Downregulation of protein kinase C gamma reduces epithelial property and enhances malignant phenotypes in colorectal cancer cells

Highlights

- Low PKCγ expression is associated with a poor prognosis in patients with CRC
- The expression of PKCγ is associated with the epithelial properties of CRC cells
- PKCγ knockdown induces malignant phenotypes in CRC cells
- PKCγ phosphorylates and stabilizes ARHGEF18, that stabilizes cell-cell junctions
Downregulation of protein kinase C gamma reduces epithelial property and enhances malignant phenotypes in colorectal cancer cells

Reiko Satow,1,3,* Yudai Suzuki,1 Shinobu Asada,1 Sae Ota,1 Masashi Idogawa,2 Shiori Kubota,1 Noi Ikeo,1 Atsuko Yoneda,1 and Kiyoko Fukami1

SUMMARY
Loss of epithelial integrity is associated with colorectal cancer (CRC) aggressiveness. Protein kinase C (PKC) is frequently implicated in human cancers, but the role of PKCγ in CRC remains poorly understood. Here, we show that PKCγ, a conventional PKC, is expressed in normal colonic epithelium, but this is lower in dedifferentiated CRC. PKCγ expression was downregulated by SNAI1 overexpression, and low PKCγ expression was associated with poor prognosis in patients with CRC. Transient or stable knockdown of PKCγ reduced E-cadherin expression in CRC cells. PKCγ knockdown enhanced proliferation, anchorage-independent cell growth, resistance to anti-cancer drugs, and in vivo tumor growth of DLD-1 cells. We have also identified phosphorylation substrates for PKCγ. Among them, ARHGEF18, a RhoA activator that stabilizes cell-cell junctions, was phosphorylated and stabilized by PKCγ. Thus, these results suggest that the downregulation of PKCγ decreases the epithelial property of CRC cells and enhances its malignant phenotypes.

INTRODUCTION
Although several drugs targeting specific molecules have been developed and are used clinically for colorectal cancer (CRC) treatment, the clinical outcome of patients with CRC with metastasis is still poor.1 Therefore, further research is required to determine the molecular mechanisms of CRC progression to develop more effective drugs. Cancer metastasis is often driven by the epithelial–mesenchymal transition (EMT), which is a crucial cellular program that enables epithelial cells to acquire an invasive phenotype for metastatic progression.2 EMT permits epithelial cells to acquire a mesenchymal morphology accompanied by the loss of the apical–basal polarity and disassembly of epithelial cell–cell contacts including tight junctions and adherens junctions. Recent studies found that migrating cancer cells frequently display a partial or transient EMT state in which various combinations of epithelial and mesenchymal properties coexist.3

E-cadherin is a significant mediator of cell-cell adhesion in epithelial tissue, and loss of E-cadherin is a critical step in the loss of epithelial property. In CRC cells, loss of E-cadherin is a hallmark of EMT, and E-cadherin is a modulator of cell biological traits, as the depletion of E-cadherin by small interfering RNA (siRNA) promotes cell growth, invasion, and drug resistance through the induction of β-catenin nuclear translocation.4 In addition, the loss of cell–cell contact promotes β-catenin signaling, which then activates EMT-inducing transcription factor to repress E-cadherin transcription.5 Thus, E-cadherin loss and EMT are mutually related.

EMT-inducing transcription factors such as SNAI1 (also known as SNAIL), SNAI2 (also known as SLUG), ZEB1, and TWIST repress the genes associated with the epithelial phenotype. SNAI1 binds to E-box sequences in the promoter region of CDH1 (encoding E-cadherin) and recruits the polycomb repressive complex to repress transcription.6–8 SNAI1 also represses the expression of genes regulating tight junction and apical–basal polarity.9 In CRC, SNAI1 induces features of cancer stem cells, including chemoresistance, radioresistance, and the ability to initiate tumor formation.10 EMT also enhances cancer stemness and resistance to therapeutic agents.2
Previously, we have shown that phospholipase C delta 1 (PLCδ1) contributes to E-cadherin expression to suppress CRC aggressiveness.\textsuperscript{11} During our attempt to identify PKC isoforms that can be regulated by PLCδ1 in CRC, we noticed the novel role of PKC\textsubscript{g} in CRC cells. PKCs are serine/threonine kinases that can be classified into three groups: "conventional" (cPKCs), "novel" (nPKCs), and "atypical" (aPKCs). Of these three groups, only cPKCs are activated by both calcium ions and DAG. The cPKCs comprise PKC\textsubscript{a} (PRKCA), two splice variants of PKC\textsubscript{b} (PRKCB), and PKC\textsubscript{g} (PRKCG).\textsuperscript{12} Some previous reports have suggested tumor-suppressive roles for PKC\textsubscript{a} and PKC\textsubscript{b},\textsuperscript{13,14} however, the role of PKC\textsubscript{g} in CRC is not completely characterized. In this study, we have characterized the roles of PKC\textsubscript{g} in CRC.

