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Human Cancer Biology

Clinical Cancer Research

Epigenetic Alteration of *PRKCDBP* in Colorectal Cancers and Its Implication in Tumor Cell Resistance to TNFα-Induced Apoptosis

Jin-Hee Lee[1], Min-Ju Kang[1], Hye-Yeon Han[1], Min-Goo Lee[1], Seong-In Jeong[1], Byung-Kyu Ryu[1], Tae-Kyu Ha[1], Nam-Goo Her[1], Jikhyon Han[1], Sun Jin Park[2], Kil Yeon Lee[2], Hyo-Jong Kim[3], and Sung-Gil Chi[1]

Abstract

Purpose: *PRKCDBP* is a putative tumor suppressor in which alteration has been observed in several human cancers. We investigated expression and function of PRKCDBP in colorectal cells and tissues to explore its candidacy as a suppressor in colorectal tumorigenesis.

Experimental Design: Expression and methylation status of *PRKCDBP* and its effect on tumor growth were evaluated. Transcriptional regulation by NF-κB signaling was defined by luciferase reporter and chromatin immunoprecipitation assays.

Results: PRKCDBP expression was hardly detectable in 29 of 80 (36%) primary tumors and 11 of 19 (58%) cell lines, and its alteration correlated with tumor stage and grade. Promoter hypermethylation was commonly found in cancers. PRKCDBP expression induced the G_1 cell-cycle arrest and increased cellular sensitivity to various apoptotic stresses. PRKCDBP was induced by TNFα, and its level correlated with tumor cell sensitivity to TNFα-induced apoptosis. PRKCDBP induction by TNFα was disrupted by blocking NF-κB signaling while it was enhanced by RelA transfection. The *PRKCDBP* promoter activity was increased in response to TNFα, and this response was abolished by disruption of a κB site in the promoter. PRKCDBP delayed the formation and growth of xenograft tumors and improved tumor response to TNFα-induced apoptosis.

Conclusions: *PRKCDBP* is a proapoptotic tumor suppressor which is commonly altered in colorectal cancer by promoter hypermethylation, and its gene transcription is directly activated by NF-κB in response to TNFα. This suggests that PRKCDBP inactivation may contribute to tumor progression by reducing cellular sensitivity to TNFα and other stresses, particularly under chronic inflammatory microenvironment. *Clin Cancer Res; 17(24); 7551–62. ©2011 AACR.*

Introduction

PRKCDBP (also known as Cavin3/hSRBC) is a putative tumor suppressor in which genetic and epigenetic alterations have been found in several human malignancies (1–3). The *PRKCDBP* gene encodes for a protein of 261 amino acids, which contains a leucine zipper, a protein kinase C (PKC)-binding site, a PKC phosphorylation site, a phosphatidylserine-binding site, and 2 PEST domains (1). These structural motifs are also found in serum deprivation protein response (SDPR) and pol I and transcription release factor (PTRF), which have similar expression patterns under various growth conditions (4–9).

Caveolae are flask-shaped vesicular invaginations of the plasma membrane characterized by the existence of integral membrane proteins termed caveolins (10). Caveolae modulate cross-talk between distinct signaling cascades, and many cellular functions have been attributed to caveolae, including membrane trafficking, endocytosis, cell adhesion, and apoptosis (10, 11). A recent study showed that PRKCDBP binds to caveolin-1 (CAV1) and traffics with CAV1 to different locations in the cells and directs the formation of caveolar vesicles, indicating that PRKCDBP plays as a caveolin adapter molecule that regulates caveolae function (12). It was also known that PTRF and SDPR are required for the formation and elongation of caveolae, respectively (13–15). On the basis of their roles as regulators for caveolae dynamics, these molecules were named as cavin (PTRF/Cavin1, SDPR/Cavin2, and PRKCDBP/Cavin3) and have been classified as being part of the cavin family with a newly identified muscle-specific member MURC (Cavin4; refs. 15–18).

Authors' Affiliations: [1]School of Life Sciences and Biotechnology, Korea University; and Departments of [2]Surgery and [3]Internal Medicine, School of Medicine, Kyung Hee University, Seoul, Korea

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Corresponding Author: Sung-Gil Chi, School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Republic of Korea. Phone: 82-2-3290-3443; Fax: 82-2-927-5458; E-mail: chi6302@korea.ac.kr

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Translational Relevance

PRKCDBP is a putative tumor suppressor gene which is inactivated in some cancers by genetic or epigenetic alteration. This article shows first that PRKCDBP plays a growth suppressive role in colorectal tumorigenesis. We evaluated the expression status of PRKCDBP in a large set of cancer cell lines and primary carcinomas and found that PRKCDBP expression is commonly lost or decreased in colorectal cancers by aberrant promoter CpG sites hypermethylation and its alteration is associated with malignant tumor progression. Our work also shows that PRKCDBP is directly activated by NF-κB signaling in response to TNFα and its inactivation contributes to tumor growth and the increased resistance to TNFα-induced apoptosis. This work could lead to further investigation of PRKCDBP as a potential target in the treatment of colorectal cancer.

The chromosomal region 11p15 shows frequent deletion in many types of human cancer, including breast, lung, and bladder cancers (1, 19, 20) In particular, 11p15.5-p15.4, in which the *PRKCDBP* gene is located, is a critical region of loss of heterozygosity (LOH) for chromosome 11 in several cancers, suggesting that *PRKCDBP* might be a target tumor suppressor gene in this region (21–23). Moreover, several frameshift and truncation mutations of *PRKCDBP* were found in a few ovarian and lung cancer cell lines, and aberrant promoter hypermethylation leading to downregulation of its expression was observed in a large fraction of breast, lung, and ovarian cancers (1, 2, 24). We recently reported that PRKCDBP expression is commonly reduced in gastric cancers by aberrant promoter CpG sites hypermethylation, and its alteration correlates with stage and grade of tumors (3).

Although several lines of evidence suggest that PRKCDBP may function as a tumor suppressor, the molecular basis of its action has been poorly understood (1–3). PRKCDBP was originally identified in screens looking for PKCδ-binding protein, and found to be phosphorylated *in vivo* by PKCδ, a potential tumor suppressor involved in the regulation of cell proliferation, differentiation, and apoptosis (5, 25). The mRNA for *PRKCDBP* is induced in response to serum deprivation and downregulated during G_0–G_1 transition, suggesting that it may be involved in cell-cycle control (5, 6). Through a yeast 2-hybrid screen, PRKCDBP was also identified as a BRCA1-interacting protein, raising the possibility that PRKCDBP may participate in DNA-damage response, and its inactivation may compromise BRCA1-mediated tumor suppression functions (1). We have shown that PRKCDBP increases the protein stability of p53, and its proapoptotic effect stems partially from the p53-enhancing activity, suggesting that dysregulation of PRKCDBP may attenuate p53 response to stresses and thus contribute to malignant tumor progression (3).

In this study, we found that PRKCDBP expression is lost or reduced in a substantial fraction of colorectal cancers by aberrant promoter hypermethylation, and its altered expression is associated with malignant tumor progression. It was also found that PRKCDBP is a transcription target of TNFα, which plays a crucial role in TNFα-induced apoptosis. We hypothesized that PRKCDBP inactivation contributes to colorectal tumorigenesis by enhancing cellular resistance to various apoptotic stresses including TNFα.

