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Prox1 overexpression of Hela cells inhibits PKC beta II transcription through promoter DNA methylation

(子宮頸癌細胞株 Hela における Prox1 過剰発現はプロモーター領域のメチル化を介して PKC-beta II の転写を抑制する)

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Abstract

Prospero-related homeobox 1 (*PROX1*) is important for embryonic organ formation and differentiation, and changes in PROX1 activity were recently associated with cancer. To address the PROX1 roles in tumorigenesis, we established cells stably overexpressing PROX1 using the human cervical cancer cell line, HeLa. Overexpression of PROX1 reduced cell proliferation and the rate of tumor formation as compared with controls. Comparison of gene expression profiles between PROX1-overexpressing and mock-transfected cells revealed that the expression of protein kinase C βII (*PRKCB2*) is down-regulated in PROX1-overexpressing cells. A PRKCB inhibitor suppressed cell growth of control cells more than PROX1-expressing cells. Analysis of the 5'-promoter of *PRKCB* revealed that a region between -110 bp and the first exon contains two Sp1 binding sites and is important for transcriptional regulation of *PRKCB*. The inhibition of Sp1 transcription factor resulted in down-regulation of PRKCB2 protein levels. Treatment with a demethylating agent, 5-aza-2'-deoxycytidine, restored *PRKCB2* mRNA expression in PROX1-expressing cells, suggesting that the 5'-promoter of *PRKCB* is methylated in these cells. Actually, it was found that a CpG island in this region, in particular a CpG site overlapping with the distal Sp1 site, was hypermethylated and direct Sp1 binding to this region was inhibited in PROX1-overexpressing cells. Thus, the suppressive effect of PROX1 on cell growth and tumor formation might be partially mediated by PRKCB2 via altered methylation of its promoter.

INTRODUCTION

Homeobox proteins play essential roles in the determination of cell fate and the development of the body plan, and are often aberrantly expressed in cancer. It was originally thought that homeobox genes were transcriptional activators that are up-regulated in cancer cells, promoting oncogenesis. However, it is now clear that the true picture is far more complex, as both reduction and up-regulation of homeobox gene expression can be associated with tumorigenesis (Abate-Shen, 2002).

One of the homeobox genes in *Drosophila*, *prospero,* regulates the switch from stem cell self-renewal to differentiation in the nervous system. Previous studies have demonstrated that *prospero* represses genes required for the self-renewing neural stem cell fate, such as cell cycle regulatory genes, as well as activating genes required for neuronal differentiation. These observations suggest that *prospero* acts as a tumor suppressor (Li and Vaessin, 2000; Betschinger et al., 2006; Choksi et al., 2006).

The vertebrate homolog of *prospero*, *Prox1*, was originally identified in mice (Oliver et al., 1993). Analysis of *Prox1*-null mice indicated its potential roles in lens fiber elongation, development of the lymphatic systems, and hepatocyte migration (Wigle et al., 1999; Wigle and Oliver, 1999; Sosa-Pineda et al., 2000). Moreover, these reports also suggested the possibility that *PROX1* in mammals can act as a tumor suppressor, similar to what was observed for the *Drosophila prospero* gene.

In human cancers, the role of *PROX1* is heterogeneous and appears to be dependent upon cellular context. We observed several point mutations

in the *PROX1* gene in hematologic malignancies, for example, and found that *PROX1* expression is silenced by DNA methylation in both cancer cell lines and clinical samples (Nagai et al., 2003). Similar epigenetic silencing of *PROX1* has been found in sporadic breast cancer and carcinomas of the biliary system (Laerm et al., 2007; Versmold et al., 2007). Moreover, RNA mutation of *PROX1* leading to loss of function has been reported in some human cancer cell lines, including pancreatic cancer (Takahashi et al., 2006). Thus, decreased PROX1 levels have been observed in various types of cancer. In addition, Shimoda *et al.* reported that knockdown of PROX1 accelerates in vitro cell growth, whereas its overexpression suppresses cell growth of hepatocarcinoma cells, and demonstrated the tumor-suppressive effect of PROX1 (Shimoda et al., 2006). By contrast, it has been recently reported that PROX1 is overexpressed in colorectal cancers and promotes dysplasia, tumor growth, and malignant progression (Petrova et al., 2008). These results are apparently contradictory; however, this complexity might suggest that the role of PROX1 is highly dependent upon the specific signaling pathways active in different cellular contexts. Understanding by what mechanism PROX1 regulates target gene expression could help shed light on the different roles for PROX1 observed in different cancerous or normal cell types.

In this work, we sought to identify new PROX1 target genes and uncover mechanisms underlying regulation of target genes by PROX1. To this end, we overexpressed PROX1 in HeLa cells, in which there is no detectable endogenous PROX1 expression. With this approach, we have

identified a novel PROX1 target, *PRKCB2*. We investigated the mechanisms of *PRKCB2* regulation and discuss our results in the context of human carcinogenesis.

MATERIALS AND METHODS

Cell Culture

The human cervical cancer cell line, HeLa, two human colorectal cancer cell lines (DLD-1, HCT116) and two hematologic cell lines (K562 and KMS-12-PE) were cultured either in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) or RPMI1640 (Sigma) supplemented with 10% fetal bovine serum (FBS; Sigma) in a humid atmosphere with 5% CO_2 at 37°C. All of the cell lines were kindly provided by Dr. T. Murate (Nagoya University, Japan).

Construction of a *PROX1* Expression Vector and Establishment of HeLa Cells Stably Overexpressing PROX1

The full-length coding region of *PROX1* was amplified by PCR. The PCR-amplified product was cloned into the pT$_{ARGE}$T Mammalian Expression Vector (Promega, Madison, WI). Its sequence was confirmed. A total of 24 μg of DNA were transfected into HeLa cells using Lipofectamine™2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. At 48 hours post-transfection, the cells were grown in the selection medium containing 800 μg/ml G418 (Sigma). PROX1 expression was confirmed by immunoblotting. Two subclones that constitutively express PROX1 were selected for further experiments (Prox1-HeLa sc8, sc55). HeLa cells transfected with empty vector were also established and used as a control (Mock-HeLa sc1 and sc2).

Immunoblotting

Whole cell extracts were prepared in RIPA Buffer (Pierce, Rockford, IL) and protein concentrations were measured using a BCA Protein Assay Kit (Pierce). Cell extracts were separated by SDS-PAGE. After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes, blocked with 5% nonfat milk, and probed with primary antibodies. The membranes were then washed with TBS containing 0.05% Tween 20 and exposed to horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Detection was performed using the ECL Plus Western Blotting Detection System (GE Healthcare UK Ltd., Buckinghamshire, UK). The primary antibodies used in this study were anti-Prox1 (Upstate, Lake Placid, NY), anti-PKCβII, anti-Sp1, and anti-Sp3 (Santa Cruz Biotechnology), and anti-β-actin (Sigma). Relative protein levels were quantified by using ImageJ software (http://rsb.info.nih.gov/ij/).