RESULTS

The expression of PKC\textsubscript{g} is associated with the epithelial properties of colorectal cancer cells

To assess the expression of PKC\textsubscript{g} and E-cadherin in CaCo-2, WiDr, DLD-1, SW480, SW620, Lovo, and HCT116 cells was quantified using western blotting. The correlation between PKC\textsubscript{g} and E-cadherin expression was assessed using Pearson's product-moment correlation test.

Western blot analyses of PKC\textsubscript{g} and other marker proteins in DLD-1 and SW480 cells expressing SNAI1. The relative expression levels of CDH1 (E-cadherin) and PRKCG (PKC\textsubscript{g}) normalized to that of GAPDH, were determined by qPCR analyses in DLD-1 and SW480 cells expressing SNAI1 (n = 6, each). Data represent means ± SD. Statistical analysis was performed using Student's t test. **p < 0.01; ***p < 0.001.

See also Figures S1 and S2, and Table S1.

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RESULTS

The expression of PKC\textsubscript{g} is associated with the epithelial properties of colorectal cancer cells

To assess the expression of PKC\textsubscript{g}, western blot analysis of the CRC cell lines Caco-2, WiDr, DLD-1, SW480, SW620, Lovo, and HCT116 was performed. The specificity of the PKC antibodies used in this study was confirmed (Figure S1). The characteristics of the CRC cell lines used in this study are summarized in Table S1. Among these cell lines, only SW620 showed pronounced expression of the mesenchymal marker, vimentin. The expression of both PKC\textsubscript{g} and E-cadherin was the lowest in the SW620 cells (Figure 1A). The other cell lines showed little or no vimentin expression, suggesting that they did not undergo EMT, although the possibility of partial EMT cannot be excluded. The results revealed that the amount of PKC\textsubscript{g} and E-cadherin proteins in these cells were significantly correlated (\(r = 0.928, p = 0.00259\)) (Figure 1A).

To explore the relationship between epithelial state and PKC\textsubscript{g} expression, DLD-1 and SW480 cells (used mainly because both these cell lines have mutations in both KRAS and TP53, which are frequently observed in CRC, and they retain epithelial traits) were stably overexpressed with SNAI1, an EMT-inducible transcription factor associated with CRC.\textsuperscript{15} SNAI1 induced EMT, as demonstrated by a reduction in E-cadherin and an upregulation of vimentin expression (Figures 1B and 1C). SNAI1 transcriptionally repressed PKC\textsubscript{g} (Figure 1C). Furthermore, six putative SNAI1-binding sites (E-box; CANNTG) were identified in the proximal
promoter region of PRKCG (encoding PKC\(\gamma\)). To assess whether SNAI1 regulates PRKCG expression via the E-box region, we performed reporter assays using reporter constructs containing the PRKCG proximal promoter region. SNAI1 downregulated PRKCG promoter activity (Figure S2A), and mutagenesis in each E-box (CANNTG to ATNNTG) revealed two E-boxes (E1 and E3) that were essential for the suppression (Figure S2B). Because loss of epithelial property enhances additional EMT transcriptional factors, such as SNAI2, which also binds to the E-box, SNAI1 could regulate PRKCG directly and/or indirectly.5,16 These results suggest that PKC\(\gamma\) expression is associated with the epithelial property of CRC.

PKC\(\gamma\) expression is suppressed in dedifferentiated colorectal cancers

Using human CRC tissue microarrays, we next analyzed the expression of PKC\(\gamma\) in clinical samples. Immunohistochemical staining with anti-PKC\(\gamma\) antibody (positive control staining is shown in Figure 2A) revealed that PKC\(\gamma\) is expressed in normal colonic epithelium and that its expression is lower in CRCs, especially in dedifferentiated CRC (Figures 2B–2D, and Table S2).

PKC\(\gamma\) knockdown reduces E-cadherin expression and induces malignant phenotypes in colorectal cancer cells

To investigate the role of PKC\(\gamma\) in CRC, we stably knocked down PKC\(\gamma\) in the CRC cell line DLD-1 (Figure 3A). The knockdown of PKC\(\gamma\) reduced E-cadherin expression (Figure 3B). Transient knocked down of PKC\(\gamma\) in CRC cell lines, SW480, WiDr, Caco-2, and DLD-1 also caused E-cadherin reduction (Figures 3C and 3D). Furthermore, because PKC isozymes can affect each other,14 the expression of PKC\(\alpha\), which is expressed in the colon epithelium, was also assessed (Figures 3C and 3D). In some cases, PKC\(\alpha\) expression was partially reduced by transfection with PKC\(\gamma\) siRNA (Figure 3, WiDr). However, the knockdown of PKC\(\alpha\) in these cells did not cause a reduction in E-cadherin (Figure S3). Thus, PKC\(\gamma\), but not PKC\(\alpha\), is responsible for E-cadherin expression.