Materials and Methods

Tissue specimens and cancer cell lines

A total of 160 colorectal tissues including 80 primary carcinomas were obtained by surgical resection in the Kyung Hee University Medical Center (Seoul, Korea). Signed informed consent was obtained from each patient. Bits of primary tumors and adjacent portions of each tumor were fixed and used for hematoxylin and eosin staining for histopathologic evaluation. Tumor specimens composed of at least 70% carcinoma cells and adjacent tissues found not to contain tumor cells were chosen for molecular analysis. Nineteen human colorectal cancer cell lines were obtained from Korea Cell Line Bank or American Type Culture Collection

Expression analysis

RNA extraction, cDNA synthesis, and quantitative PCR were carried out as described previously (3, 26). Reverse transcriptase PCR (RT-PCR) for *PRKCDBP* expression was carried out with primers SRBC-4 (sense 5′-TTCTGCTCTT-CAAGGAGGAG-3′) and SRBC-7 (antisense 5′-CCAAGGC-GAGGCGGCTTGAC-3′). For quantitative DNA-PCR, intron 2 region of *PRKCDBP* was amplified with intron-specific primers SRBC-IN1 (sense 5′-CGTCCGCA-GAATTTGGTCTG-3′) and SRBC-5 (antisense 5′-AAGGG-CTCTGGTGCCTTCTG-3′). Western blot analyses were conducted with antibodies specific for CDKN1A/$p21^{Waf1}$ (Santa Cruz Biotechnology), pAKT (Cell Signaling), pGSK3β (Cell Signaling), total AKT (Santa Cruz Biotechnology), RelA/p65 (Santa Cruz Biotechnology), CAV1 (BD Transduction Laboratories), and tubulin (Sigma). The polyclonal antibody against PRKCDBP was generated as described previously (3).

Methylation analysis

Tumor cells were exposed to 5-aza-dC for 4 days, and *PRKCDBP* expression was analyzed by RT-PCR. For methylation-specific PCR analysis, 200 ng of bisulfite-modified DNA was subjected to PCR amplification of the *PRKCDBP* promoter region with methylation-specific primers M04 (sense 5′-GAAATAAAAATTTTCGTGATTC-3′) and M03 (antisense 5′-CTTAAAAACGTTTCGCCTTCCG-3′) and unmethylation-specific primers U04 (sense 5′-GTTGT-GTTAATATAGTTTTTGT-3′) and U03 (antisense 5′-AAAATCTCTTAAAAACATTTCA-3′). For bisulfite sequencing analysis, 50 ng of bisulfite-modified DNA was subjected to PCR amplification of the *PRKCDBP* promoter region with primers seq-1 (sense 5′-CCATCTTCACTAATATAAAAAA-3′)

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and seq-2 (antisense 5′-GTTTTAGTTGTGATTTAGGTAG-3′). The PCR products were cloned into pCR^{II} vectors (Invitrogen Corporation), and 10 clones of each specimen were sequenced by automated fluorescence-based DNA sequencing to determine the methylation status.

Expression plasmids, siRNAs, and transfection

PRKCDBP expression vectors used in this study were described previously (3). Transfection was carried out with Lipofectamine 2000 (Invitrogen Corporation) according to the manufacturer's protocol. To generate stable sublines, RKO cells were transfected with 4 μg of PRKCDBP expression vector and colonies were isolated by G418 selection (1,600 μg/mL). siRNA duplex against PRKCDBP (siGENOME SMART pool reagent, M-016416-00-0005) and CAV1 (5′-AACCAGAAGGGACACACAGUU-3′) were synthesized by Dharmacon Research. Transfection of siRNA was carried out with siRNA-Oligofectamine mixture. A plasmid encoding wild-type or dominant-negative mutant CAV1 (*P132L*) was cloned into the pcDNA3.1-V5-His (Invitrogen Corporation) and the pEGFP-N3 vector (Clontech) using the Expand High Fidelity PCR system (Roche Molecular Biochemicals).

Cell growth and apoptosis assays

Cells (0.5×10^5) were transfected with expression vector or siRNA, and cell numbers were counted with a hemocytometer for 4 days at 24-hour intervals. [^{3}H]thymidine uptake and flow cytometry analyses were conducted as described previously (3, 27). For colony formation assay, cells were transfected with expression vectors encoding wild type (WT)-PRKCDBP or si-PRKCDBP and maintained in the presence of G418 (1,600 μg/mL) for 3 to 4 weeks. Colonies were fixed with methanol for 15 minutes and stained with 0.05% crystal violet in 20% ethanol. TUNEL assay was conducted to evaluate apoptosis induction. Briefly, cells transfected with PRKCDBP expression vectors or si-PRKCDBP were exposed to TNFα (40 ng/mL) for 48 hours. The cells were fixed with 4% paraformaldehyde in PBS, and the buffer containing 3% bovine serum albumin and 0.1% Triton X-100 was added and incubated for 15 minutes at 4°C. The cells were labeled by TUNEL reaction mixture using the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals).

Promoter luciferase and chromatin immunoprecipitation assay

The *PRKCDBP* promoter regions were cloned into the pGL3-basic vector (Promega). The putative NF-κB binding element was mutated (5′-G*TT*ATTG*AAA*-3′) by site-directed mutagenesis. Cells were transfected with 500 ng of the promoter constructs using Lipofectamine 2000 (Invitrogen Corporation). After normalization of each extract for protein content, luciferase activity was measured by Luciferase assay system (Promega). For chromatin immunoprecipitation, cells were incubated in 1% formaldehyde solution for 20 minutes. The cells were lysed, and the pellet was resuspended in nuclei lysis buffer and sonicated. Immunoprecipitation was carried out with p65/RelA antibody (Santa Cruz Biotechnology).

Animal studies

Four-week-old immunodeficient female nude mice (nu/nu; Orient Bio Inc.) were maintained in pressurized ventilated cages. To test PRKCDBP induction by TNFα, 2 μg of recombinant TNFα (R&D systems) or 100 μL of saline for control were injected intravenously, and PRKCDBP level was measured in the small intestine and the spleen after 12-hour treatment. BAY11-7082 (100 μg) was injected 1 hour before TNFα treatment. For xenograft assay, RKO-pcDNA or RKO-PRKCDBP cells (1×10^7) were injected subcutaneously into 6 mice for each group. Tumor growth was monitored periodically and volume (V) was calculated by the formula $V = 1/2 \times \text{length} \times (\text{width})^2$. To evaluate PRKCDBP effect on tumor response to TNFα, xenograft tumors generated at the left and right flanks were exposed to saline and TNFα (0.5 μg), respectively, by intratumoral injection at days 8 and 12. Tumor volume was measured at day 24. All animal studies were carried out with the approval of Korea University Institutional Animal Care and Use Committee and Korea Animal Protection Law.

Statistical analysis

The results of apoptosis and colony forming assays were expressed as mean ± SD. The Student t test was used to determine the statistical significance of the difference. The χ^2 test was used to determine the statistical significance of expression and methylation levels between tumor and normal tissues. A $P < 0.05$ was considered significant.

Results

Frequent reduction of PRKCDBP expression in cancer cell lines and primary tumors

To explore the candidacy of PRKCDBP as a tumor suppressor, we initially characterized its expression status in cancer cell lines. PRKCDBP expression was not detected in 31.6% (6 of 19) of cancer cell lines at both mRNA and protein levels and another 5 cell lines showed low mRNA but no protein expression (Fig. 1A). Although *PRKCDBP* expression was easily detectable in all normal colonic tissues we tested, a substantial fraction of primary tumors showed no or markedly decreased expression (Fig. 1B). Moreover, 55 of 80 (68.8%) matched tissue sets showed significant reduction of *PRKCDBP* in cancers compared with adjacent noncancerous tissues (Fig. 1C and Supplementary Fig. S1A). Overall, *PRKCDBP* mRNA was significantly low in carcinoma tissues (0.00–1.46; mean: 0.71) compared with adjacent normal tissues (0.81–1.49; mean: 1.15; $P < 0.01$; Fig. 1D). Moreover, 36.3% (29 of 80) of tumors showed *PRKCDBP* levels less than a half (<0.575) of normal means (1.15). *PRKCDBP* reduction was highly frequent in advanced tumor (25 of 54, 46.3%) compared with early-stage tumors (4 of 26, 15.4%; $P < 0.05$) and more

