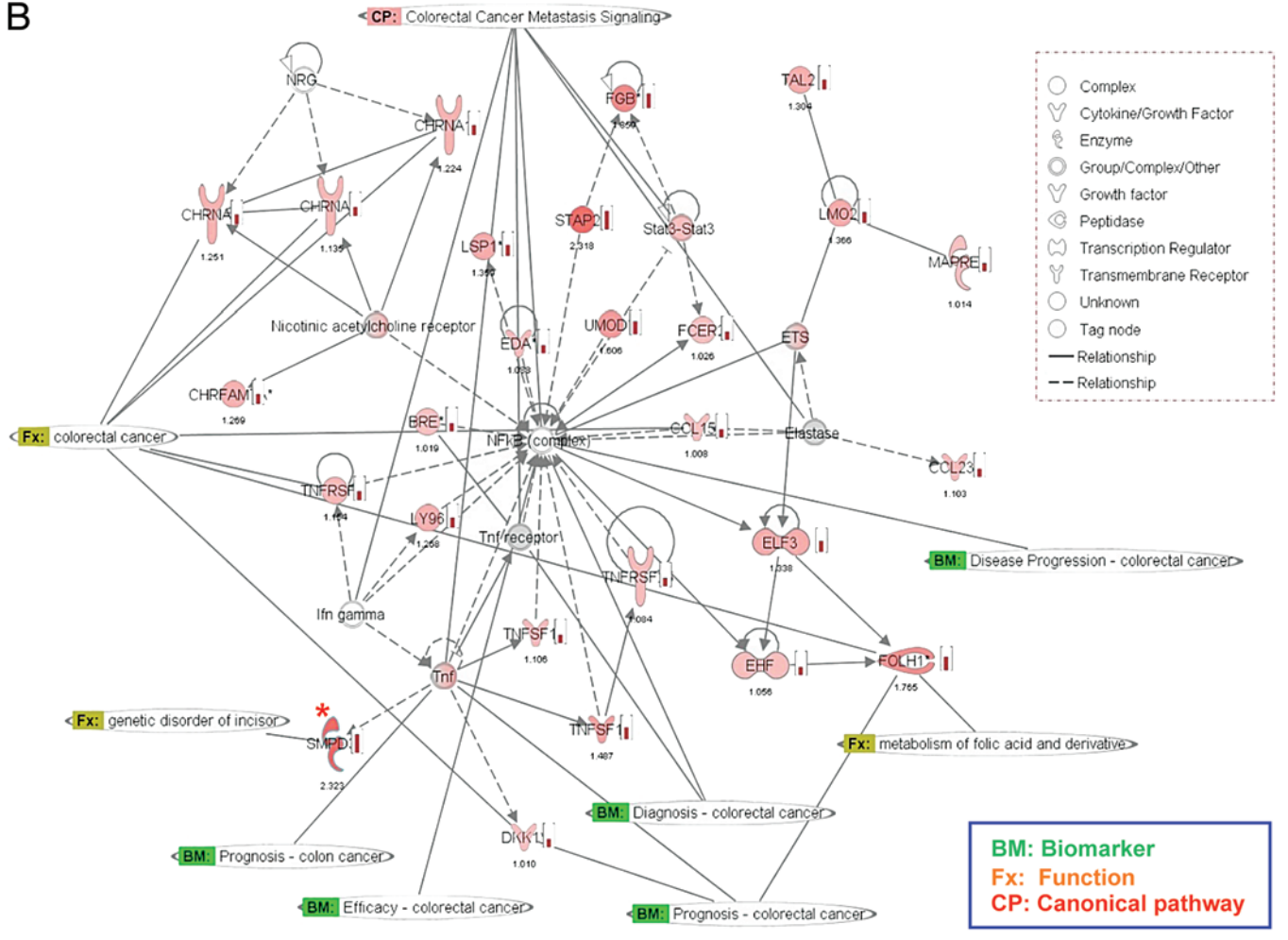
## Discussion

The data presented in this study provides the first evidence for the involvement of epigenetic mechanisms underlying the chemopreventative effects of boswellic acids in CRC. Using a series of genome-wide DNA methylation and gene expression microarray analyses, we discovered that AKBA induced demethylation of a large number of CpG loci, and the demethylation was associated with a concurrent upregulation of genes that may be involved in CRC carcinogenesis. Our results build upon the existing knowledge of boswellic acid-induced inhibition of proteins involved in other intracellular signaling pathways, such as NF $\kappa$ B and STAT-3, that lend additional support in favor of the anticancer effects of this compound in CRC.