Because a reduction in E-cadherin promotes morphological change, cell growth at high cell density, drug resistance, and survival through the induction of \(\beta\)-catenin nuclear translocation,4,17 we next assessed
Figure 3. PKC\(\gamma\) knockdown reduces E-cadherin expression in CRC cells

(A) DLD-1 cells suppressing PKC\(\gamma\) (shPKC\(\gamma\) #1, #2) or negative control clones (shNeg#1, #2) were assessed by western blotting.

(B) DLD-1 cells transfected with shPKC\(\gamma\) (shPKC\(\gamma\) #1, #2) or negative control clones (shNeg#1, #2) were assessed by western blotting for E-cadherin. Immunofluorescence staining of E-cadherin (green) with an anti-E-cadherin antibody and counter nuclear staining using Hoechst (blue) is shown. The bar indicates 25 \(\mu\)m.

(C) SW480, WiDr, and Caco-2 cells were transfected with siPKC\(\gamma\)#1 and #2 and assessed using western blotting with the indicated antibodies (n = 3).

(D) DLD-1 cells were transfected with siRNA for PKC\(\gamma\), and after 6 days, the cells were assessed by western blotting using the indicated antibodies.

(E) DLD-1 cells were transfected with siPKC\(\gamma\)#1 and assessed for the TOP/FOP FLASH assay.

(F) Phase contrast images of DLD-1 cells transected with indicated siRNA and cultured on collagen-coated plates. The bar indicates 50 \(\mu\)m.
β-catenin-TCF/LEF-mediated transcription reporter assay (TOP/FOP FLASH). Knockdown of PKCγ in DLD-1 cells led to significant activation of TOP FLASH activity (Figure 3E). Indeed, the knockdown of PKCγ induced mesenchymal morphology to DLD-1 cells (Figure 3F). Mesenchymal marker fibronectin and the phosphorylation of related survival factor AKT were markedly upregulated upon PKCγ knockdown (Figure 3G). However, vimentin expression was not induced in these cells (data not shown), suggesting knockdown of PKCγ-induced partial EMT. Knockdown of PKCγ had a minimal effect on cell proliferation by 48 h, but the proliferation rate of PKCγ-suppressed cells increased after 3–4 days (Figure 4A). Soft agar assays revealed that PKCγ knockdown enhanced the anchorage-independent growth of DLD-1 cells (Figure 4B). Furthermore, PKCγ-suppressed cells showed less oxaliplatin-induced cell death, suggesting that PKCγ knockdown also enhances the resistance to oxaliplatin in DLD-1 cells (Figure 4C). Scratch migration assay revealed that PKCγ-knockdown enhanced the migration of DLD-1 cells (Figure 4D). We next performed in vivo experiments to evaluate the roles of PKCγ in tumor malignancy. Xenograft assays showed that PKCγ knockdown significantly enhances in vivo tumor growth of DLD-1 cells (Figure 4E). These results indicate that PKCγ has suppressive roles in CRC cell proliferation, anchorage-independent cell growth, resistance to anti-cancer drugs, migration, and in vivo tumor growth.

Low PKCγ expression is associated with poor prognosis in patients with colorectal cancer

To determine whether PKCγ expression affects prognosis, we examined gene expression datasets from patients with CRC, and constructed survival curves using the Kaplan–Meier method. In colon cancer datasets, the incidence of disease-free survival was significantly lower in patients whose tumors had low PKCγ expression than in those who had high PKCγ-expressing tumors (Figure 4F). These results support the idea that PKCγ has suppressive roles in CRC progression.