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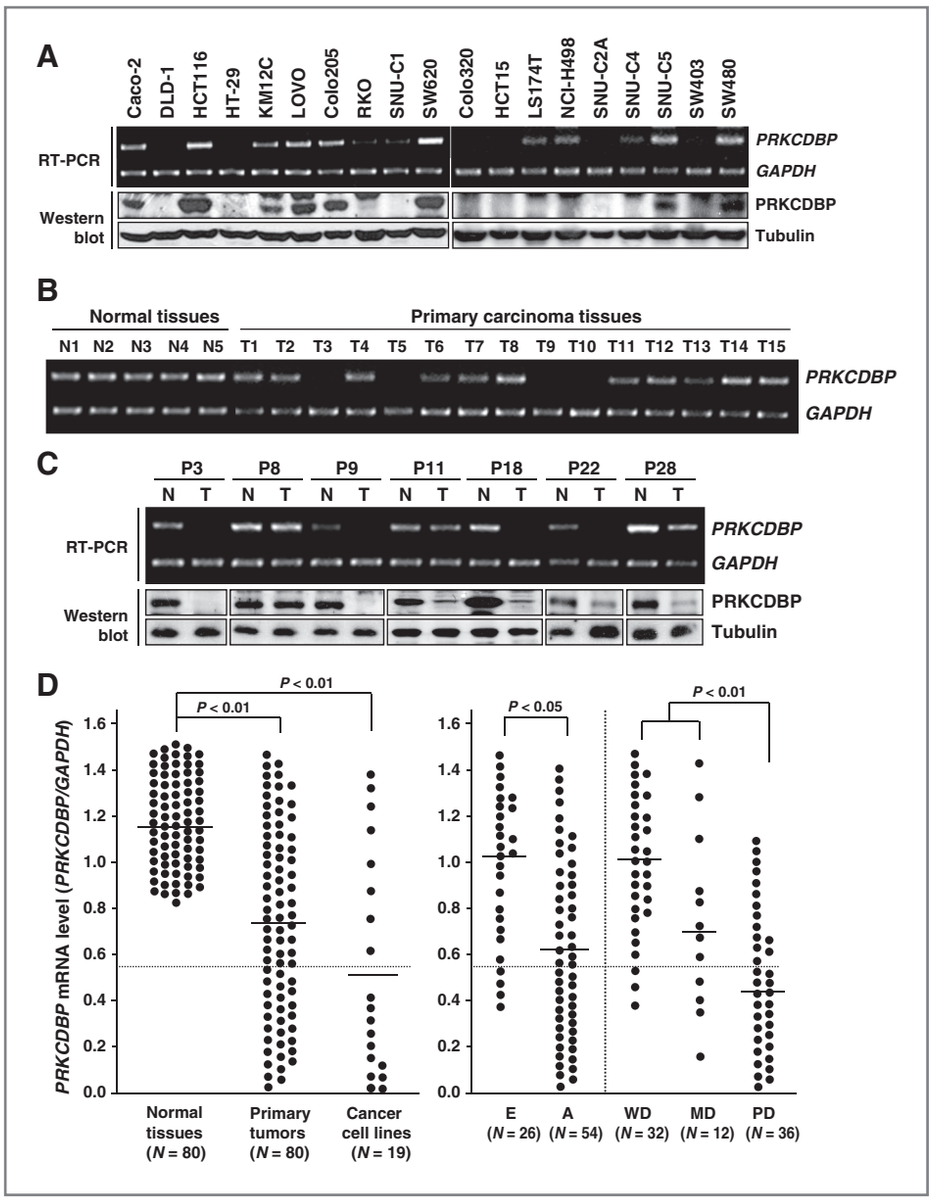

Figure 1. Expression status of PRKCDBP in colorectal cancer cell lines and tissues. A, semiquantitative RT-PCR and immunoblot assays of *PRKCDBP* in cell lines. B, *PRKCDBP* mRNA expression in normal and primary tumor tissues. N, normal tissues; T, tumor tissues. C, tumor-specific reduction of PRKCDBP. PRKCDBP expression was compared between cancer and adjacent noncancerous tissues obtained from the same patients. D, correlation of *PRKCDBP* downregulation with tumor progression. Data represent means of triplicate assays. Bar indicates the mean expression level of each group. *PRKCDBP* levels were compared between early (E) and advanced (A) tumors and well-differentiated (WD), moderately differentiated (MD), and poorly differentiated (PD) tumors. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

common in poorly differentiated tumors (22 of 36, 61.1%) than well- and moderately differentiated tumors (7 of 44, 15.9%; $P < 0.05$). However, *PRKCDBP* expression showed no association with age and gender of the patients (data not shown).

Absence of *PRKCDBP* gene mutations in colorectal cancer

To define whether low expression is caused by gene deletion, we examined genomic level of *PRKCDBP*. Although no or low level of *p53* was clearly seen from 6 (60%) of 10 cell lines harboring allelic deletion of *p53*, none of these cell lines and primary tumors displayed detectable reduction of *PRKCDBP* gene level (Supplementary Figs. S2A and S2B). Next, we surveyed 80 tumors for LOH with an intragenic single-nucleotide polymorphism (T690A) located in exon 2, which leads to the disruption of a recognition site (5′-AGC↓GCT-3′) for the endonuclease *Afe*I. Among 80 matched sets, 21 (26.3%) were heterozygous for this marker. However, none of these informative cases displayed LOH, indicating that allelic loss of *PRKCDBP* is not a common event in colon cancer (Supplementary Fig. S2C). Mutation analysis of *PRKCDBP* for 19 cell lines and 80 tumor tissues failed to find any types of mutation leading to amino acid substitutions except for previously described polymorphisms (1). Mutations of *KRAS* and *TP53* were found in 27 (33.8%) and 28 (35%) of the 80 primary tumors, respectively, but showed no significant correlation with altered expression of *PRKCDBP*.

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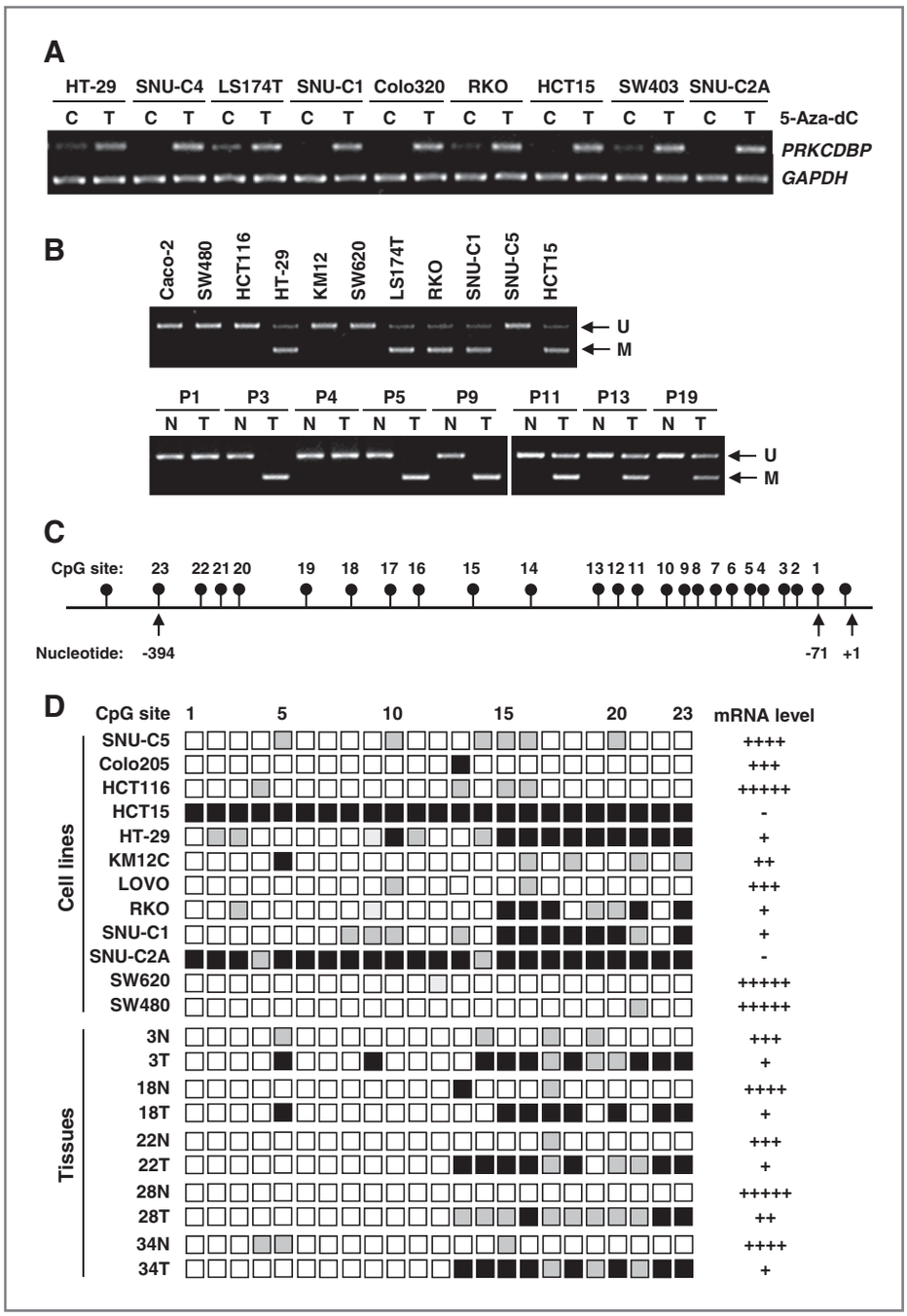