<sup>13,14</sup> Our observations that AKBA caused demethylation and re-activation of putative tumor suppressor genes such as *SAMD14* and *SMPD3*, provide a novel mechanism by which boswellic acid exerts its anticancer effects in cancer cells.

DNA hypermethylation of CpG sites in promoter regions of tumor suppressor genes is one of the key mechanisms that cause

**A****Top Canonical Pathways**

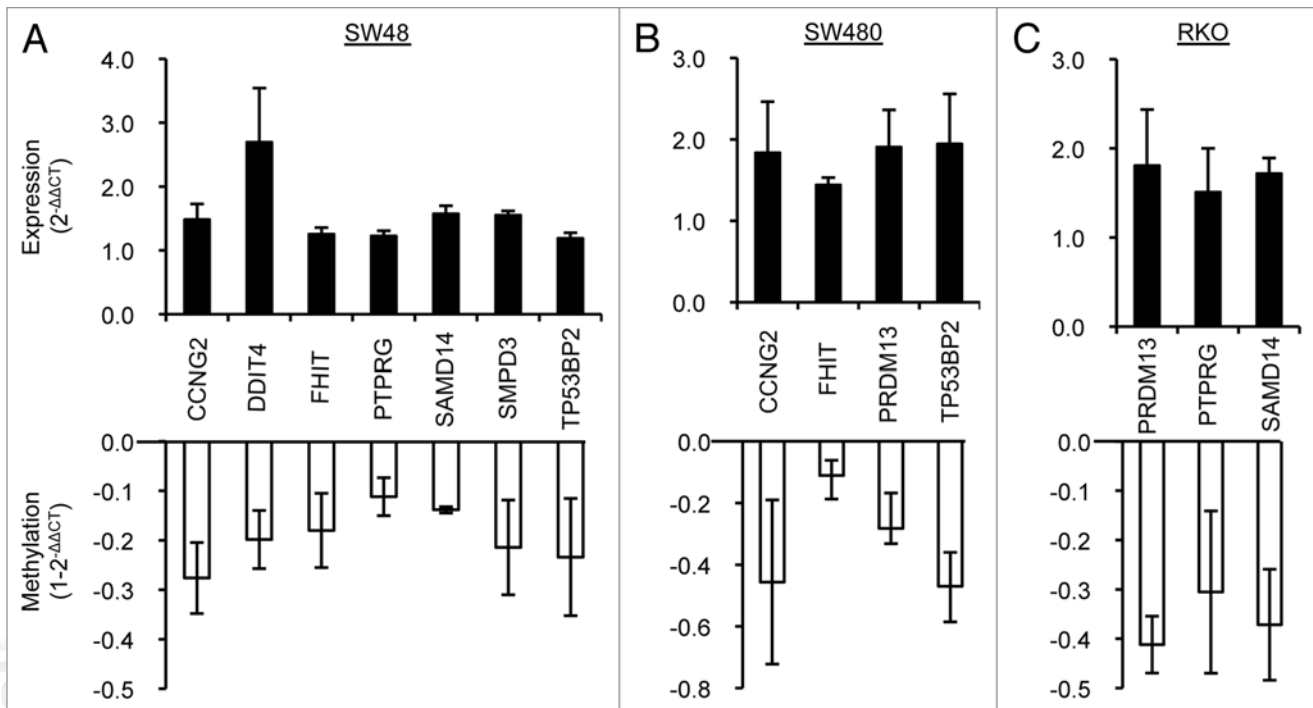
Pathway Name	P-value	Ratio
Molecular Mechanisms of Cancer	2.18E-36	319/375 (0.851)
Axonal Guidance Signaling	3.51E-24	331/410 (0.807)
G-Protein Coupled Receptor Signaling	2.91E-23	442/531 (0.832)
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	4.88E-21	207/243 (0.852)
<b>Colorectal Cancer Metastasis Signaling</b>	<b>1.07E-19</b>	<b>220/259 (0.849)</b>