Identification of the phosphorylation substrates of PKCγ

To identify phosphorylation substrate proteins for PKCγ, we explored the phosphorylated PKC substrates in DLD-1 using an anti-phosphorylated PKC substrate antibody. Western blot analysis revealed several candidate fractions, which are decreased by PKCγ knockdown (Figure 5A). The immunoprecipitation of phosphorylated PKC substrate proteins using an anti-phosphorylated PKC substrate antibody from both DLD-1 and SW620 also revealed the candidate fraction which is observed more intensely in DLD-1 than in SW620 (Figure 5A). Subsequent nano-liquid chromatography (LC)–mass spectrometry (MS)/MS of the fraction identified the candidate proteins (Table S3). Among them, we assessed proteins with a putative PKC recognition motif (serine residues surrounded by arginine or lysine at the –2 and +2 positions and a hydrophobic residue at the +1 position), whether they are phosphorylated by PKCγ. Because the candidate fraction identified in SDS-PAGE (Figure 5A) could be degradation products, we did not take the molecular weight into account. When Rho/Rac guanine nucleotide exchange factor 18 (ARHGEF18) or BICD cargo adaptor 2 (BICD2) were co-overexpressed with PKCγ in DLD-1 cells, the phosphorylation of ARHGEF18 or BICD2 was enhanced (Figures 5B–5D). To determine whether these proteins can be directly phosphorylated by PKCγ, we performed in vitro kinase assays using the isolated proteins. These results revealed that ARHGEF18 and BICD2 can be directly phosphorylated by PKCγ (Figure 5E).

Consensus phosphorylation site motifs for PKCγ typically contain basic amino acids ([Arg] or [Lys]) at positions –2 and +2 from the phosphorylation sites (serine [Ser] or threonine [Thr]), although this is not absolute. To determine the possible phosphorylation sites of the identified substrates, the Ser residues in the motifs were mutated to Ala, and the phosphorylation by PKCγ was assessed. When Ser-256 of BICD2 or Ser-581 of ARHGEF18 was mutated to Ala, the phosphorylation of these substrates by PKCγ was reduced in cells and in vitro (Figures 5B, 5C, and 5E). These results indicate that these Ser residues are the phosphorylation sites for PKCγ or affect its phosphorylation.

To elucidate whether these proteins are phosphorylated by endogenous PKCγ, we knocked down PKCγ in SW480 cells, and the phosphorylation of the relevant substrates was evaluated. The phosphorylation of
BICD2 and ARHGEF18 was diminished by PKCγ knockdown (Figure 5F). In DLD-1 cells, phosphorylation of ARHGEF18 also decreased upon the knockdown of PKCγ (Figure 5G).

PKCγ stabilizes ARHGEF18 protein, which maintains epithelial morphogenesis

We observed a significant reduction in the amount of Halo-ARHGEF18 upon PKCγ knockdown (Figure S4A). This prompted us to examine whether PKCγ enhances the stability of ARHGEF18. The rate of ARHGEF18 degradation was measured in DLD-1 cells following treatment with cycloheximide, a protein synthesis inhibitor. Upon the siRNA-mediated suppression of PKCγ, the Halo-ARHGEF18 degradation rate increased.

Figure 4. PKCγ knockdown enhances malignant phenotypes in CRC cells

(A) The cell number was determined at the indicated times and the relative proliferation is shown (n = 3). (B) Cells were seeded in 6-well plates with soft agar and after 2–3 weeks, the numbers of colonies were counted (n = 3). Representative images of the colonies are also shown. (C) Cells were treated with 10 μM oxaliplatin (oxa) for 48 h and then incubated with FITC-labeled annexin V, and the percentage of annexin V-positive cells was determined (n = 3). (D) Confluent cells were starved for 24 h and the cell layers were scratched. Images at the same position were obtained before and after 19 h incubation. (E) Cells were inoculated into the flanks of nude mice. Representative images of the xenografts 28 days after inoculation are shown in the upper panels, when the xenografts were weighed (shNeg#1, n = 5; shNeg#2, shPKCγ#1, #2, n = 6, each). Data represent means ± s.e. (F) The relationship between PKCγ expression (GSE39582; Affymetrix microarray probe:206,270_at) and survival was determined and plotted using the Kaplan–Meier method. The disease-free survival rate for patients with high or low PKCγ expression is plotted as red and blue lines, respectively. (A–D) Data represent means ± S.D. Statistical analysis was performed using Dunnett’s multiple comparison of means test (A, B, and D), or Student’s t test (C). *p < 0.05; **p < 0.01; ***p < 0.001.
Figure 5. Identification of phosphorylation substrates of PKCγ

(A) Lysates of DLD-1 cells transfected with siPKCγ or siNeg were analyzed by western blotting using an anti-phosphorylated PKC substrate antibody (phos) (left panel). Lysates of DLD-1 and SW620 cells were immunoprecipitated using an anti-phosphorylated PKC substrate antibody, separated by SDS-PAGE, and analyzed by silver staining (right panel). The indicated bands were analyzed using nano-LC–MS/MS.

(B) FLAG-tagged BICD2 or (C) Halo-tagged ARHGEF18 was co-transfected with PKCγ in DLD-1 cells. Cell lysates were immunoprecipitated using anti-FLAG or Halo antibodies, and the phosphorylation levels were assessed using the anti-phosphorylated PKC substrate antibody (phos). The cell lysate before the commencement of immunoprecipitation served as the input (n = 3).