Figure 2. Epigenetic inactivation of *PRKCDBP* in tumors. A, reexpression of *PRKCDBP* by 5-aza-dC treatment. Cells were treated with 5-aza-dC (5 μmol/L) for 4 days. C, control; T, treated. B, methylation-specific PCR analysis of *PRKCDBP*. Bisulfite-modified DNA was subjected to PCR amplification of the *PRKCDBP* promoter region with unmethylation (U)- and methylation (M)-specific primers. C, a map of the CpG sites of the *PRKCDBP* promoter. Twenty-three CpGs analyzed are represented by vertical lines and numbered 1 to 23. The transcription start site is indicated by an arrow at +1. D, methylation status of 23 CpG sites in cancer cell lines and tissues. The region comprising 23 CpGs was amplified by PCR. The PCR products were cloned and 5 plasmid clones were sequenced for each specimen. Black, gray, and white squares represent complete methylation (4–5 clones), partial methylation (1–3 clones), and unmethylation, respectively. N, adjacent noncancerous tissue; T, tumor tissue.

Epigenetic alteration of *PRKCDBP* by aberrant promoter hypermethylation

To define whether DNA methylation is involved in altered expression, we tested effect of the demethylating agent 5-aza-dC using 9 cell lines with no or low expression. *PRKCDBP* expression was elevated in all 9 cell lines following 5-aza-dC treatment (Fig. 2A). In methylation-specific PCR analysis of the promoter sequences, methylation-specific products were detected from all of 5 non- or low expressor cell lines tested, whereas 6 normal expressors showed only unmethylation-specific products (Fig. 2B). Methylation-specific products were detected in 38 of 80 (47.5%) primary tumors and 93% (27 of 29) of tumors with low *PRKCDBP* level but only 11% (6 of 51) of tumors with normal level showed methylation. We next determined the methylation status of 23 CpGs sites in the promoter region using sodium bisulfite sequencing analysis (Fig. 2C). Five PCR clones were sequenced to determine methylation

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frequency at individual CpG sites. As summarized in Fig. 2D, 100% and 35% to 65% (8–15 sites) of the 23 CpGs were methylated in 2 nonexpressor and 3 low expressor cell lines, respectively, whereas 0% to 26% (0–6 sites) were methylated in 7 normal expressor cell lines. Likewise, primary tumors with low mRNA level displayed complete or partial methylation at 8 to 12 sites (35%–52%), whereas the adjacent noncancerous tissues or tumors with normal level showed methylation at 0 to 4 sites (0%–17%). In particular, methylation status of 9 sites (numbers 15–23 in Fig. 2C) within nucleotides −201 to −394 was most tightly associated with mRNA level in both cell lines and primary tumors. Approximately 78% to 100% (7–9 sites) of these 9 sites were completely or partially methylated in non- or low expressor cell lines, whereas only partial methylation at less than 4 of these sites were found in normal expressors, suggesting that hypermethylation of CpG sites within this region might be critical for the transcriptional silencing of *PRKCDBP*. Collectively, these results indicate that abnormal reduction of *PRKCDBP* in cancers is caused by epigenetic gene silencing due to aberrant promoter hypermethylation.

PRKCDBP suppression of tumor cell growth by inhibition of cell proliferation

We investigated whether PRKCDBP affects tumor cell growth. As shown in Fig. 3A, transient transfection of WT-PRKCDBP caused approximately 35% to 41% reduction of HT-29 cell growth and siRNA-mediated knockdown

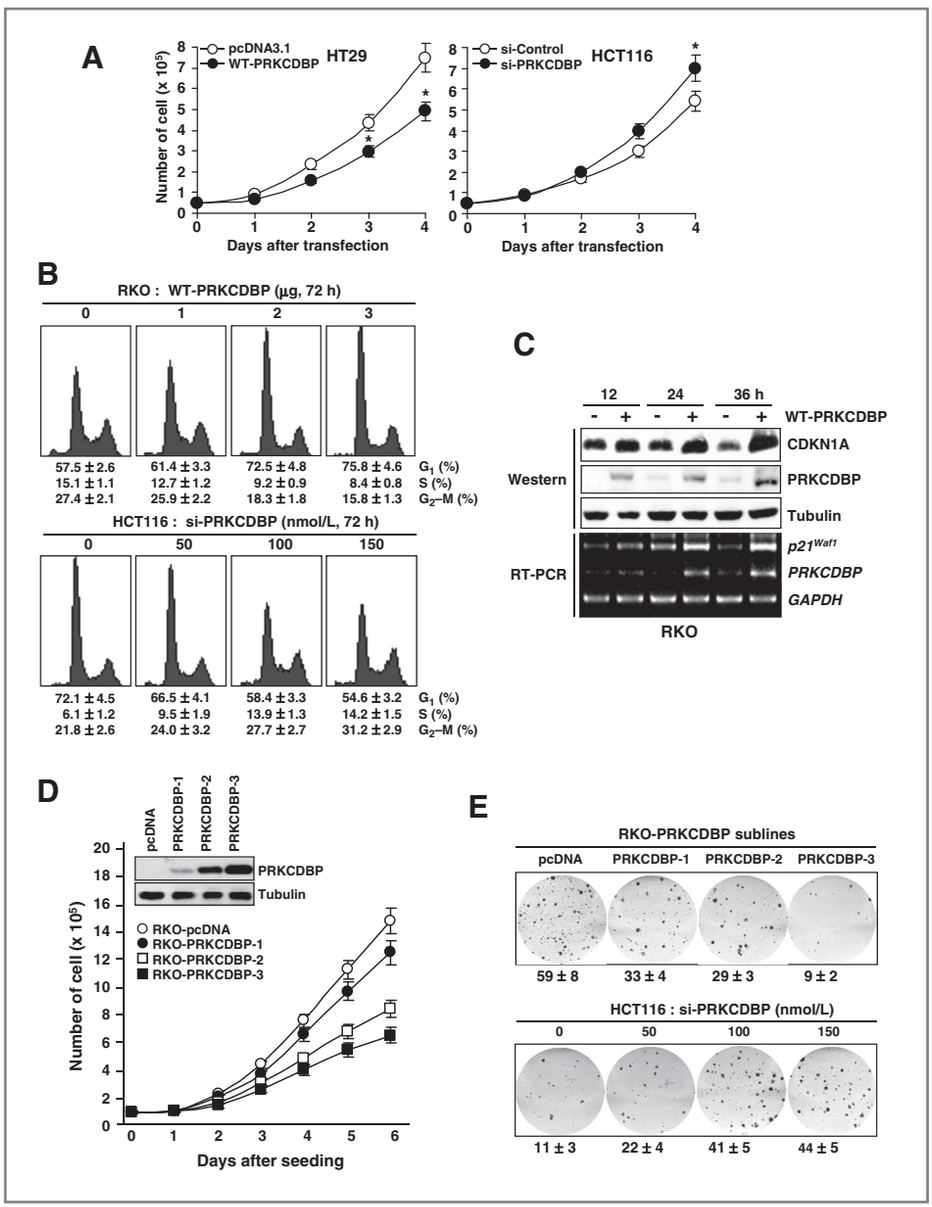

Figure 3. Effect of PRKCDBP on tumor cell growth. A, inhibition of cell growth by PRKCDBP. Cellular growth was determined by cell number counting using a hemacytometer. Data represent means of triplicate assays (bars, SD; *, $P < 0.05$). B, PRKCDBP induction of G_1 cell-cycle arrest. Percentage of the G_1 phase cells was measured by flow cytometry. Cells were transfected with increasing doses of WT-PRKCDBP or si-PRKCDBP and cell-cycle progression was analyzed at 48 hours after transfection. C, PRKCDBP induction of CDKN1A. D, cellular growth of RKO sublines expressing different levels of PRKCDBP. E, PRKCDBP suppression of colony forming ability of tumor cells. Cells were maintained in the presence of G418 (1,600 μg) for 3 weeks, and colonies were stained with crystal violet. Assays were conducted in triplicate.