**B**

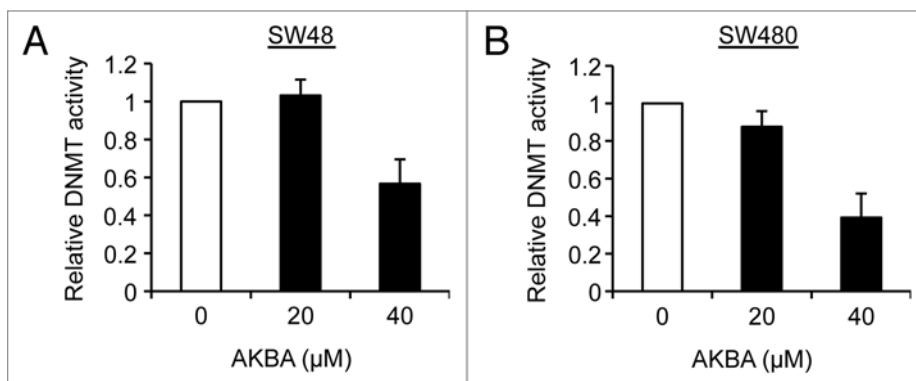
**Figure 4.** Pathway analysis revealed those most affected molecular pathways by AKBA. (A) Pathway analysis revealed that CRC metastasis signaling was one of the top five canonical pathways that served as a target of AKBA-induced demethylation in CRC cells. (B) An illustration from Ingenuity Pathway Analysis (IPA) demonstrating the biological relationship between demethylated and overexpressed gene from the three genes clusters. Bold green “BM” represents the genes that are described as biomarkers of CRC, orange “Fx” represents the genes that have direct function in CRC, and “CP” represents the canonical pathway involved in CRC metastasis signaling. One of the genes, *SMPD3*, which was found within the most demethylated gene group, “cluster 1” in the methylation microarray analysis and subsequently confirmed in our validation analysis (expression by qRT-PCR and methylation by qMSP), is marked with \* in red. Density of red color of icons and the number below the icons represents the fold change in gene expression induced by AKBA obtained in our genome-wide gene expression analysis.

transcriptional silencing of the corresponding genes in cancer cells. A subset of 30–40% colorectal tumors can be characterized by the presence of CIMP, in which several tumor suppressor genes with CpG islands in their promoters are frequent targets

of aberrant hypermethylation.<sup>27,28</sup> CIMP-positive CRCs associate with proximally located tumors, the presence of microsatellite instability (MSI) and *BRAF* mutations. Although CIMP CRCs significantly associate with sporadic MSI-high tumors due to



**Figure 5.** Validation of DNA methylation and gene expression status of selected putative tumor suppressor genes in three CRC cell lines. Re-expression and demethylation of nine putative tumor suppressor genes were confirmed by real-time RT-PCR and quantitative MSP in three CRC cell lines: SW48 (A), SW480 (B) and RKO (C) treated with 20  $\mu$ M AKBA (SW48 and RKO) or 30  $\mu$ M AKBA (SW480) for 6 d. Six of the nine genes (*CCNG2*, *FHIT*, *PTPRG*, *PRDM13*, *SAMD14* and *TP53BP2*) were upregulated along with demethylation in at least in two of the cell lines. Data are represented as mean  $\pm$  SEM from two or three independent experiments.