Cell Proliferation Assay

Cell proliferation was analyzed using the MTT assay. Cells were seeded at a density of 1×10^3 cells/well in 96-well plates. Next, the cells were incubated for the indicated times at 37°C in a humidified chamber at 5% CO_2, and 20 μl/well of MTS reagent (CellTiter 96 AQ$_{UEOUS}$ One Solution Cell Proliferation Assay; Promega) were added. After 1 hour of incubation, the absorbance was measured using a plate reader (Bio-Rad Laboratories, Inc., Berkeley, CA) at 490 nm. To determine the effects of the PRKCB inhibitor Enzastaurin (LC

Laboratories, Woburn, MA) on cell proliferation, cells were incubated with 10 μM of Enzastaurin or dimethyl sulfoxide (DMSO) for 12 hours in media containing 1% FBS. We used low serum conditions because high concentrations of FBS in culture medium reduced the potency of Enzastaurin due to its high protein-binding affinity. The assay was done in triplicate and the mean ± SD was determined.

Tumor Formation Assay

Male 5-week-old Balb/c mice were used. Sub-confluent and exponentially growing monolayer cells from each cell line (original HeLa, Mock-HeLa sc1, Prox1-HeLa sc8 and Prox1-HeLa sc55) were collected, washed and resuspended in culture medium without serum or antibiotics. Cells (1×10^6 cells/200 μl/mouse) were inoculated subcutaneously into the flanks of a mouse. Eight mice were used for each cell type. The mice were sacrificed 42 days post-inoculation and tumor formation was examined.

Microarray Analysis of Gene Expression Profiles

Microarray analysis was used to compare the gene expression profile of Prox1-HeLa sc8 cells with that of mock-transfected control cells. The RNA extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) was amplified into cRNA and biotinylated by in vitro transcription using the Illumina® TotalPrep RNA Amplification Kit (Ambion, Austin, TX) according to the manufacturer's protocol. Biotinylated cRNAs were purified, fragmented, and subsequently hybridized to Sentrix™ Human-6 V2 Expression BeadChip

(Illumina, Inc., San Diego, CA), which encompasses 48000 human gene transcripts. Normalization and all analysis of microarray data were performed using GeneSpring software (Agilent Technologies, Inc., Santa Clara, CA).

Reverse Transcriptase (RT)-PCR and Quantitative Real-time PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's protocol. First strand cDNA was synthesized from 1 μg of total RNA using random hexamers as primers and SuperScript™III reverse transcriptase (Invitrogen). We used 1 μl cDNA as a template for RT-PCR and quantitative real-time PCR. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified as an internal control. Primer sequences for RT-PCR were as follows: *PROX1* forward 5'-TTCAGATGGAGAAGTACGCA-3', reverse 5'-GGACTGCTACTCTTCATACA-3'; *GAPDH* forward 5'-CCATGGAGAAGGCTGGGG-3', reverse 5'-CAAAGTTGTCATGGATGACC-3'; *PRKCB2* forward 5'-TGGGGTGACAACCAAGACATTC-3', reverse 5'-GTCAATATTCCTGATGACTTCCTG-3'. Quantitative real-time PCR was performed with TaqMan® Gene Expression Assays and TaqMan® Universal PCR Master Mix using ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was repeated three times, with expression levels normalized to respective *GAPDH* expression of each sample.

Construction of Reporters Containing *PRKCB* Promoter Fragments and Luciferase Assay of Reporter Activity

The 5'-promoter of *PRKCB* (*i.e.* the region between -1968 bp to +110 bp) was obtained by PCR from human genomic DNA. The PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen) and confirmed by DNA sequencing. Deletion mutants were constructed using a PCR-based approach. The following forward primers were used for each promoter construct.

-1968 bp: 5'-GGGCTCGAGGGCTCTTTTCCATCCACAGCC-3',

-807 bp: 5'-GGGCTCGAGTCTTGCTGTTCAAACTCAGACC-3',

-215 bp: 5'-GGGCTCGAGCGAGGCTCGGGTCCGACGAC-3',

-110 bp: 5'-GGGCTCGAGAGCAGCTGGCAGCGCTGGG-3',

-48 bp, 5'-GGGCTCGAGGGGGCTCCGCCTCCCTC-3',

-18 bp, 5'-GGGCTCGAGGCCAGCGGTGCCAAGCGC-3'. For construction of all promoter constructs a common reverse primer was used: 5'-CCCAAGCTTCGGCCGCTGCTGCACCC-3'. Single and double underlines indicate *Xho*I and *Hind*III site, respectively. Each PCR product was purified, digested with *Xho*I and *Hind*III, and then ligated into the pGL3-Basic vector (Promega) treated with *Xho*I and *Hind*III similarly. HeLa and the stably transfected cell lines (Mock-HeLa sc1, Prox1-HeLa sc8 and Prox1-HeLa sc55) were cultured in triplicate and transfected using Lipofectamine™2000 (Invitrogen) with 1 μg luciferase vector and 50 ng pRL-TK vector (Promega) as an internal control. Twenty-four hours after transfection, cells were harvested, lysed, and assayed for luciferase activity using the

Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The promoter activity of the -18 bp construct was normalized to 1.0. The mean ± SD was calculated.

Inhibition of Sp1 Transcription Factor by the Treatment with Mithramycin A or siRNA of Sp1

To suppress Sp family transcription factors, mithramycin A (Fluka, Buchs, Switzerland) was used. HeLa cells were treated with mithramycin A (200 or 500 nM) for 24 hours. In siRNA experiments, siRNAs directed against Sp1, Sp3, or a scrambled siRNA was transfected into HeLa cells using Lipofectamine™2000 (Invitrogen). Seven hours after transfection, the medium was changed and treated cells were incubated for a total of 72 hours. The siRNAs directed against human Sp1 and Sp3 had the sequences 5'-GGAUGGUUCUGGUCAAAUATT-3' and 5'-GGGAUAGGAACUGUUAAUATT-3', respectively. We used the Non-specific Control Duplex XI (36% GC content, Dharmacon, Lafayette, CO) as a scrambled control, considering GC content of respective target siRNA described above. At the end of the treatment period, cells were collected and their RNA or protein lysates were extracted.

5-aza-2'-deoxycytidine and/or Trichostatin A Treatment

Cells were seeded at a low density in 60 mm diameter culture plates. Two days after plating, the cells were incubated with 10 μM 5-aza-2'-deoxycytidine (5-aza-dC; Sigma) for 72 hours or 330 nM trichostatin

A (TSA) for 24 hours. Because cells cannot survive by the simultaneous treatment with both reagents, cells were cultured in the presence of 10 μM 5-aza-dC (72 hours) followed by treatment with 330 nM TSA (24 hours). The medium and reagents were replaced daily. At the end of the treatment period, cells were collected and their RNA or DNA was extracted for further experiments.