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of endogenous PRKCDBP led to 22% to 29% increase of HCT116 cell growth. Similar results were obtained from RKO and SW620 cells (Supplementary Fig. S3A). Consistently, the percentage of G_1 phase cells is elevated by WT-PRKCDBP transfection (RKO) but decreased by si-PRKCDBP transfection (HCT116) in a dose-associated manner, indicating that PRKCDBP induces a G_1 cell-cycle arrest (Fig. 3B). [^{3}H]thymidine uptake assay also revealed that DNA synthesis is inhibited and stimulated by restoration and knockdown of PRKCDBP, respectively (Supplementary Fig. S3B). In addition, PRKCDBP upregulated expression of a cyclin-dependent kinase inhibitor CDKN1A ($p21^{Waf1}$), supporting that PRKCDBP has an antiproliferative function (Fig. 3C). We generated several RKO sublines (RKO-PRKCDBP) which stably express different levels of PRKCDBP and observed that these sublines display a PRKCDBP level-associated decrease in cellular growth and colony formation (Figs. 3D and E).

Proapoptotic function of PRKCDBP and its role for TNFα-induced apoptosis

To elucidate the mechanistic basis for PRKCDBP-mediated growth suppression, we examined its effect on signaling factors involved in cell growth regulation. Although phospho extracellular signal-regulated kinase (ERK)1/2 and phospho–c-*jun*-NH_2-kinase (JNK) levels were not affected by PRKCDBP expression, phospho-AKT level was substantially down- and upregulated by transfection of WT-PRKCDBP and si-PRKCDBP, respectively, suggesting that PRKCDBP may inhibit phosphoinositide 3-kinase (PI3K)-AKT signaling (Fig. 4A). Consistently, we observed that AKT phosphorylation by insulin-like growth factor (IGF) and subsequent GSK3β phosphorylation is attenuated by WT-PRKCDBP transfection (Supplementary Fig. S4A). As suggested by these findings, both baseline (0.9% versus 1.6%) and etoposide-induced apoptosis (5.5% versus 29.7%) are remarkably high in PRKCDBP-expressing RKO subline cells (RKO-PRKCDBP-2) compared with control, and that

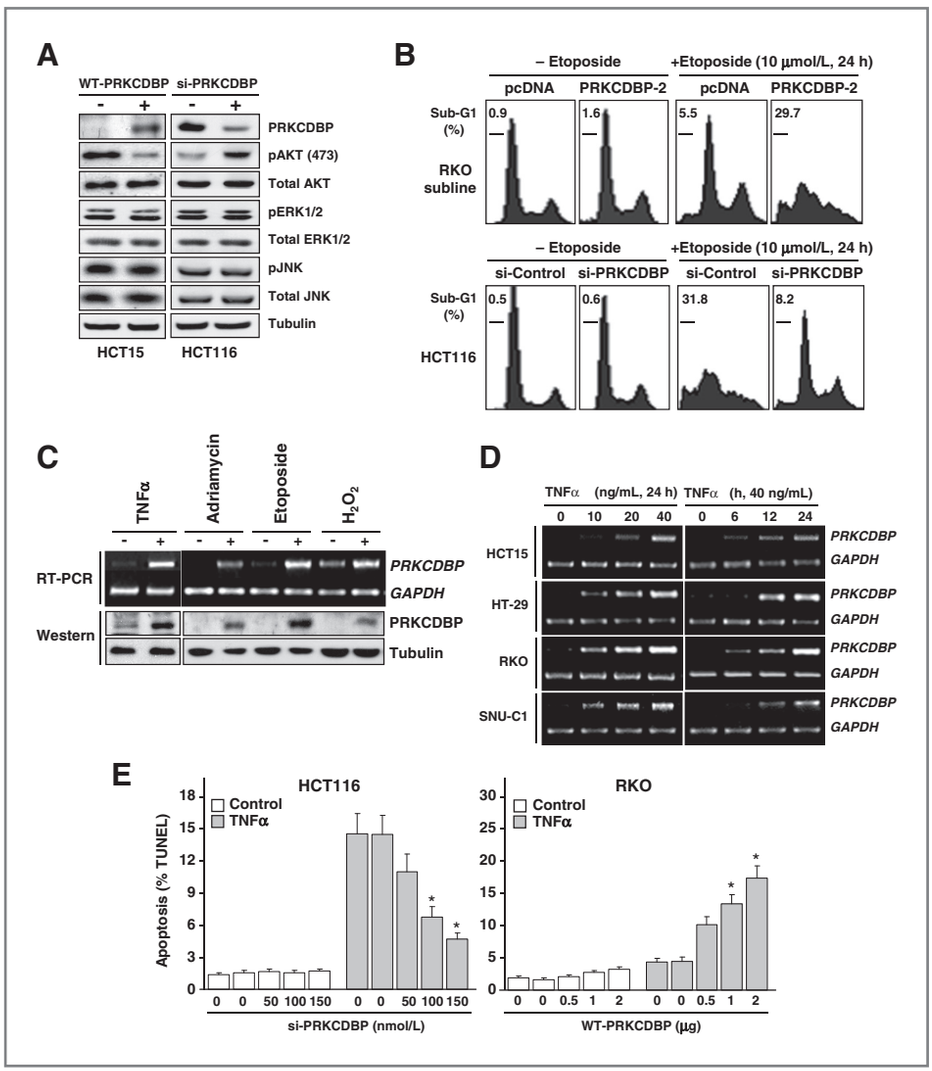

Figure 4. Proapoptotic function of PRKCDBP and its role in TNFα-induced apoptosis. A, PRKCDBP effect on signaling components. Cells were transfected with WT-PRKCDBP (2 μg) or si-PRKCDBP (50 nmol/L). Total and phospho-AKT, phospho-ERK1/2, and phospho-JNK level was examined by immunoblot assays at 48 hours after transfection. B, apoptosis-promoting effect of PRKCDBP. Cells were exposed to etoposide (10 μmol/L) for 24 hours and distribution of sub-G_1 cells was analyzed with flow cytometry. C, PRKCDBP induction by various stresses. HT-29 cells were exposed to TNFα, adriamycin, etoposide, and H_2O_2, and PRKCDBP level was examined by RT-PCR and immunoblot assays. D, a time- and dose-associated induction of *PRKCDBP* by TNFα. E, PRKCDBP effect on cellular sensitivity to TNFα. Cells were transfected with si-PRKCDBP or WT-PRKCDBP, and apoptotic response to TNFα was determined by TUNEL assay. Data represent means of triplicate assays (bars, SD; *, $P < 0.05$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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PRKCDBP ablation decreases apoptotic response of HCT116 cells to etoposide (Fig. 4B). MTT assays also showed that under stress conditions, cell viability is decreased by PRKCDBP (Supplementary Fig. S4B). Interestingly, we found that PRKCDBP is induced by various apoptotic stimuli, including TNFα, adriamycin, etoposide, and H_2O_2 (Fig. 4C). Considering a critical role for TNFα in colonic inflammation and tumorigenesis, we further defined PRKCDBP implication in TNFα signaling. *PRKCDBP* was induced in many colon tumor cells by TNFα in a dose- and time-dependent manner (Fig. 4D). An mRNA decay assay showed that TNFα induction of PRKCDBP is due to increased transcription rather than enhanced mRNA stability (Supplementary Fig. S4C). We next evaluated whether PRKCDBP affects tumor cell response to TNFα with HCT116 and RKO cells, which is sensitive and resistant to TNFα, respectively. As shown in Fig. 4E, restoration and blockade of PRKCDBP markedly up- and downregulated cellular sensitivity to TNFα-induced apoptosis. In addition, *PRKCDBP* expression levels in cell lines showed a correlation with tumor cell sensitivity to TNFα in 16 colorectal cancer cell lines (Supplementary Fig. S4D). These results show that PRKCDBP induction by TNFα contributes to TNFα-induced apoptosis.