**Figure 6.** AKBA inhibits DNMT activity in CRC cells. DNMT activity was measured in SW48 (A) and SW480 (B) cells treated with DMSO, 20  $\mu$ M AKBA and 40  $\mu$ M AKBA for 3 d. Data are represented as mean  $\pm$  SEM from three independent experiments.

*MLH1* hypermethylation, aberrant methylation is not limited to these tumors and can be observed in other subsets of CRCs including ones with chromosomal instability (CIN) phenotype.<sup>29</sup> In view of this, we studied CRC cell lines that were representative of all three subsets of CRCs, which included SW48 and RKO cells (CIMP and MSI-high) and SW480 cells (CIN). Our initial hypothesis was that we would perhaps observe the most noticeable changes in AKBA-induced reversal of methylation in CIMP cell lines due to the high frequency and levels of methylated

genes in these cells. But at the same time, we also included a non-CIMP or CIN cell line for our validation analysis, to ascertain the demethylating potential of AKBA in these cells, which represent about 50–60% of all CRCs. Indeed, our screening results were supportive of our hypothesis, wherein genome-wide methylation analysis revealed AKBA-induced demethylation of a large number of CpG loci in the CIMP-positive SW48 cells, although the level of demethylation was relatively modest compared with DAC. These findings were further corroborated by our observations that DAC also induced hypomethylation of LINE-1 elements, which are considered to be markers of global methylation changes, while AKBA did not (data not shown). Another strength of our study is that we successfully validated the preferential demethylation of various tumor suppressor genes along with their upregulation following AKBA treatment in multiple cell lines, in spite of the genetic and epigenetic diversity of these CRC cells. In SW48 cells, the validation results for most genes (7 of 9) were concordant with our screening array data from independently treated cell lines. More importantly, the majority of genes (6 of 9) were confirmed to be demethylated and upregulated in at least

two of the three cell lines following multiple independent treatments. Furthermore, it was interesting to observe AKBA-induced demethylation in some genes even in the non-CIMP SW480 cells, since non-CIMP-related genes are frequently methylated in tumors with a CIN phenotype.<sup>28,29</sup> Our observations of definitive demethylation of a subset of genes in the face of modest generalized demethylation induced by AKBA may be of significance from a cancer chemoprevention perspective, in which long-term drug use may be required, to avoid secondary tumor occurrence resulting from excess, continuous genome-wide hypomethylation as observed in the DNMT1-deficient mice model.<sup>30,31</sup> In addition, we noticed that, compared with the modest effect seen with 20  $\mu\text{M}$  AKBA, treatment at 40  $\mu\text{M}$  concentrations resulted in marked inhibition of DNMT activity. These results are not entirely surprising considering that, in comparison to direct DNMT inhibitors such as DAC, AKBA and other natural dietary compounds may induce DNMT inhibition through an indirect mechanism. Consequently, the precise mechanisms by which AKBA treatment causes demethylation and DNMT inhibition, as well as the optimal doses and/or frequencies of AKBA administration for its demethylating activity in preclinical animal models, need to be clarified in future studies.

Although a number of attempts have been made for chemoprevention of CRC, such efforts have met with limited success, and as a result no chemopreventive drugs are widely accepted for clinical use. One of the best-studied drugs for chemoprevention of CRC is aspirin. Aspirin's antitumor effects are primarily attributed to its ability to inhibit cyclooxygenase-2 (COX-2), and some data suggest that it may have a preventive effect on adenoma formation and CRC occurrence in humans in long-term observational studies.<sup>32</sup> However, the long-term use of aspirin can also cause toxic effects including gastroduodenal ulcers and bleeding.<sup>32</sup> Selective COX-2 inhibitors, such as celecoxib, have more potent chemopreventive effects and fewer gastrointestinal toxicities than aspirin, however, these drugs associate with an increased risk of cardiovascular events.<sup>33</sup> Consequently, specific COX-2 inhibitors have not been accepted for chemopreventive use in general populations either. One of the biggest hurdles for chemoprevention in general populations is finding a compound that has no toxicity. In this regard, boswellic acids, like several other botanical-derived compounds, seem to be extremely safe, and hence may be more rational for use in cancer chemoprevention.