Bisulfite PCR and DNA Sequencing

We carried out bisulfite modification of genomic DNA. Briefly, 200 ng of genomic DNA extracted from cells was processed using a Methylamp™ DNA Modification Kit (Epigentek, Brooklyn, NY) according to the manufacturer's protocol. Methyl Primer Express® software v1.0 (Applied Biosystems) was used to search for CpG islands in the 5' region of the *PRKCB* gene and to design primers for bisulfite PCR amplification. Two μl of modified DNA with bisulfite was subjected to PCR to amplify the CpG island sequence in the *PRKCB* promoter. The following primer pair was used for PCR: forward 5'-TTGTAGAGTTGCGGTTGTGTA-3', reverse 5'-CAACCGAATCAACCATCTTA-3'. To sequence bisulfite-PCR products, fragments were cloned into pCR4-TOPO vector using a TOPO TA Cloning® Kit for Sequencing (Invitrogen). DNA sequencing of each clone was carried out using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). DNA methylation status and statistical significances between the two groups were analyzed using the web-based tool QUMA (QUantification tool for Methylation Analysis; http://quma.cdb.riken.jp/top/quma_main_j.html,

(Kumaki et al., 2008)).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed with a ChIP-IT™ Express Enzymatic Kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. In brief, DNA and protein complexes were cross-linked with formaldehyde. The DNA was sheared into small and uniform fragments by enzymatic digestion. Anti-Sp1 or anti-Sp3 antibody (Santa Cruz Biotechnology) was added to precipitate specific transcription factor protein-DNA complexes. Mouse IgG (Active Motif) was used as a negative control antibody. Cross-linked chromatin without immunoprecipitation with specific antibodies was used as an input control. Following immunoprecipitation, cross-linking was reversed and proteins were digested with proteinase K. Next, DNA was isolated for PCR analysis. The *PRKCB* promoter region containing Sp1 binding sites was amplified by PCR using the following primers: forward 5'-GGCGCCGATATGTAAAGCA-3' and reverse 5'-GAGCCGGGGCTGTCACTC-3' (producing a 169 bp PCR product).

Statistical Analysis

All experiments were performed at least three times. Results are expressed as the mean ± SD. Excel-Toukei 2010 (Social Survey Research Information Co., Ltd., Tokyo, Japan) was used for the statistical analysis. A value of $p<0.05$ was considered statistically significant.

RESULTS

Characterization of PROX1-overexpressing Cells

Two representative PROX1-overexpressing clones (Prox1-HeLa sc8 and sc55) were selected and used for subsequent experiments. In the original HeLa cell line, endogenous PROX1 is barely detectable, whereas in the two subclones we selected, PROX1 is clearly detected by immunoblotting (Fig. 1A). To examine the effects of PROX1 overexpression on cell proliferation, cell growth of Prox1-HeLa and control cells was monitored using the MTT assay. Cell proliferation in Prox1-HeLa cells was significantly inhibited as compared with those of the original HeLa or mock-transfected cells (Fig. 1B). We also evaluated tumor formation in vivo using nude mice. Whereas mice inoculated with original HeLa cells or mock-transfected cells developed a considerable number of subcutaneous tumors, during our observation period, no solid tumors were seen in mice inoculated with Prox1-HeLa cells (Fig. 1C).

Gene Expression Profile of PROX1-overexpressing Cells

To search for PROX1 target genes, we compared the gene expression profiles of Prox1-HeLa sc8 cells with that of mock-transfected control cells. Table 1 lists the top 10 genes exhibiting up- or down-regulation by PROX1. We focused on protein kinase C βII (PRKCB2), which showed the highest level of down-regulation in Prox1-HeLa cells (undetectable under our microarray conditions) as compared with control cells. Although the PKC family consists of 9 isoforms, no other isoforms were included on this list. To validate the

gene expression profile results for *PRKCB2*, we examined *PRKCB2* mRNA and protein levels. In Prox1-HeLa cells, mRNA expression of *PRKCB2* was undetectable after 35 PCR cycles; however, after 40 cycles, we could detect a very small amount of PCR product (Fig. 2A, RT-PCR). Similarly, PRKCB2 protein levels in Prox1-HeLa cells were significantly decreased as compared with those in mock-transfected cells (Fig. 2A, IB). We further analyzed the relationship between PROX1 and PRKCB2 expression using other cancer cell lines. As shown in Fig. 2B, among examined colon cancer cell lines, DLD-1 showed high PROX1 and low PRKCB2 protein levels. In contrast, HCT116 exhibited the inverse expression pattern, namely, lack of PROX1 and high expression of PRKCB2. These data suggests the possibility that PROX1 suppresses the expression of PRKCB2 in some colon cancer cell lines.

Effects of the PRKCB Inhibitor Enzastaurin on Cell Proliferation

Protein kinase C (PKC) is a family of serine/threonine kinases whose members have been implicated in the regulation of cell proliferation, differentiation and survival. PRKCB, one of the isoforms in PKC family, has been reported to be involved in oncogenesis, and is considered an important target for antitumor drug development. To address the PRKCB function for cell growth of mock-transfected and Prox1-HeLa cells, we treated these cell lines with the PRKCB specific inhibitor Enzastaurin. As shown in Fig. 3, treatment with Enzastaurin for 12 hours reduced viability of mock-transfected control cells to about 50% of levels observed for vehicle (DMSO)-treated cells. These data show that Prox1-HeLa cells are more

resistant to Enzastaurin treatment than control cells. Notably, Enzastaurin inhibits proliferation of Prox1-HeLa cells, which have very low PRKCB2 expression, suggesting another activity of Enzastaurin in addition to inhibition of PRKCB2. Consistent with this idea, Enzastaurin is known to affect other signaling pathways, such as the PI3K/AKT pathway (Moreau et al., 2007).

Analysis of the *PRKCB* Promoter

The *PRKCB* gene encodes two alternative splice variants, PRKCB1 and PRKCB2, which are transcribed from the same start site and differ only in the 3' region, generating different carboxyl termini in the resulting proteins (Ono et al., 1987). Previous studies have identified a putative 5'-promoter region for *PRKCB* (Niino et al., 1992; Obeid et al., 1992) and suggested that there was heterogeneity in the active promoter region dependent upon the cellular context (Niino et al., 1992). The results of our gene expression profiling analysis, as reported above, suggest that *PRKCB* might be a target of PROX1. To help understand the mechanism of transcriptional control of *PRKCB*, we prepared a series of luciferase reporter vectors that contain various regions of the 5'-promoter of *PRKCB* and measured their reporter activity. We found that *PRKCB* promoter activity is maintained in the -110 bp/Luc reporter vector, suggesting that the region between -110 bp and the first exon is sufficient for basal *PRKCB* promoter activity in HeLa cells (Fig. 4A). The results of a computational analysis using the TFSEARCH program (http://mbs.cbrc.jp/research/db/TFSEARCH.html) indicated that this region

contains two putative Sp1 binding sites. Furthermore, previous studies identified two different consensus sequences as putative *prospero* binding sites [*i.e.* C(a/t)(c/t)NNC(t/c) and (T)AAGACG] (Hassan et al., 1997; Cook et al., 2003). We were also able to identify two putative PROX1 binding sites in this region (Fig. 4A left). Further promoter deletion analysis suggests that the Sp1 sites are more important for *PRKCB* transcription than the predicted PROX1 binding sites (Fig. 4B). We found that Prox1-HeLa, mock, and original HeLa cells do not exhibit differences in promoter activity as assayed using the luciferase reporter vectors. This finding was further analyzed below (see also Discussion).