(D) Halo-tagged ARHGEF18 and PKCγ was transfected in DLD-1, performed pull-down using Halo resin, treated with Halo TEV protease to elute ARHGEF18 (GEF18), and then assessed using anti-phosphorylated Ser/Thr antibody (pSer/Thr) and indicated antibodies (n = 3).

(E) In vitro kinase assays were performed using purified PKCγ and substrate proteins as indicated (n = 3).

(F) FLAG-tagged BICD2 or Halo-tagged ARHGEF18 was co-transfected with control siRNA (siNeg) or siPKCγ. Cell lysates were immunoprecipitated using anti-FLAG or Halo antibodies, and then the phosphorylation levels were assessed using the anti-phosphorylated PKC substrate antibody (phos) (n = 3).
compared to that in cells transfected with a control siRNA (Figure 6A). Endogenous ARHGEF18 also degraded faster when PKCγ was knocked down (Figure 6B). In contrast, the knockdown of PKCα did not significantly affect ARHGEF18 degradation (Figure S4B). Furthermore, the degradation rate of ARHGEF18 (SS81A) was greater than that of the wild type (Figure 6C), suggesting the significance of ARHGEF18 phosphorylation by PKCγ in its stability. When PKCγ expression was suppressed, endogenous ARHGEF18 was also reduced (Figure 6D). Because ARHGEF18 is a guanine nucleotide exchange factor for RhoA, which activates RhoA at cell–cell junctions and promotes cell–cell junction assembly,23 we next assessed ARHGEF18 localization in DLD-1 cells. In control cells, ARHGEF18 localization at the cell–cell junction close to E-cadherin and apical actin filaments was observed (Figure 6E). In cells transfected with PKCγ-siRNA, E-cadherin and actin remained partially localized at the junction at day five post-transfection, but ARHGEF18 was diffusely distributed in the cells (Figure 6E). ARHGEF18 knockdown in CRC cells results in aberrant appearance of the junctional staining of peri-junctional F-actin, which is required for normal junction formation.23 F-actin staining away from cell junctions and redistribution of F-actin throughout the cells in PKCγ knockdown cells was also observed (Figure 6F). Because ARHGEF18 contributes to the maintenance of epithelial morphogenesis, such as 3D cyst formation observed in Caco-2 cells,23 we next assessed cyst formation. Caco-2 cells cultured in Matrigel form cysts with apical constricted actin.24 Although Caco-2 cells transfected with negative control siRNA formed normally polarized cysts with apically constricted actin, Caco-2 cells transfected with siRNA for PKCγ barely formed normal cysts (Figure 6G). These results suggest that PLCγ phosphorylates and stabilizes ARHGEF18 and regulates epithelial morphogenesis in CRC (Figure 6H).

**DISCUSSION**

To date, the role of PKCγ has not been well characterized in the field of cancer, although the role of PKCγ in neuronal diseases is well documented.25 Among them, SCA14, which is a type of spinocerebellar ataxia (a group of cerebellar diseases characterized by progressive ataxia and cerebellar atrophy), is caused by missense mutations in PRKCG. PKCγ dysregulation leads to the abnormal dendritic development of Purkinje cells, which induces the onset of SCA14.25 Because PRKCG expression in intestinal epithelium is much lower than that in neuronal tissues, the role of PKCγ in colorectal cancer progression is not well documented. In several human cancers, most PKC mutations result in their loss of function. Correction of a heterozygous PKCβ mutation via genome editing suppressed anchorage-independent growth and reduced CRC growth in a xenograft model, indicating that PKCβ has a tumor suppressive function.12 PKCα-knockout APCmin mice model display an elevated risk of intestinal tumors,13 suggesting that PKCα suppresses colon cancer development, whereas multiple studies revealed that PKCα confers drug resistance to human CRC cells.26,27 In human CRC tissues, PKCα and PKCβ expressions were downregulated compared with the normal colonic epithelium.27 Moreover, disease progression is not associated with the protein levels of PKCβII, and there is no reduction in the disease–free survival time associated with low PKCβII expression in colorectal cancer epithelial tissue.27