Moreover, this botanical has the ability to target a wide range of genes/pathways that affect the malignant properties of cancer cells including demethylating activity against a subset of tumor suppressor genes as demonstrated in our present study. Molecular mono-targeted drugs, such as antibodies against EGFR and VEGF, have recently been used in the treatment of patients with metastatic CRC. However, benefits drawn from such mono-targeted agents are limited, and furthermore resistance to these antibodies always occurs with prolonged treatment duration.<sup>34</sup> CRC arises as a consequence of the stepwise accumulation of a multitude of genetic and epigenetic alterations; therefore, multi-targeted therapies that target both genetic and epigenetic pathways could be more beneficial in this disease compared with

mono-targeted therapies.<sup>35,36</sup> In light of accumulating evidence and our findings in this study, clinical validation of the efficacy of boswellic acids for both chemoprevention and therapy of CRC could be promising.

In conclusion, we have shown that AKBA induces demethylation and concurrent upregulation of a subset of tumor suppressor genes, which may contribute to its anticancer effects in CRC cells. Thus, boswellic acid could be useful not only as an epigenetic modulator for the treatment of patients with CRC, it may be effective against other malignancies. Our data provide the rationale for further investigation of this compound, as well as other botanicals, for therapeutic interventions against human cancers.

## Materials and Methods

**Cell culture.** Human CRC cell lines RKO, SW48 and SW480 were obtained from the American Type Culture Collection (ATCC). Cells were cultured in Iscoves modified Dulbeccos medium (IMDM, Invitrogen) with 10% fetal bovine serum. Cells were treated with 0.1% DMSO (vehicle controls) or AKBA (Sigma Chemical Co.), at a dose range of 0–40  $\mu\text{M}$  for 6 d. The medium containing DMSO or AKBA was changed every 2 d and in the demethylation experiments, cells were treated with DMSO, AKBA or DAC (2.5  $\mu\text{M}$ ) for a total of 72 h.

**MTT assay.** The growth inhibitory effect of AKBA on cells was evaluated by the use of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) assay. Human CRC cell lines were plated in 96-well plates at a density of  $5.0 \times 10^3$  cells/well in 100  $\mu\text{l}$  of IMDM, and incubated for 24 h. Cells were then treated with AKBA for 72 h with the final concentrations of 5, 10, 20 or 40  $\mu\text{M}$ . Following treatment with AKBA, cells were incubated with 100  $\mu\text{l}$  of 0.5 mg/ml MTT in medium for 3 h at 37°C. The cells were lysed for 12 h at room temperature in a buffer containing 10% SDS and 0.01 N HCl. For each sample, the optical density of the reduced intracellular formazan product was read at 570 nm in a spectrophotometer. Each assay was performed in triplicate in three independent experiments.

**BrdU cell proliferation assay.** The proliferation index was measured by bromodeoxyuridine (BrdU) incorporation in CRC cells 96 h after AKBA treatment as described above, following the manufacturer's instructions (Cell Proliferation ELISA, BrdU, Roche Diagnostics). Each experiment was performed in triplicate, and the data were calculated based upon three independent experiments.

**Immunofluorescence.** Cells were cultured on coverslips placed in 12-well plates at a density of  $0.5 \times 10^5$  cells in 1 ml of IMDM. Once attached, cells were treated for an additional 48 h with 0, 20 or 40  $\mu\text{M}$  AKBA. Cells cultured on coverslips were then fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.3% Triton X-100 in PBS for 5 min, and blocked with 5% goat serum for 1 h. Cells were incubated with a rabbit anti-active caspase 3 antibody (1:500; Promega) at room temperature for 1 h, washed with PBS for three times, followed by a goat anti-rabbit IgG secondary antibody for



immunofluorescence staining (1:800) for 30 min at room temperature. Thereafter, slides were washed with PBS three times, followed by nuclear staining with DAPI and mounted in an appropriate medium for fluorescence microscopy. The images were taken with an AxioSkop2 multichannel epifluorescence microscope equipped with AxioVision software (Carl Zeiss).