Involvement of Sp Proteins in *PRKCB* Transcription

The results of our promoter analysis suggested a positive role for the Sp1 binding sites in transcription of *PRKCB*. To further examine the possible involvement of Sp family transcription factors in *PRKCB* regulation, we treated original HeLa cells with mithramycin A, a relatively specific inhibitor of Sp family proteins, as well as with siRNA directed against Sp1 or Sp3. As shown in Fig. 5, mithramycin A and RNAi knockdown of Sp1 resulted in down-regulation of PRKCB2 protein expression levels. In contrast, treatment with siRNA directed against Sp3 did not have a significant effect. We sometimes observed a slight increase in cellular Sp3 following treatment with siRNA directed against Sp1. The reason is not clear at the moment. The effect of siRNA treatment on *PRKCB2* mRNA expression was also confirmed by RT-PCR (Supplementary Fig. 1). Taken together, these

data suggest that Sp1 transcription factor activity is important for PRKCB2 expression.

Chromatin Remodeling by PROX1 Expression

It has recently been recognized that chromatin remodeling is an important regulatory mechanism for transcription. DNA methylation and histone modifications regulate target gene expression through changes to chromatin structure and subsequent transcriptional activation. To ask if these mechanisms are associated with down-regulation of *PRKCB2* gene expression in Prox1-HeLa cells, we treated Mock- and Prox1-HeLa cells with the demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC, 10 μM) and/or the histone deacetylase inhibitor trichostatin A (TSA, 330 nM). Exposure of cells to TSA alone did not induce *PRKCB2* mRNA expression. However, 5-aza-dC treatment significantly restored *PRKCB2* mRNA expression in Prox1-HeLa cells (Fig. 6). We next asked if a similar effect of 5-aza-dC could be observed for the colon cancer cell line DLD-1, which exhibits the same protein expression pattern of PROX1 and PRKCB2 as Prox1-HeLa cells. Treatment with 5-aza-dC did induce *PRKCB2* mRNA expression in DLD-1 cells (Supplementary Fig. 2A). These data suggest that the 5'-promoter of the *PRKCB* gene is methylated in PROX1-expressing cells.

Methylation Status of the *PRKCB* Promoter

Based on these results, we searched the *PRKCB* promoter region for putative CpG islands using Methyl Primer Express® software and found a predicted

1115 bp CpG island in this region (Fig. 7A). To characterize the site, we performed bisulfite sequencing in order to determine the methylation status of the CpG island. We primarily analyzed the methylation status of the region surrounding the transcription start site, similar to what was described for our promoter analysis (see above). The region containing 52 CpG sites was more methylated in Prox1-HeLa cells as compared to that of mock cells (left side of Fig. 7B). The CpG site in the distal Sp1 binding site described in Fig. 4 was hypermethylated in Prox1-HeLa cells (illustrated as CpG 10 in Fig. 7B). As 5-aza-dC is capable of inducing re-expression of *PRKCB2* in Prox1-HeLa cells, we also examined the methylation status of this region following 5-aza-dC treatment. Treatment with 5-aza-dC led to a significant reduction of DNA methylation levels in Prox1-HeLa cells, whereas in mock cells, the same treatment did not lead to detectable changes in the methylation status of this promoter region (right side of Fig. 7B). In addition, we assessed the methylation status of the CpG sites in the Sp1 binding motif in colon cancer cell lines that do or do not express PROX1 endogenously. As expected, the CpG site was hypermethylated in DLD-1 cells, which express PROX1, whereas that of HCT116 cells was not (Supplementary Fig. 2B).

ChIP Assay

We performed a ChIP assay to directly test Sp1 transcription factor binding to the 5'-promoter region of *PRKCB* as suggested by our promoter analysis and bisulfite sequencing. As indicated in Fig. 7C, the results of the ChIP

assay show that Sp1 does bind to the *PRKCB* promoter region in mock-transfected cells. Additionally, the level of Sp1 binding to this region in Prox1-HeLa cells was significantly less than what was observed in mock-transfected cells.

DISCUSSION

In this study, we demonstrated that the overexpression of PROX1 reduces cell proliferation and tumor formation in part via down-regulation of PRKCB2. We also showed that *PRKCB2* is silenced by promoter hypermethylation in PROX1-expressing cells but not in cells lacking endogenous expression of PROX1. Thus, PROX1 might affect the DNA methylation status of the *PRKCB* promoter region.

PROX1 was originally thought to be a tumor suppressor based on the finding that in *Drosophila*, loss of *prospero* shifts the balance toward uncontrolled proliferation through the deregulation of cell cycle-related genes, leading to the formation of tumors with neuroblastoma-like features (Li and Vaessin, 2000; Caussinus and Gonzalez, 2005; Betschinger et al., 2006; Choksi et al., 2006). The characteristics of our Prox1-HeLa subclones are consistent with this idea (Fig.1). Moreover, a similar inhibitory effect of PROX1 on cell growth and tumor formation in nude mice has been reported previously for other tumor cells (Shimoda et al., 2006; Takahashi et al., 2006). Given the potential clinical impact of identifying factors that promote cancer cell growth and tumorigenicity, we were interested to identify target genes down-regulated in the presence of PROX1. In addition, identifying novel targets might help clarify the molecular mechanisms underlying how PROX1 regulates target genes in an apparently complex and cell type-specific manner.

Several recent reports have argued against the classical idea of *PROX1* as a tumor suppressor. Petrova *et al.* reported a tumor promoting

activity of *Prox1* in $Apc^{min/+}$ mice models (Petrova et al., 2008). Others have also demonstrated that PROX1 overexpression promotes aggressive behavior in mouse models of kaposiform hemangioendotheliomas (Dadras et al., 2008). These apparently contradictory results, wherein PROX1 can act as a transcriptional activator or repressor, suggest that PROX1 activity is highly dependent on the cellular context, including the presence or absence of co-factors.

In our microarray analysis, we found that *PRKCB2* mRNA levels showed the largest degree of inhibition when PROX1 was overexpressed. PRKCB enhances expression of cyclin D1 in human breast cancer cells, leading to enhanced cell cycle progression and cell proliferation (Li and Weinstein, 2006). Overexpression of PRKCB2 induces colonic hyperproliferation and increased sensitivity to colon carcinogenesis (Murray et al., 1999; Gokmen-Polar et al., 2001). PRKCB also plays an important role in hematologic malignancies, especially lymphoid malignancies. It is one of the most overexpressed genes and levels of expression are linked to poor prognosis in patients with diffuse large B-cell lymphoma (Shipp et al., 2002). Its overexpression in chronic lymphocytic leukemia has also been reported (Abrams et al., 2007). These findings suggested that PRKCB is an important target for anticancer therapy. Enzastaurin (LY317615), initially developed as an ATP-competitive selective inhibitor of PRKCB (Faul et al., 2003), also targets the phosphatidylinositol 3-kinase/AKT pathway and inhibits GSK3β and ribosomal protein S6 phosphorylation (Graff et al., 2005). Enzastaurin inhibits proliferation and induces apoptosis in cancer cell lines and xenograft

mouse models (Graff et al., 2005; Moreau et al., 2007). Following these promising pre-clinical results, encouraging results have recently been obtained in several phase II studies (Robertson et al., 2007; Morschhauser et al., 2008). In our study, PROX1-expressing cells, in which PRKCB2 levels are down-regulated, appear to be more resistant to Enzastaurin treatment than control cells, although some inhibitory effects were still observed in PROX1-overexpressing cells that do exhibit a very low level of PRKCB2 expression (Fig. 3). This suggests that PROX1-induced cell growth suppression might be explained in part by down- regulation of PRKCB2.