In this study, we explored the role of PKCγ in several CRC cell lines harboring no PRKCG mutation.26 We clarified that PKCγ expression is lower in CRC tissues, especially in dedifferentiated CRC (Figure 2). PKCγ knockdown reduces E-cadherin expression and enhances malignant phenotypes in several CRC cell lines (Figures 3 and 4). As a possible mechanism of PKCγ-mediated E-cadherin regulation, we suggested ARHGEF18 modulation by PKCγ. PKCγ phosphorylates and stabilizes ARHGEF18 (Figures 5 and 6), which maintains junctional assembly and epithelial morphogenesis via RhoA activation.22 Because cell–cell junctions maintain E-cadherin expression and suppress EMT progression in CRC cells,28 downregulation of PKCγ might result in E-cadherin reduction because of junctional disassembly. In DLD-1 cells, vimentin expression was not induced by PKCγ knockdown (data not shown), while fibronectin expression was markedly induced (Figure 3G); we thus speculated that PKCγ knockdown initially induces partial reduction of epithelial traits and partial induction of mesenchymal traits, which also enhances malignant properties.
Figure 6. PKCγ stabilizes ARHGEF18 and maintains epithelial integrity

(A) DLD-1 cells were transfected with Halo-ARHGEF18 and the indicated siRNAs. After 3 days, the cells were treated with cycloheximide (200 μg/mL) and then harvested at the indicated time points. The relative levels of Halo-ARHGEF18 normalized to ACTB were analyzed using western blotting (n = 3).

(B) DLD-1 cells were transfected with indicated siRNAs. After 3 days, the cells were treated with cycloheximide (200 μg/mL) and then harvested at the indicated time points. The relative levels of ARHGEF18 normalized to ACTB were analyzed using western blotting (n = 3).

(C) DLD-1 or SW480 cells were transfected with Halo-ARHGEF18 (WT or S581A), following which the cells were treated with cycloheximide (200 μg/mL) and harvested at the indicated time points. The relative levels of Halo-ARHGEF18 normalized to ACTB were analyzed using western blotting (n = 3).

(D) DLD-1 cells were transfected with the indicated siRNAs. After 4 days, endogenous ARHGEF18 was detected using western blotting (n = 4).

(E) DLD-1 cells were transfected with the indicated siRNAs. After 5 days, the cells were fixed with methanol and subjected to immunofluorescence staining with E-cadherin and ARHGEF18 antibodies (upper panels) or F-actin (Acti-stain 488 phalloidin; green) and ARHGEF18 antibodies (red) (lower panels). Scale bar = 10 μm.

(F) DLD-1 cells were transfected with the indicated siRNAs. After 6 days, the cells were fixed with methanol and subjected to immunofluorescence staining with F-actin. Scale bar = 50 μm.

(G) Caco-2 cells were transfected with the indicated siRNA, mounted in Matrigel, and then examined using immunostaining with Hoechst (blue) and phalloidin (red). The percentage of normally polarized cysts per total cell cluster...
Several LOF PKC mutations act in a dominant-negative manner. This means that they decrease global endogenous PKC activity, which could be a sign of the interrelationships between the different isoforms of PKCs. Since PKCα is expressed in the colon epithelium and has a suppressive function in colon cancer development,13 we also assessed whether knockdown of PKCα affects E-cadherin expression. Knockdown of PKCα in CRC cells did not result in a reduction in E-cadherin level (Figure S3), suggesting a functional difference between PKCα and PKCγ. The precise functional comparison of cPKCs remains to be fully resolved.

**Limitations of the study**

In this study, we did not rescue the PKCγ knockdown phenotype by ARHGEF18 overexpression, because it is speculated that many other phosphorylation substrates for PKCγ might regulate cellular phenotype coordinately. In that situation, the importance of ARHGEF18 modulation by PKCγ remains obscure. Further investigations are required to identify many other substrate proteins for PKCγ and to elucidate the importance of their phosphorylation by PKCγ. Appropriate regulation of PKCγ activity at the cell-cell junction may also be a prerequisite for its tumor suppressive effect and the role of PKCγ in other cellular components may be different. Further analysis of PKCγ with respect to other identified binding proteins is required.

**STAR+METHODS**

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Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105501.
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AUTHOR CONTRIBUTIONS