**DNA and RNA extraction.** DNA was extracted from RKO, SW48 and SW480 cells treated with DMSO alone or with AKBA by use of the QIAmp DNA mini kit (Qiagen). Total RNA was extracted from the cell extracts with the RNeasy Mini kit (Qiagen). The cDNA was obtained using the Advantage RT-for-PCR kit (Clontech).

**Genome-wide DNA promoter methylation and gene expression analyses.** Genome-wide DNA promoter methylation status in control and AKBA treated SW48 cells were analyzed using the Infinium HumanMethylation27 BeadChip microarrays (Illumina, Inc.). These methylation microarrays span across 28,000 CpG sites that are located within the promoter regions of ~14,475 genes based upon the NCBI Database (Genome Build 36). On an average, two assays are designed for each gene that includes anywhere between 3–20 CpG sites. The methylation level in each CpG site was given a  $\beta$ -value ranging from 0 (indicating no methylation) to 1 (100% methylation). Genome-wide gene expression status was analyzed using Illumina's HumanHT-12 v4 BeadChips, which included more than 47,000 probes. SW48 cells treated with 2.5  $\mu$ M DAC (Sigma-Aldrich) were used as positive controls for demethylation.

**Quantitative methylation-specific polymerase chain reaction.** The DNA methylation status of *CCNG2*, *DDIT4*, *DEDD*, *FHIT*, *PRDM13*, *PTPRG*, *SAMD14*, *SMPD3*, *TP53BP2* and the collagen type II  $\alpha 1$  (*COL2A1*:reference gene<sup>37</sup>) were determined by quantitative methylation-specific polymerase chain reaction (MSP) using primers specific for methylated sequences on a bisulfite-modified genomic DNA template (EZ DNA methylation Gold Kit, Zymo Research). Each reaction was performed in duplicate and the results were obtained from two or three independent assays. The  $2^{-(\Delta\Delta CT)}$  method was used to present the data from the genes of interest relative to the controls. Table 1 shows the primers' sequences and the putative function of genes.<sup>24-26,38-46</sup>

**Quantitative real-time reverse transcription-polymerase chain reaction.** Expression levels of *CCNG2*, *DDIT4*, *DEDD*, *FHIT*, *PRDM13*, *PTPRG*, *SAMD14*, *SMPD3*, *TP53BP2* genes, and the reference  $\beta$ -actin gene were determined by use of SYBR Green based-quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed in a 25- $\mu$ L total volume containing 10 ng of cDNA, 12.5  $\mu$ L of SYBR Green mix (Applied Biosystems), and 10 pmol of each primer. The following PCR cycle conditions were used: initial denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 15 sec, and 58°C for 1 min. The relative amounts of the genes of

interest and  $\beta$ -actin were measured in two independent assays. The  $2^{-(\Delta\Delta CT)}$  method was used to present the data on the genes of interest in treated cells relative to controls. The primers used are listed in Table 1.

**DNMT inhibition assay.** DNMT activity of CRC cell lines was measured in a DNMT activity/inhibition assay (Active Motif) according to the manufacturer's protocol. In brief, SW48 and SW480 cells were treated with DMSO alone, 20 or 40  $\mu$ M AKBA for 72 h. The medium with the drug was changed every 24 h. The nuclear extracts were prepared with the Nuclear Extract Kit (Active Motif). Each 10  $\mu$ g of nuclear extract was incubated with the enzymatic buffer containing AdoMet for 2 h, followed by incubation with His-MBD2 protein (1:200 dilution) for 45 min. After another 45 min incubation with anti-polyHis-HRP antibody (1:1,000 dilution), the developing solution was added to the extracts and the absorbance was read on a spectrophotometer at 450 nm. The data were obtained from three independent experiments.