The 5'-promoter of the human *PRKCB* gene has been investigated previously (Niino et al., 1992; Obeid et al., 1992), and these studies showed that a small region of the 5'-proximal promoter is particularly important for promoter activity of the *PRKCB* gene. Results presented in this study are compatible with these data. We also demonstrated that the region between -110 bp and -48 bp, which contains two Sp1 sites, is important for the transcription activity (Fig. 4). Moreover, an important role for the Sp1 transcription factor was suggested by the results of tests using a chemical inhibitor of Sp family transcription factors and RNAi against Sp1 (Fig.5). Although the results of our promoter analysis suggest that PROX1 binding sites are not essential for *PRKCB* transcription, their possible contribution should not be overlooked (Fig. 4). A possible explanation for this finding is that the inhibitory effects of PROX1 on PRKCB2 protein level are not exerted at the transcriptional level. However, another possibility is that the

luciferase reporter is not sensitive enough to detect epigenetic changes such as DNA methylation and histone acetylation.

In support of this second possibility, we found that treatment with an inhibitor of DNA methyltransferase, 5-aza-2'-deoxycytidine, restored *PRKCB2* mRNA levels in Prox1-HeLa cells (Fig. 6), suggesting that PROX1 overexpression normally suppresses *PRKCB2* transcription via enhanced methylation of CpG islands in the 5'-promoter of the *PRKCB* gene. The results of bisulfite sequencing clearly showed that the area around the transcription start site was more densely methylated in Prox1-HeLa cells as compared with control cells (Fig. 7AB). Treatment with 5-aza-dC relieved DNA methylation in this area as well in other regions (Fig. 7B). Notably, those regions showing changes in DNA methylation status coincided with the regions detected as relevant in the promoter analysis (see Fig. 4). The results of a comparison of DNA methylation status in DLD-1 versus HCT116 cells support our hypothesis that methylation status of the area containing Sp1 binding sites affects *PRKCB2* transcription (Supplementary Fig. 2). Furthermore, the results of our ChIP assay showed reduced binding of Sp1 to the *PRKCB* promoter in PROX1-overexpressing cells (Fig 7C). These findings suggest that methylation of the CpG site within the Sp1 binding site inhibits binding of Sp1 to the promoter, leading to down-regulation of *PRKCB* transcription.

The results of our present study using PROX1-overexpressing cells demonstrate, for the first time, an inverse relationship between PROX1 and PRKCB2 expression. Furthermore, our results suggest the possibility that

PRKCB2 inhibition is at least partially due to DNA methylation at the 5'-promoter of *PRKCB*, providing evidence for a novel aspect of PROX1 function. Our present data should be interpreted cautiously, as these results were mainly obtained from HeLa cell lines, and as the pathophysiological role of PROX1 is cellular context-dependent. Nevertheless, our analysis of colon cancer cell lines with differences in endogenous PROX1 expression suggests that the inverse relationship between PROX1 and PRKCB2 exists in some tumor cells, in addition to being observed in HeLa cells in which PROX1 expression was artificially modulated. Particularly given the possible clinical relevance of Enzastaurin to cancer treatment, gaining a better understanding of PROX1 through the use of clinical tumor samples, as well as further mechanistic studies in cell lines, seems warranted.

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Figure Legends

Figure 1: PROX1-overexpressing cells show reduced cell proliferation and tumorigenicity.

(A) Establishment of HeLa cell clones stably overexpressing PROX1. Two cell clones constitutively expressing PROX1 were established (Prox1-sc8, sc55). PROX1 expression was confirmed by immunoblotting. β-actin was shown as an internal control. (B) Effect of PROX1 overexpression on cell proliferation as measured by the MTT assay. Original HeLa, mock-transfected control cells (Mock), and PROX1-overexpressing cells (Prox1-HeLa) were cultured and their cell growth was examined. Three independent experiments were carried out and representative data are illustrated. (C) In vivo tumor formation assay. Transfected or original HeLa cells were injected subcutaneously into the flanks of 8 nude mice /group according to the Materials and Methods. Tumor formation was surveyed 42 days after injection.

Figure 2: The down-regulation of PRKCB2 expression in PROX1-expressing cells.

(A) Effect of PROX1 overexpression on *PRKCB2* mRNA and protein levels. RT-PCR and immunoblotting were performed as described in the Materials and Methods. PCR was carried out for 40 cycles (*PRKCB2*) or 30 cycles (*GAPDH*). *GAPDH* and *β-actin* were used as respective internal control. K562 and KMS-12-PE cells were used as a positive and negative control for PRKCB2 expression, respectively. (B) PROX1 and PRKCB2 protein

expression in colon cancer cell lines were examined by immunoblotting. β-actin was used as an internal control.

Figure 3: PRKCB is required for proliferation of HeLa cells.

The effect of Enzastaurin on cell growth was determined using the MTT assay. Cells were treated with either vehicle (DMSO) or Enzastaurin (10 μM) for 12 hours. The data are expressed as a percentage of DMSO-treated cells (the mean ± SD from triplicate experiments). An asterisk indicates statistical significance between the samples (*$p<0.05$).

Figure 4: Analysis of the 5'-promoter of the *PRKCB* gene.

(A) The 5'-promoter region of *PRKCB* was examined for promoter activity using various truncated luciferase constructs as shown at the left. The transcription start site was designated as +1. The locations of putative transcription factor binding sites between -110 bp and the first exon are indicated at the lower left. Luciferase assays were performed as described in the Materials and Methods. Promoter activity of the -18 bp construct was normalized to 1.0. The mean ± SD of three independent experiments is shown. (B) Promoter analysis was performed using luciferase constructs with or without Sp1 binding sites. Luciferase assays were performed as described in the Materials and Methods. Promoter activity of the -18 bp construct was normalized to 1.0. The mean ± SD of three independent experiments is shown.

Figure 5: Effect of inhibiting Sp family transcription factor activity on PRKCB2 expression.

To inhibit Sp1 transcription factor activity, HeLa cells were treated with mithramycin A (0, 200, or 500 nM) for 24 hours (A), or transfected with 50 nM of siRNA directed against Sp1, Sp3, or a scrambled control siRNA and then incubated for 72 hours (B). The treated cells were then harvested and cell lysates were subjected to immunoblotting using the indicated antibodies. β-actin was used as an internal control. Relative PRKCB2 protein levels are expressed as the ratio of PRKCB2/β-actin as quantified using ImageJ software.

Figure 6: *PRKCB2* mRNA expression in the presence or absence of 5-aza-dC and/or TSA treatment.