Identification of PRKCDBP as a direct transcription target of NF-κB

We next examined whether NF-κB signaling is involved in TNFα induction of PRKCDBP. PRKCDBP induction by TNFα was abolished by the NF-κB inhibitor BAY11-7082 or si-RelA transfection (Fig. 5A). Moreover, PRKCDBP was upregulated by RelA transfection in the absence of TNFα treatment, suggesting that PRKCDBP is directly activated by NF-κB (Supplementary Fig. S5A). We found a putative κB site (5′-GGGATTTTCT-3′) in the *PRKCDBP* promoter region comprising nucleotides −1,295/−1,304 relative to the transcription start site and tested whether this site could confer NF-κB responsiveness to a heterologous reporter (Fig. 5B). Reporter constructs comprising a putative κB site (Pro1500-Luc and Pro1350-Luc) exhibited strong response to TNFα and this response was suppressed by BAY11-7082 treatment or si-RelA transfection (Fig. 5C and Supplementary Fig. S5B). In contrast, reporter constructs without the putative κB site (Pro1294-Luc and Pro/ΔκB-Luc) or a mutant reporter (*Pro/MTκB-Luc*) with a mutated κB site (5′-G*TT*ATT-*GAAA*-3′) exhibited no activity (Fig. 5C). Chromatin immunoprecipitation assays also revealed that RelA interacts with PRKCDBP chromatin in TNFα-treated cells, and this interaction is disrupted by pretreatment with BAY11-7082, indicating that RelA occupies the κB site in living cells (Fig. 5D). Collectively, these results show that *PRKCDBP* is a direct transcription target of NF-κB signaling.

Caveolin-independent function of PRKCDBP

A recent study showed that PRKCDBP is a CAV1 adapter protein that regulates caveolae function (12). We thus examined whether CAV1 is implicated in PRKCDBP-mediated growth suppression using RKO and SNU-C2A cells, which have low and high levels of CAV1, respectively. It was found that CAV1 expression does not affect PRKCDBP regulation of CDKN1A and AKT and PRKCDBP induction of cell-cycle arrest and apoptosis (Supplementary Figs. S6A–S6C). Likewise, ectopic overexpression of CAV1/P132L, a dominant-negative mutant *CAV1*, did not influence PRKCDBP effect on etoposide- or TNFα-induced apoptosis, indicating that PRKCDBP has caveolin-independent growth suppression function (Supplementary Fig. S6C).

Effect of PRKCDBP on *in vivo* tumor growth and response to TNFα

To explore PRKCDBP effect on tumor growth and TNFα-induced apoptosis *in vivo*, we initially tested whether PRKCDBP is induced in response to TNFα. As shown in Fig. 6A, elevated expression of *PRKCDBP* mRNA was observed in the small intestines and spleens of TNFα-treated mice, and this induction was blocked by pretreatment of BAY11-7082 (Fig. 6B). We next analyzed PRKCDBP effect on xenograft tumor growth. The identical numbers (1×10^7) of RKO-pcDNA or RKO-PRKCDBP cells were injected subcutaneously into the flank of nude mice, and tumor formation and growth were monitored regularly up to 20 days. RKO-pcDNA cells generated visible tumors at day 8 after injection and formed continuously growing tumor mass from all 6 mice we tested. However, RKO-PRKCDBP cells generated detectable tumors after 12 days from 5 of 6 mice, and their growth rate was significantly low compared with those of controls (Fig. 6C). To examine PRKCDBP effect on tumor response to TNFα, xenograft tumors generated at the left and right flanks of mice were exposed to saline and TNFα (0.5 μg), respectively, at days 8 and 12, and tumor volume was compared at day 24. RKO-PRKCDBP tumors displayed dramatic response to TNFα (83% reduction) whereas RKO-pcDNA tumors showed only slight decrease (11% reduction; Fig. 6D). In addition, cleaved caspase-3 and PARP were higher in RKO-PRKCDBP versus RKO-pcDNA tumors and further elevated by TNFα only in RKO-PRKCDBP tumors, indicating that PRKCDBP plays a critical role in tumor response to TNFα (Fig. 6E).

Discussion

In this study, we show first that *PRKCDBP* expression is frequently lost or downregulated in human colorectal cancers by aberrant promoter hypermethylation, and its alteration correlates with tumor progression. We also found that PRKCDBP induces the G_1 cell-cycle arrest and enhances cellular sensitivity to various apoptotic stresses. Furthermore, PRKCDBP was found as a transcription target of TNFα–NF-κB signaling, which acts a crucial role in TNFα-induced apoptosis both *in vitro* and *in vivo*. This study thus suggests that epigenetic inactivation of PRKCDBP may contribute to colorectal tumor progression by attenuating tumor cell response to TNFα and other apoptotic stresses.

The 11p15.4 region, in which the *PRKCDBP* gene is located, undergoes frequent allelic losses in a variety of human malignancies including breast, lung, and gastric

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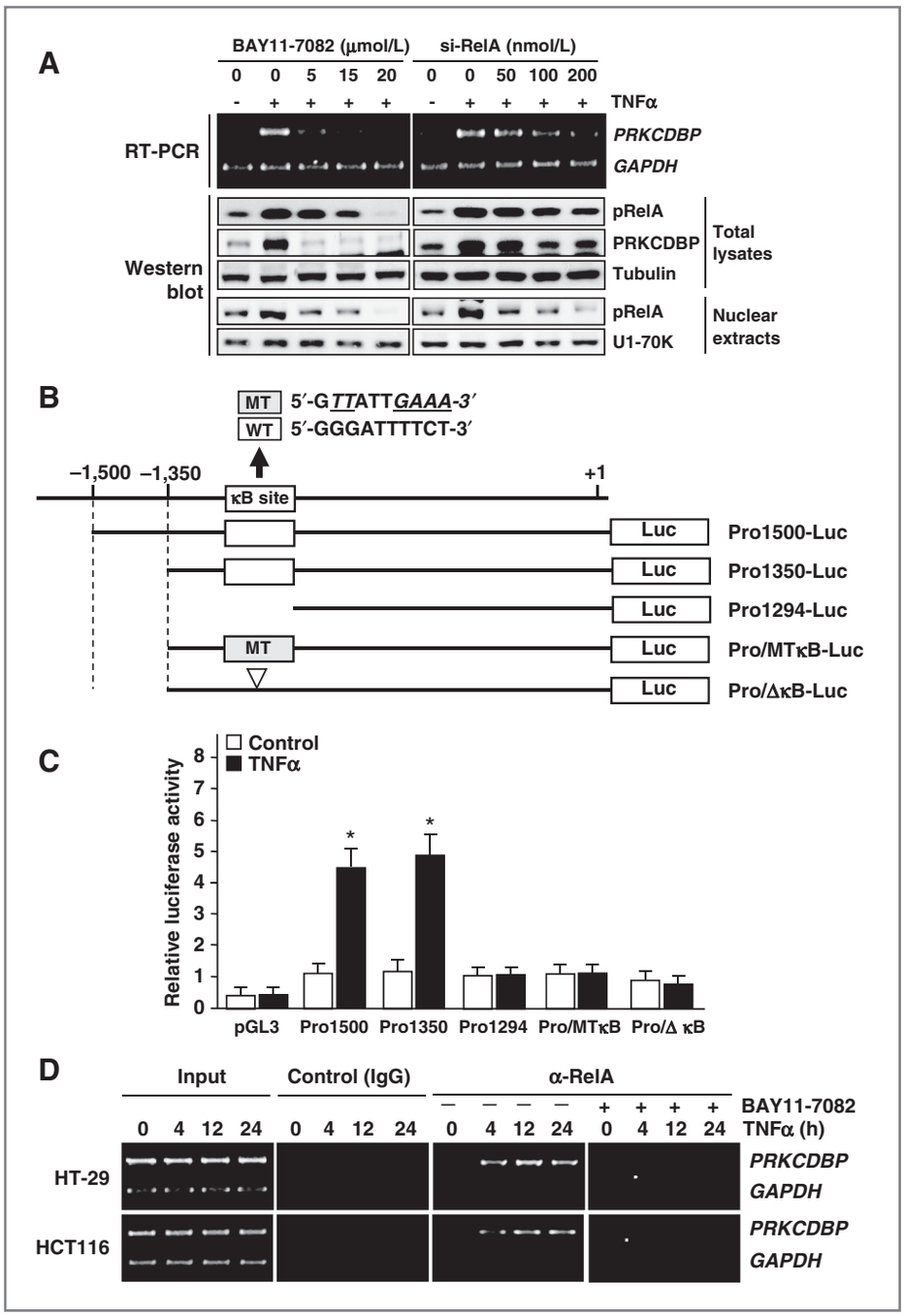