**Data analysis.** The Illumina DNA methylation data were analyzed using the open-source programs Cluster 3.0 and Java TreeView. Cluster 3.0 was used to perform an unsupervised hierarchical cluster analysis by applying complete linkage clustering with a Euclidean distance metric. Several analyses including the networks and functional analyses were performed through the use of Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com/). A data set containing gene identifiers and corresponding expression values was uploaded into the application. Each identifier was mapped to its corresponding object in Ingenuity's Knowledge Base Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity. Right-tailed Fishers exact test was used to calculate p values determining the probability that each biological function and/or disease assigned to that data set is due to chance alone. The basic statistic and scatter plots were done using JMP, Version 7 (SAS Institute Inc.). All microarray data reported in this article is MIAME compliant and that the raw data have been deposited in the GEO database and can be accessed at www.ncbi.nlm.nih.gov/geo/.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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**Table 1.** MSP and RT-PCR primers used in this study

Gene	Direction	Sequence of MSP primers	Amplicon size (bp)	Sequence of real-time RT-PCR primers	Amplicon size (bp)	Functions/characteristics	References*
CCNG2	F	AGG CGA GAA GGT ATA GTT TC	185	GCC TTG TGC CTT CTC AAT TT	101	Inhibits cell cycle progression	38
	R	ATT CAC CCT CGA ATC TAA AT		TCA GTG TCA TTA ATC TTG GAA TGT T			
DDIT4	F	GAT TTT GGT AGT TGT AGT CGT C	132	GGG AGG AGG GGT TTG ACC	102	Inhibits mTOR pathway; negatively feed-back HIF-1	39, 40
	R	CGT TCT CTC CTA CCC GAT A		GGT AAG CCG TGT CTT CCT C			
DEDD	F	AAT CGA TTT TCG TGG TAT TTC	179	AGC CTG AGC CGA GAG AAG A	100	Potentiates apoptosis; impedes cell mitosis	41, 42
	R	AAC AAC GCC GAT TTA TCT TA		AGG TAC GCA ATG CTT TCC AG			
FHIT	F	TTG GGG CGC GGG TTT GGG TTT TTA CGC	100	CAG CTG TCA ACA TCC TGG AA	111	Altered in various cancers	43
	R	CGT AAA CGA CGC CGA CCC CAC TA		TCT CTC CCT TCC ACC GTC T			
PRDM13	F	GGT GGG ATT TTG TAG TTG TTT C	173	CCC GGA GGA CAG ATC TTC TA	166	A candidate tumor suppressor as transcriptional regulators	44
	R	CTA CGC GCT AAC CAA AAA AC		TCC AGC AGT ACC AGC AGA TG			
PTPRG	F	GTT CGT TCG TTT TTT CGT TC	113	CCA GTT CCG TGC TCC ATT AT	145	Mutated in colorectal cancer	45
	R	AAA AAA CGA AAA ACG CGA		GCA CCA GAG TAG GCC CAG TA			
SAMMD14	F	CGC GTT TTT TGT TTT TAG C	122	AGA CGC TGA AGA TGA CCG ATG	107	Downregulated in lung adenocarcinoma; hypermethylated in early invasive pulmonary adenocarcinoma	24
	R	AAC TCG AAC CGC TCC ATC		ACG GGT TCT CGG AGC TTT G			
SMPD3	F	CGG TGG GAG AGT TTT TTT C	113	AGC AAC ACT CCC TGT TCA CC	153	Induces apoptosis in bronchial epithelial cells; hypermethylated and functions as a growth sup- pressor in breast cancer cells	25, 26
	R	AAC GAA AAA CTA TAC CCC CG		CAC TCT CCA GGA CCT TCT GC			
TP53BP2	F	TTT TAT TTA GTT TGC GGT GTA C	161	GAT GCC GAT GTT TCT TAC CG	109	Enhances p53-induced apoptosis	46
	R	CCC GTA TAA CTT CAA CTC G		TTC TTT GCA CAG ATC CAC CA			
COL2A1	F	GTA ATG TTA GGA GTA TTT TGT GGG TA	86				37
	R	CTA CCC CAA AAA AAC CCA ATC CTA					

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