(A) *PRKCB2* mRNA levels in PROX1-overexpressing or control cells with or without 5-aza-dC and/or TSA treatment were examined by RT-PCR. *GAPDH* was used as an internal control. PCR was carried out for 35 cycles (*PRKCB2*) or 30 cycles (*GAPDH*). Representative data of three independent experiments is shown. (B) Quantification of *PRKCB2* mRNA expression by quantitative real-time PCR. Data were normalized to *GAPDH* mRNA levels and expression of *PRKCB2* in untreated mock-transfected control cells was normalized to 1.0. The mean ± SD of three independent experiments is shown. N.D., not detected.

Figure 7: PROX1 expression affects the methylation status of the *PRKCB*

promoter region.

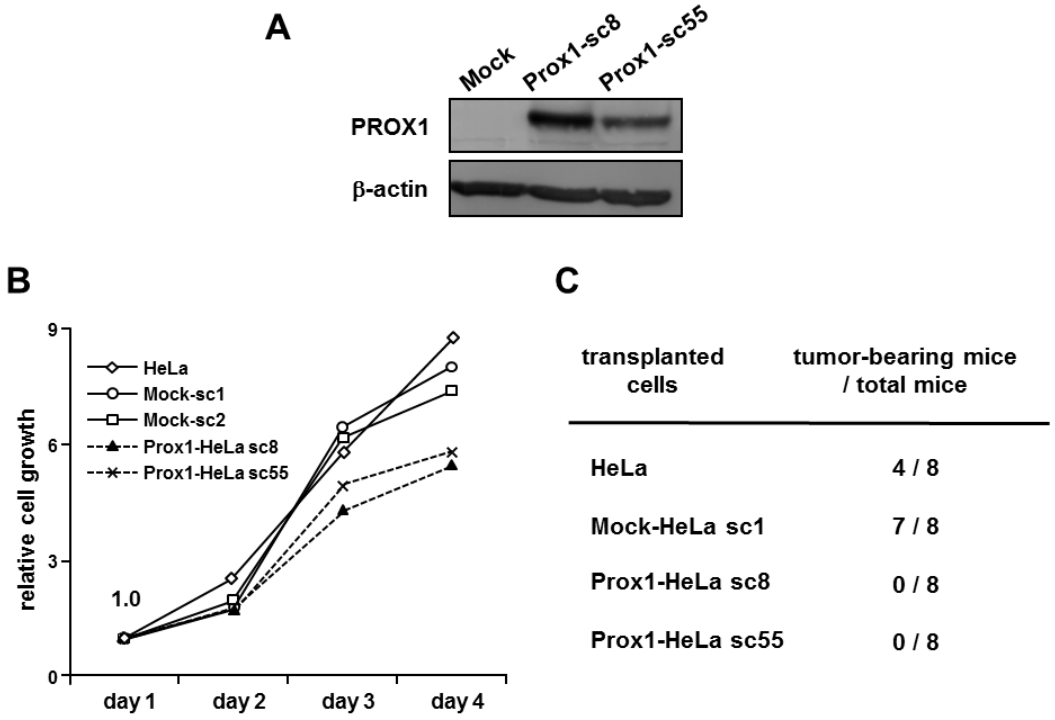
(A) The 5'-promoter region of the *PRKCB* gene. A putative CpG island predicted using Methyl Primer Express® software is indicated with a solid bar and each CpG site within this region is indicated with a vertical bar. The arrow indicates the transcription start site (+1). The CpG island around the transcription start site (underlined) was analyzed by bisulfite sequencing as described in the Materials and Methods. In total, 52 CpG sites between locations -164 bp and +207 bp were identified. The location of two relevant CpG sites, 10 and 13, are shown. Two gray boxes indicate putative Sp1 binding sites as shown in Fig. 4. (B) Results of bisulfite sequencing. Diagrams show the methylation status of 13 CpG sites (CpG 9–21 shown in (A)) in the PRKCB promoter CpG island. We performed bisulfite sequencing using control cells (upper panel) and Prox1-HeLa sc8 cells (lower panel) with or without 5-aza-dC treatment. One horizontal row of circles denotes each PCR clone analyzed. Solid and open circles represent methylated and unmethylated CpGs, respectively. Arrowheads indicate CpG sites that include the Sp1 binding sites as shown in (A). (C) ChIP assay was performed using mock-transfected (M) and Prox1-HeLa sc8 (P) cells as described in the Materials and Methods. A sample immunoprecipitated with mouse IgG (mIgG) was used as the negative control. Sp1 and Sp3 indicate the samples immunoprecipitated with anti-Sp1 and anti-Sp3 antibody, respectively.

Table 1. Top 10 down- and up-regulated genes identified in this study

Symbol	Gene	Genbank ID
Prox1-down-regulated genes		
PRKCB1	protein kinase C, beta 1, transcript variant 2	NM_002738
EPB41L3	erythrocyte membrane protein band 4.1-like 3	NM_012307
EVA1	epithelial V-like antigen 1, transcript variant 1	NM_005797
COL4A3	collagen, type IV, alpha 3, transcript variant 1	NM_000091
LXN	latexin	NM_020169
CXCR4	chemokine (C-X-C motif) receptor 4, transcript variant 2	NM_003467
EGFLAM	EGF-like, fibronectin type III and laminin G domains, transcript variant 1	NM_152403
UTS2D	urotensin 2 domain containing	NM_198152
PCDHA4	protocadherin alpha 4, transcript variant 1	NM_018907
CSS3	chondroitin sulfate synthase 3	NM_175856
Prox1-up-regulated genes		
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	NM_000927
LZTFL1	leucine zipper transcription factor-like 1	NM_020347
KIAA1914	KIAA1914, transcript variant 1	NM_001001936
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	NM_004827
CCRL1	chemokine (C-C motif) receptor-like 1, transcript variant 1	NM_178445
ROR1	receptor tyrosine kinase-like orphan receptor 1	NM_005012
SPANXB1	SPANX family, member B1	NM_032461
ICAM1	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	NM_000201
FAM20A	family with sequence similarity 20, member A	NM_017565
ZNF230	zinc finger protein 230	NM_006300

NOTE: Microarray analysis was performed to compare the gene expression profiles between Prox1-HeLa sc8 and mock-transfected cells as described in the Materials and Methods. Among the genes showing a greater than 1.4-fold difference, the ten most up- or down-regulated genes in Prox1-overexpressing cells as compared with control cells are shown. The genes on this list display a 200-fold lower (Prox1-down-regulated group) or higher (Prox1-up-regulated group) expression between Prox1-HeLa and mock-transfected cells in our assay conditions.