R. S. and K. F. conceptualized and designed the study. R. S., Y. S., S. A., S. K., S. O., N. I., and A. Y. conceived, designed, and performed most of the experiments. M.I. analyzed the gene expression datasets. R. S. supervised all the research.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Mouse monoclonal anti-E-cadherin | BD Biosciences | Cat#610182, RRID:AB_397581 |
| Rabbit polyclonal anti-Flag | Sigma | Cat#F7425, RRID:AB_439687 |
| Mouse monoclonal anti-Flag | Sigma | Cat#F1804, RRID:AB_262044 |
| Rabbit polyclonal Fibronectin | Merck | Cat#Ab1954, RRID:AB_11213226 |
| Mouse monoclonal anti-GAPDH | Santa Cruz | Cat#sc-32233, RRID:AB_627679 |
| Mouse monoclonal anti-β-actin | Sigma | Cat#A5441, RRID:AB_476744 |
| Mouse monoclonal anti-Halo | Promega | G921A |
| Rabbit polyclonal anti-Halo | Promega | G928A |
| Rabbit polyclonal anti-phospho Akt (Ser473) | Cell Signaling | Cat #2971, RRID:AB_329625 |
| Rabbit polyclonal anti-phospho PKC substrate | Cell Signaling | Cat#2261, RRID:AB_330310 |
| Mouse monoclonal anti-PKCα | BD Biosciences | Cat#610107, RRID:AB_397513 |
| Mouse monoclonal anti-PKCβ | Santa Cruz | Cat#sc-13149, RRID:AB_628144 |
| Mouse monoclonal anti-PKCγ for Figures 1A and 1B (SW480), Figures 2, 3, and 6 | Abnova | Cat#H00005582-A01, RRID:AB_463355 |
| Rabbit polyclonal anti-PKCγ for Figure 1B (DLD-1) | Santa Cruz | Cat#sc-211, RRID:AB_632234 |
| Mouse monoclonal anti-PKCγ for Figures 5B–5D | Santa Cruz | Cat#sc-166385, RRID:AB_2018059 |
| Mouse monoclonal anti-Vimentin | Santa Cruz | Cat#sc-32322, RRID:AB_628436 |
| Goat anti-ARHGEF18 | Everest BIOTECH | Cat#EB06163, RRID:AB_2227541 |
| Chemicals, peptides, and recombinant proteins |        |            |
| Bryostatin 1 | Sigma | B7431 |
| Cycloheximide | Sigma | C4859 |
| Oxaliplatin | Wako | 156-02691 |
| Puromycin | Sigma | P8833 |
| Critical commercial assays |        |            |
| Dual-luciferase Reporter Assay System | Promega | E1910 |
| TMB Super Sensitive HRP Substrate | Surmodics | TMBS-0100-01 |
| Deposited data |        |            |
| Mendeley dataset | https://data.mendeley.com/datasets/zzdr4k8fj4/draft?a=c8f44734-9ace-4306-8860-2e71ef2cb12 | N/A |
| Experimental models: Cell lines |        |            |
| CaCo2 | RIKEN BRC | RCB0988, RRID:CVCL_0025 |
| SW620 | ATCC | Cat#CCL-227, RRID:CVCL_0547 |
| SW480 | ATCC | Cat#CCL-228, RRID:CVCL_0546 |
| DLD-1 | JCRB Cell Bank | Cat#JCRB9094, RRID:CVCL_0248 |
| WiDr | ATCC | Cat#CCL-218, RRID:CVCL_2760 |

(Continued on next page)
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| HEK293              | JCRB Cell Bank | Cat#JCRB9068, RRID:CVCL_0045 |
| shPKCγ expressing DLD-1 | This paper | N/A |

**Oligonucleotides**

| Oligonucleotides | SOURCE | IDENTIFIER |
|------------------|--------|------------|
| Allstars Negative Control siRNA | Qiagen | SI03650318 |
| si PKCγ#1       | Qiagen | SI04997174 |
| si PKCγ#2       | Qiagen | SI03078306 |
| si PKCα         | Qiagen | SI04997174 |
| human PRKCG-Forward; GTTTAAGGAGCCCATGCAG | FASMAC | N/A |
| human PRKCG-Reverse; CCCTCAGCATCCAGCATCAC | FASMAC | N/A |
| human ACTB-Forward; GCCCTGGGACCCAGCACAAT | FASMAC | N/A |
| human ACTB-Reverse; GGAGGGGCCGGACTGCTCAT | FASMAC | N/A |
| human GAPDH-Forward; AGCCTCCCGCTTCGCTCTCT | FASMAC | N/A |
| human GAPDH-Reverse; CCAGGCGGCACATACGACCA | FASMAC | N/A |
| human SNAI1-Forward; CTGCCGGGAAGGCCCTTCTTCT | FASMAC | N/A |
| human SNAI1-Reverse; CGCTTGCGACTGTTACTTCTT | FASMAC | N/A |

**Recombinant DNA**

| Recombinant DNA | SOURCE | IDENTIFIER |
|-----------------|--------|------------|
| pMXs-IN-Snail (SNAI1) | This paper | N/A |
| pcDNA3.1- PKCγ (PRKCG) | This paper | N/A |
| pcDNA3.1- PKCα (PKRCA) | This paper | N/A |
| pFN21A-Halo-PKRCB | Promega | FHC10533 |
| pcDNA3.1- Flag-BICD2 | This paper | N/A |
| pFN21A-Halo-ARHGEF18 | Promega | FHC01977 |
| pSUPER retro puro- shPKCγ#1 targeting; 5’GGCCATCATGGAACAAACTGT-3’ | This paper | N/A |
| pSUPER retro puro- shPKCγ#2 targeting; 5’GGCCATCATGGAACAAACTGT-3’ | This paper | N/A |
| pGL4.10-luc2 vector | Promega | E665A |
| pGL4.74-hRluc vector | Promega | E692A |
| pGL4.10- luc2-pPKCγ-Luc | This paper | N/A |