Figure 5. Identification of *PRKCDBP* as a target of TNFα–NF-κB signaling. A, effect of NF-κB depletion on TNFα induction of PRKCDBP. HT-29 cells were treated with BAY11-7082 or transfected with si-RelA, and its effect on TNFα induction of PRKCDBP was analyzed. B, a putative κB binding site in the *PRKCDBP* promoter and construction of reporter plasmids for luciferase assay. C, disruption of promoter responsiveness to TNFα by mutation or deletion of the κB binding site. HT-29 cells transfected with promoter constructs were exposed to TNFα (40 ng/mL) for 12 hours. Relative luciferase activity was normalized by the β-galactosidase activity (*, $P < 0.05$). D, chromatin immunoprecipitation assay of RelA binding to the putative κB binding site. Cells were treated with TNFα (40 ng/mL) for 4 to 24 hours. Cross-linked chromatin was immunoprecipitated with antibodies against RelA or rabbit immunoglobulin G and analyzed by PCR with primers that flank the κB binding site. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cancer (19–22). Therefore, *PRKCDBP* was predicted as a target of deletion in many cancer types displaying frequent LOH at 11p15.4, and allelic loss of the gene was suggested as a plausible mechanism underlying its low expression in tumor cells. Furthermore, several frameshift and truncation mutations of *PRKCDBP* were found in a few ovarian and lung cancer cell lines (1). In this study, however, we failed to find allelic deletion or mutations of *PRKCDBP* in colorectal cancers. Our study revealed that 26% (21 of 80) of cancer patients are heterozygous for an intraexonic polymorphic marker, but none of these informative cases displays LOH. A mutation study also failed to detect any type of mutation leading to amino acid substitutions except for previously reported polymorphisms (1). Although further comprehensive study is required, our findings suggest that genetic alteration of *PRKCDBP* might be very rare in colorectal cancers.

The short arm of chromosome 11 is subjected to widespread regional hypermethylation in various human

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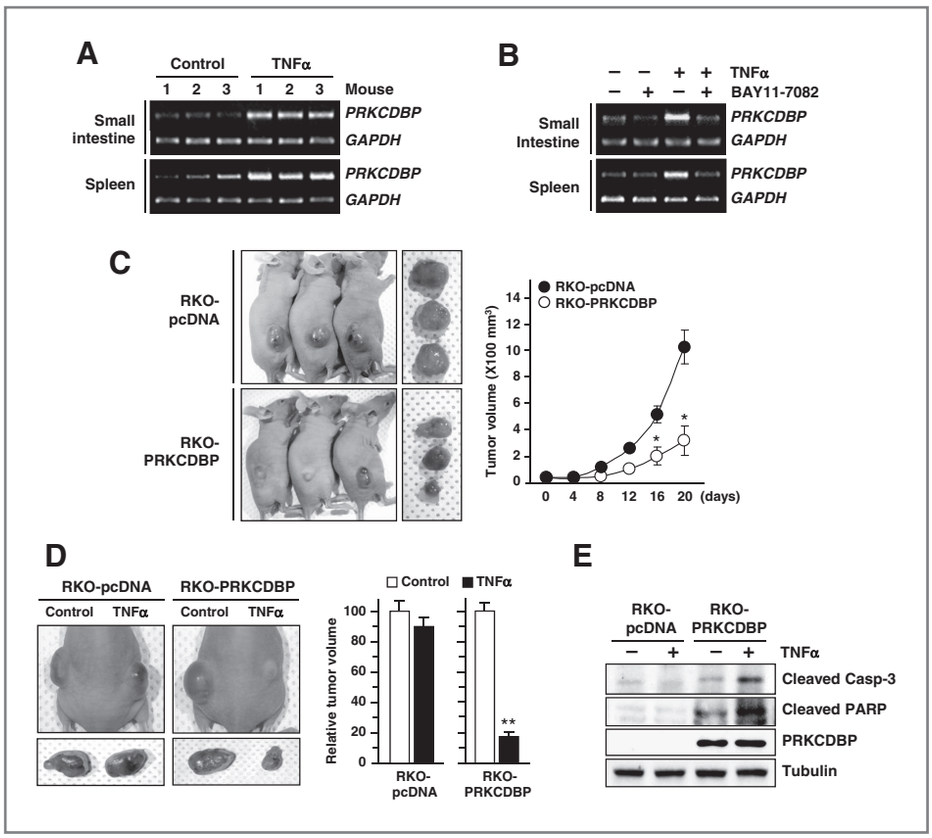

Figure 6. PRKCDBP effect on tumor growth and TNFα-induced apoptosis *in vivo*. A, TNFα induction of *PRKCDBP*. Mice were treated with 2 μg of TNFα or 100 μL of saline for control for 12 hours. *PRKCDBP* mRNA level was examined in the small intestine and the spleen by quantitative RT-PCR. B, disruption of TNFα induction of *PRKCDBP* by NF-κB blockade. Mice were treated with TNFα with or without pretreatment with BAY11-7082. C, inhibition of *in vivo* tumor growth by PRKCDBP. RKO-pcDNA or RKO-PRKCDBP were injected subcutaneously into the 4-week-old female nude mice, and tumor sizes were measured every 4 days (*, $P < 0.05$). D, implication of PRKCDBP in tumor response to TNFα. Xenograft tumors were generated at the left and right flanks of 3 mice, and the tumors were exposed to saline or TNFα (0.5 μg) at days 8 and 12. Representative photographs of mouse tumors at day 24 are illustrated. E, PRKCDBP effect on TNFα-induced apoptosis. Cleaved caspase-3 and cleaved PARP levels were examined in xenograft tumor tissues by an immunoblot assay.

neoplasms. Several genes residing at 11p, such as *WT1*, *calcitonin*, and *mucins*, have significant hypermethylation of CpG sites within their promoter regions in colorectal carcinomas compared with normal colonic mucosa (28, 29). The 7,466 bp of genomic DNA sequences containing the *PRKCDBP* gene (GenBank accession number AF408198) include 3 CpG islands, and the methylation status of 8 CpG sites between nucleotides −241 and −451 is correlated with *PRKCDBP* expression in breast and lung cancer cells (1). Silencing of *PRKCDBP* expression by epigenetic mechanism has been also suggested in studies of primary lung and gastric cancers (2, 3). Consistent with these reports, we found that the *PRKCDBP* promoter is methylated in a substantial fraction of primary colorectal cancers and loss or reduction of *PRKCDBP* expression is tightly associated with aberrant promoter hypermethylation. Methylation-specific PCR analysis revealed that promoter methylation is significantly higher in tumors with low transcript level compared with tumors with normal expression. In particular, the methylation status of 9 CpG sites within nucleotides −201 to −394 were most tightly associated with gene silencing, indicating that CpG sites in this region may play a critical role for transcription regulation. Together, our data support that *PRKCDBP* is epigenetically inactivated in a broad range of human solid tumors, and CpG sites hypermethylation of the 5′ proximal region of the promoter is crucial for the transcriptional silencing of *PRKCDBP* in human cancers.