Figure 1



C

transplanted cells	tumor-bearing mice / total mice
HeLa	4 / 8
Mock-HeLa sc1	7 / 8
Prox1-HeLa sc8	0 / 8
Prox1-HeLa sc55	0 / 8

Figure 2

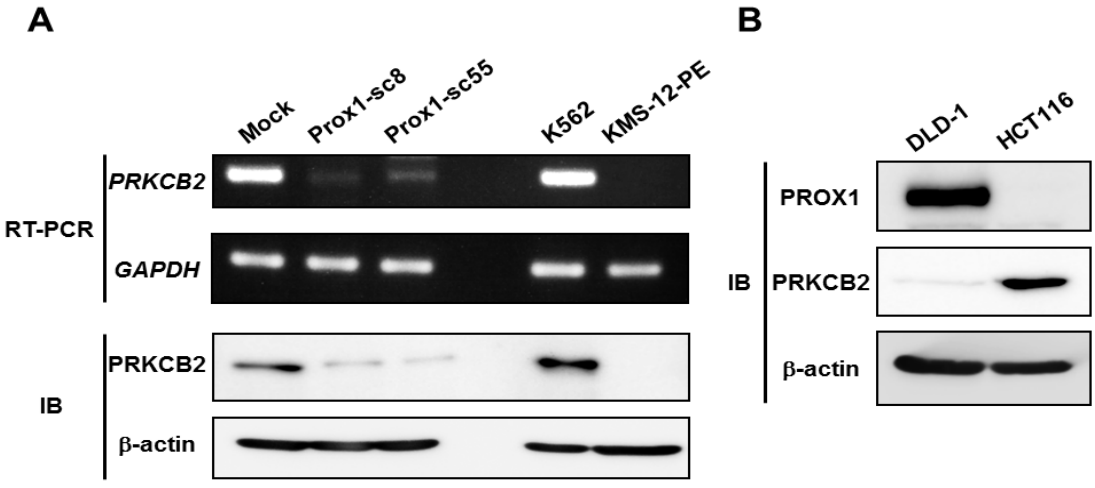


Figure 3

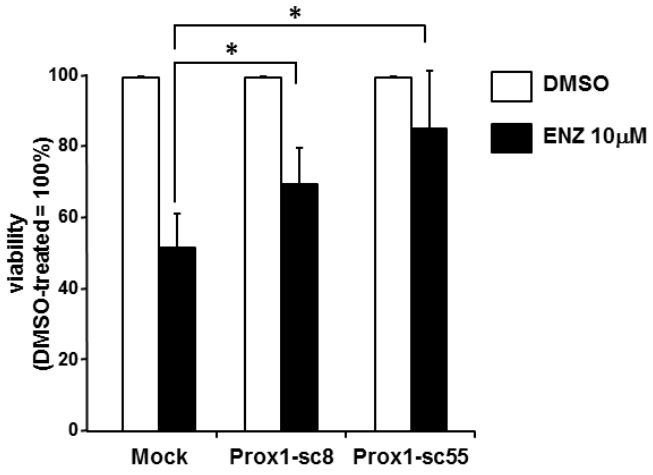


Figure 4

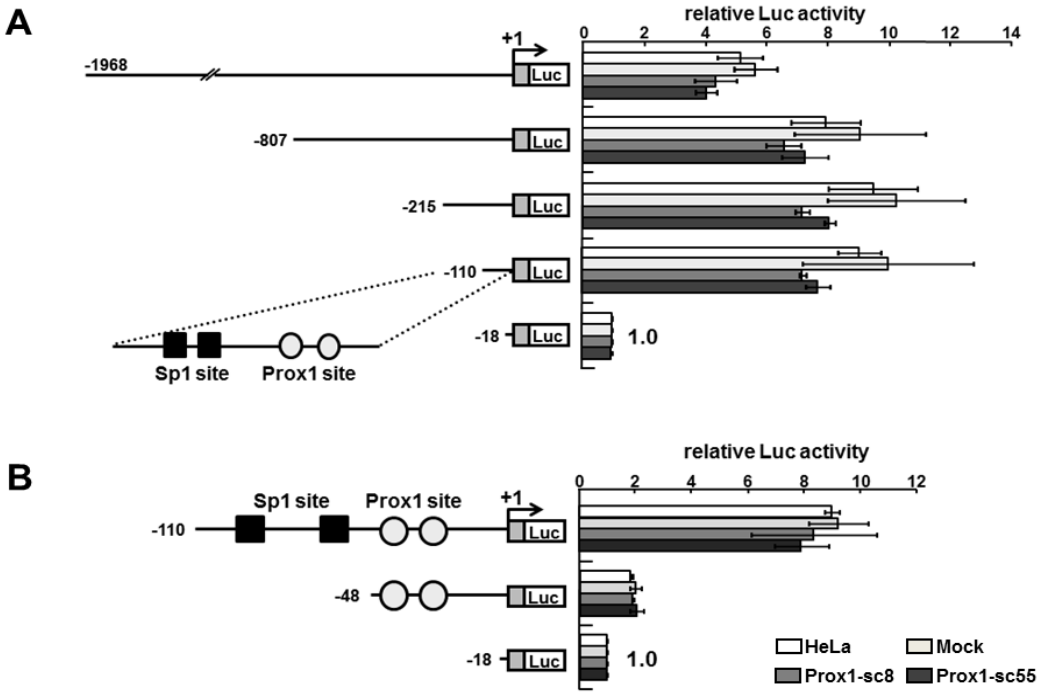


Figure 5

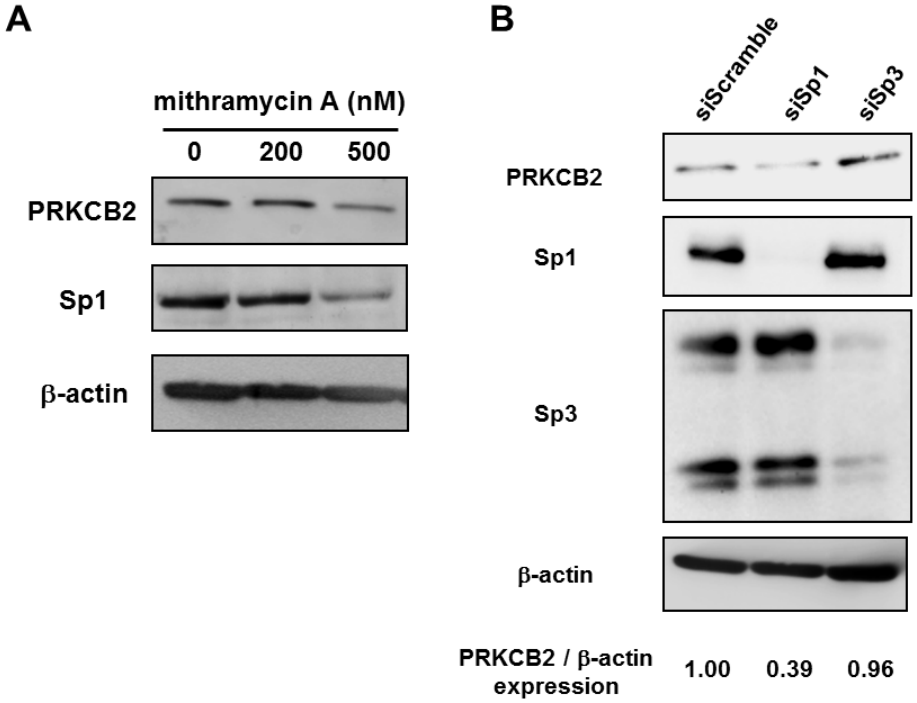


Figure 6

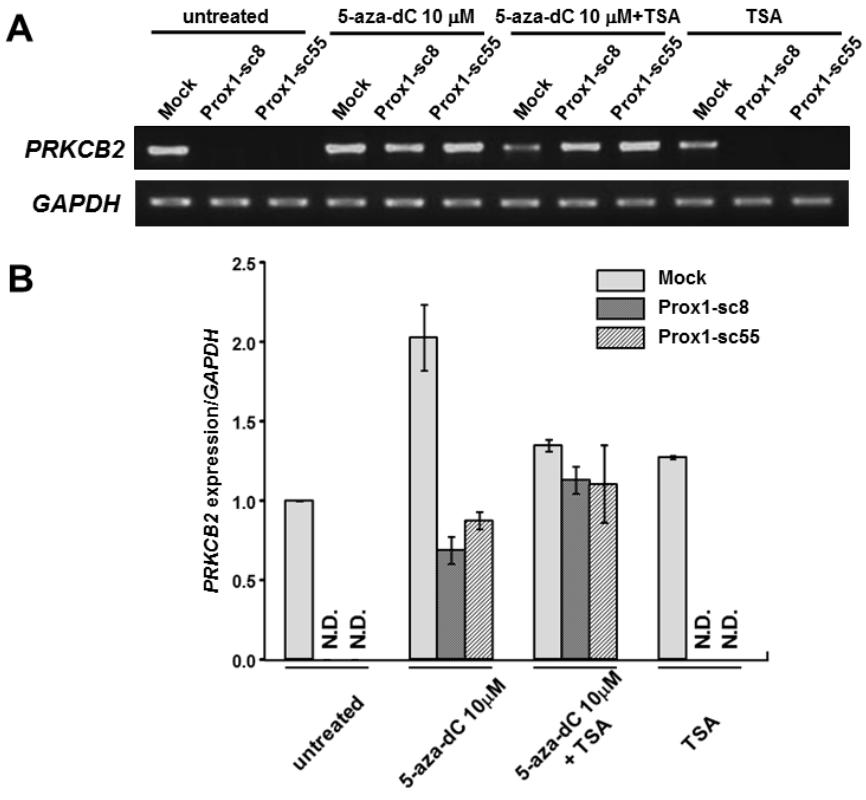
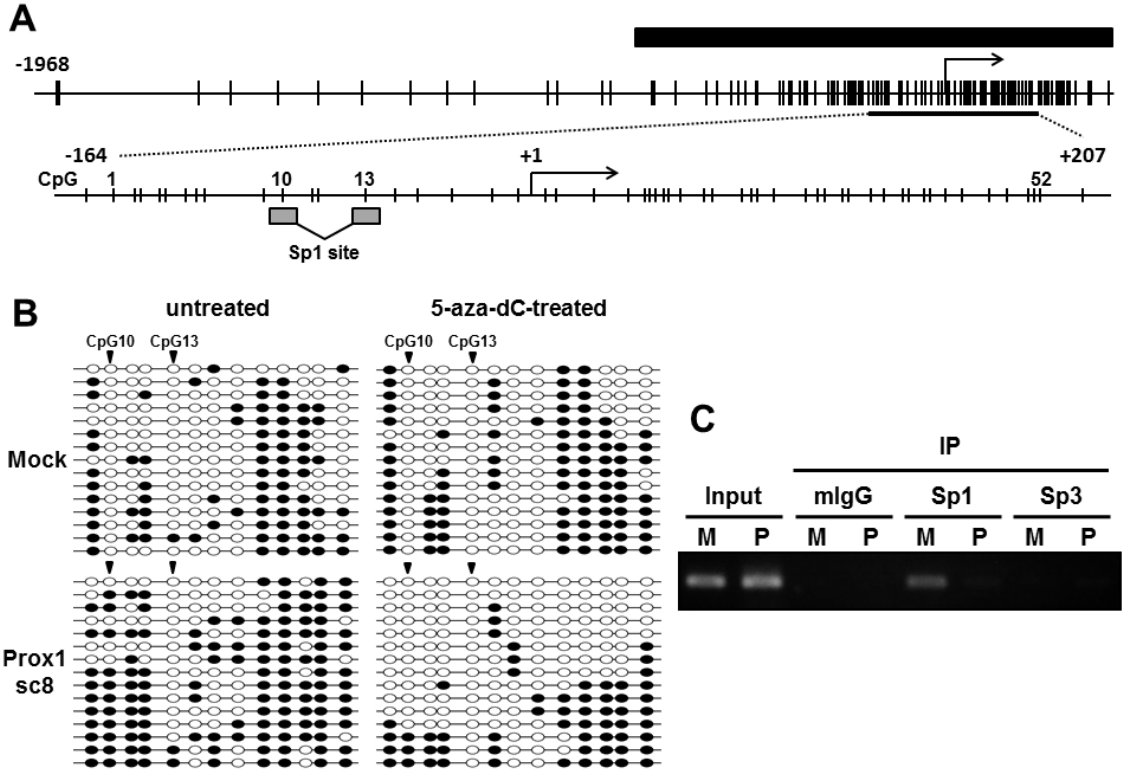


Figure 7


A
-1968
-164
+1
+207
CpG
1
10
13
52
Sp1 site
B
untreated
5-aza-dC-treated
CpG10
CpG13
Mock
Prox1
sc8
C
IP
Input
mIgG
Sp1
Sp3
M
P

Supplementary Figure Legends

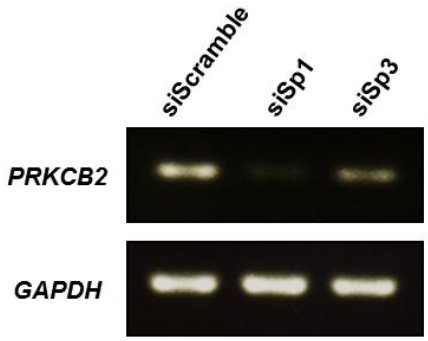
Supplementary Figure 1: Effect of inhibiting Sp protein family transcription factor activity on *PRKCB2* mRNA expression.

HeLa cells were transfected with siRNA directed against Sp1 or Sp3 or a scrambled siRNA (50 nM) for 72 hours and mRNA expression of *PRKCB2* was examined by RT-PCR. *GAPDH* was used as an internal control.

Supplementary Figure 2: The methylation status of *PRKCB* promoter in colon cancer cells that do or do not express PROX1 endogenously.

(A) *PRKCB2* mRNA levels following 5-aza-dC treatment in DLD-1 cells were determined by RT-PCR. *GAPDH* was used as an internal control. (B) Results of bisulfite sequencing using two colon cancer cell lines, DLD-1 (upper panel) and HCT116 (lower panel). These two cell lines exhibited different expression levels of PROX1; high PROX1 in DLD-1, whereas no detectable PROX1 in HCT116 (see Fig. 2B). Diagrams show the methylation status of 13 CpG sites (CpG 9-21 shown in Fig. 7A and B) in the *PRKCB* promoter CpG island. One horizontal row of circles denotes each PCR clone analyzed. Solid and open circles represent methylated and unmethylated CpGs, respectively. Arrowheads show CpG sites included Sp1 binding sites shown in Fig. 7A, CpG 10 and 13.

Supplementary Figure 1



Supplementary Figure 2