Despite several lines of evidence for PRKCDBP's tumor suppression role, the molecular mechanism underlying its functions in tumorigenesis has not been understood. PRKCDBP was originally identified as a binding protein of PKCδ, which has been known as a potential tumor suppressor involved in the regulation of cell proliferation, differentiation, and apoptosis (5, 25). The possible implication of PRKCDBP in cell-cycle control was suggested based on observation that its mRNA expression is induced in response to serum deprivation and downregulated during G_0–G_1 transition of the cell cycle (5, 6). PRKCDBP was also identified as a BRCA1-interacting protein, raising the possibility that it may participate in DNA-damage response including DNA repair processes (1). Recently, we have shown that PRKCDBP increases the stability of p53 and its target gene expression, and its loss or reduction in tumor cells attenuates p53 response to stresses (3). In this study, we found that PRKCDBP induces a G_1 arrest of the cell cycle partially through CDKN1A induction and increases apoptotic response of tumor cells to various stresses, such as etoposide, 5-FU, γ-irradiation, H_2O_2, and serum deprivation. Consistent with these effects, PRKCDBP significantly decreased the colony forming ability of tumor cells and delayed the formation and growth of xenograft tumors. It was also found that *PRKCDBP* mRNA is strongly elevated in response to genotoxic or nongenotoxic stimuli, raising the possibility that PRKCDBP is controlled by stress signaling and implicated in damage response. Moreover, we found

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that PRKCDBP suppresses both basal- and IGF-induced phosphorylation of AKT, whereas it exerts no detectable effect on ERK and JNK. Considering that AKT controls p53 stability via Mdm2 phosphorylation, it is plausible that the p53-enhancing function of PRKCDBP might be associated with its regulatory role for AKT (30, 31). Therefore, our findings suggest that PRKCDBP-mediated tumor suppression might stem, at least in part, from its ability to inhibit the PI3K-AKT signaling pathway, which plays a crucial role in the development and progression of a variety of human tumors.

TNFα is a pleiotropic cytokine that has an important role in inflammation, angiogenesis, tissue remodeling, and tumor growth (32). TNFα was originally described as a protein factor capable of killing tumor cells *in vitro* and causing hemorrhagic necrosis of transplantable tumor in mice (33). However, many studies showed that TNFα acts as a tumor-promoting cytokine in a variety of cancers (34–36). TNFα is produced during the initiation of inflammatory responses and plays a causative role for the pathogenesis of various forms of inflammatory bowel disease, an underlying condition for colon cancer development (37). Moreover, TNFα expression is elevated during colonic tumorigenesis and mutational inactivation of the type 1 receptor or interference with TNFα signaling using a soluble decoy receptor decreased cancer induction and growth (38). Nevertheless, the mechanistic basis for the tumor-destructive and tumor-promoting capacity of TNFα has not been fully understood. It has been known that binding of TNFα to its receptors primarily leads to 2 different end results: induction of genes involved in inflammation and cell survival or induction of apoptosis. These opposite properties of TNFα are linked to its ability to activate both AP-1 and NF-κB signaling pathways that regulate cell proliferation, survival, and apoptosis (39, 40). Several studies showed that NF-κB paradoxically suppresses and promotes apoptosis in response to TNFα and can mediate both beneficial and lethal effects of TNFα (40, 41). In this study, we found that PRKCDBP is induced by TNFα through NF-κB signaling both *in vitro* and *in vivo*. PRKCDBP induction was mediated by the RelA component of NF-κB through a κB site in its promoter. Intriguingly, tumor cell sensitivity to TNFα was associated with PRKCDBP expression status in colon cancer cell lines. Furthermore, our animal studies revealed that xenograft tumors derived from PRKCDBP-restored tumor cells are highly sensitive to TNFα-induced apoptosis, whereas tumors derived from PRKCDBP-nonexpressing parental cells display negligible response. These findings suggest that PRKCDBP might be an important mediator of TNFα-induced apoptosis. Given its frequent alteration in colon cancers, PRKCDBP could be involved in the appearance of tumor resistance to TNFα-induced apoptosis during tumor progression. Although the possible role for PRKCDBP in the proinflammatory function of TNFα was not defined, it is conceivable that loss of PRKCDBP function might provide tumor cells survival and growth advantages, particularly under chronic inflammatory microenvironment. In this context, it is noteworthy that epigenetic inactivation of PRKCDBP is highly frequent in both colon and gastric cancers in which pathogenesis is highly linked to chronic inflammation (3).

A recent study showed that PRKCDBP interacts with CAV1 and is localized to caveolae (12). PRKCDBP directs the formation of caveolar vesicles and acts as a caveolin adapter molecule that regulates caveolae dynamics. Based on these, PRKCDBP was also named as Cavin3 and classified as a cavin family member (15–17). In this context, the question arose as to whether growth suppression functions of PRKCDBP are associated with its interaction with CAV1 and action as a caveolae regulator. We found that PRKCDBP exerts its antiprolferative and proapoptotic effects in colon tumor cells irrespectively of CAV1 status. PRKCDBP activated CDKN1A expression and suppressed AKT phosphorylation and DNA synthesis similarly in both controls and CAV1-restored or CAV1-depleted cells. The apoptosis-enhancing activity of PRKCDBP controls and CAV1-restored or CAV1-depleted cells. The apoptosis-enhancing activity of PRKCDBP that PRKCDBP regulates cell proliferation and apoptosis through a caveolae-independent mechanism.

In summary, the data indicate that PRKCDBP undergoes frequent epigenetic inactivation due to aberrant promoter hypermethylation in human colorectal cancers, and its reduction is associated with the malignant progression of colorectal tumors. Our study shows first that *PRKCDBP* is directly activated by NF-κB in response to TNFα and plays a crucial role in TNFα-induced apoptosis. These findings raise the possibility that PRKCDBP alteration may render colorectal tumor cells a survival advantage by attenuating the apoptotic sensitivity to various stresses including TNFα, and thus the restoration of functional PRKCDBP could be effective in overcoming therapeutic resistance by sensitization of tumor cells to TNFα-induced apoptosis particularly in chronic inflammatory tumor environment. It will be valuable to explore the possible application of PRKCDBP as a clinically useful marker for detection and treatment of human colorectal malignancies.

Disclosure of Potential Conflicts of Interest

All of the authors are aware of and agree to the content of the article and their being listed as authors on the article. This article does not contain any information conveyed either by personal communication or release of unpublished experimental data. All authors read and approved the final manuscript.

Authors' Contributions

J-H. Lee, M-G. Lee, B-K. Ryu, and M-J. Kang carried out the expression and mutation studies and statistical analysis and drafted the manuscript. J-H. Lee, H-Y. Han, N-G. Her, and T-K. Ha carried out methylation studies. J-H. Lee, J. Han, and M-G. Lee carried out the immunoblot, cell growth, apoptosis, and colony formation assays. J-H. Lee, S-I. Jeong, and M-J. Kang carried out TNFα promoter assay. K.Y. Lee, S.J. Park, and H-J. Kim provided tissue specimens and participated in the design of the study. J.-H. Lee and S.J. Park conducted animal studies. S-G. Chi obtained funding, conceived of the study, and participated in its design and coordination.

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Epigenetic Alteration of *PRKCDBP* in Colorectal Cancers and Its Implication in Tumor Cell Resistance to TNF α-Induced Apoptosis

Jin-Hee Lee, Min-Ju Kang, Hye-Yeon Han, et al.

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