**Software and algorithms**

| Software and algorithms | SOURCE | IDENTIFIER |
|-------------------------|--------|------------|
| R (v4.0.3)              | http://www.r-project.org/ | N/A |
| CS Analyzer4 (Atto)     | https://www.atto.co.jp/site/products/geldocumentation/ | N/A |
|                         | Image-analysis-software/ | |
|                         | Image-Analysis-Software2 | |

**Deposited data**

| Deposited data | SOURCE | IDENTIFIER |
|----------------|--------|------------|
| Raw data for figures | https://data.mendeley.com/datasets/yzp4k8rfj/draft? a=c8f44734-9ace-43b6-8860-2e7f1efdcb12 | N/A |
RESOURCES AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Reiko Satow (rsatow@toyaku.ac.jp).

Materials availability
The plasmids generated in this study are available from the lead contact on reasonable request.

Data and code availability
The datasets used during the current study are available from the lead contact on reasonable request. Our dataset was also deposited as Mendeley dataset at [https://data.mendeley.com/datasets/yzp4k8frj4/draft?a=c8f44734-9ace-43b6-8860-2e7f1efdcbb12].

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture and reagents
The colorectal adenocarcinoma cell lines SW620, SW480 and WiDr were obtained from the American Type Culture Collection (Manassas, VA, USA). DLD-1 and HEK293 cell lines were obtained from JCRB Cell Bank (National Institute of Health Sciences, Tokyo, Japan). Caco-2 cells were obtained from the RIKEN Bioresource Center (RIKEN BRC) (Ibaraki, Japan). These cell lines were re-validated by short tandem repeat profiling in 2016 (Promega, Madison, WI, USA). The cells were maintained as described previously. Bryostatin 1 and cycloheximide were obtained from Sigma (B7431, C4859) and oxaliplatin was obtained from Wako (156-02691).

Animal experiments
Five million DLD-1 cells stably expressing shPKC\(\gamma\) were suspended in 0.1 mL of PBS (PBS) and subcutaneously inoculated into the flanks of 5-week-old female BALB/c nu/nu nude mice (CLEA, Tokyo, Japan). Animal experiments were approved by the institutional ethics committee and performed in compliance with the guidelines for Laboratory Animal Research of the Tokyo University of Pharmacy and Life Sciences (Tokyo, Japan).

METHOD DETAILS

Western blot analysis
Whole-cell lysate was collected using Laemmli sample buffer. Western blot analysis was performed as described previously, with some modifications. Primary antibodies targeting E-cadherin (610182, BD Biosciences, San Jose, CA, USA), FLAG tag (F7425, Sigma), Fibronectin (AB1954, Merck), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sc32233, Santa Cruz, Dallas, TX, USA), β-actin (AS441, Sigma), Halo tag (G921A, Promega), phospho PKC substrate (#2261, Cell Signaling), phospho Akt (Ser473) (#9271, Cell Signaling), PKC\(\gamma\) (sc211 for Figure 1B; DLD-1) or sc166385 (for Figures 5B–5D), Santa Cruz or H00005582, Abnova (for Figures 1A and 1B (SW480), Figures 2, 3, and 6)), vimentin (sc32322, Santa Cruz) and ARHGEF18 (EB06163, Everest Biotech) were used. Images were obtained using ImageQuant TL (GE Healthcare, Piscataway, NJ, USA) or LuminoGraph I (Atto) and quantified using CS Analyzer4 (Atto).

Plasmids, siRNA, and transfection
The open reading frame (ORF) of human SNAI1 (Gene symbol; SNAI1) was amplified by PCR and subcloned into the pmxs-IN vector. The ORFs of human PRKCA, PRKCG and BICD2 were amplified and subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). We have confirmed that none of these sequences contain mutations. Halo-tagged ARHGEF18 and PKCB (PKCB) were obtained from Promega (FHC01977 and FHC10533, respectively). The pSUPER retro puro retroviral vector (Oligoengine) was used for short hairpin RNA (shRNA) expression with the following targeting sequences: shPKC\(\gamma\)-#1, 5′- GGCCATCATGGAAAATG -3′; and shPKC\(\gamma\)-#2, 5′- GCCGAAGCTTTGAAGTCT -3′. To obtain stable cell lines, transfected cells were selected with 1 μg/mL puromycin (P8833, Sigma) or 1,000 μg/mL G418 (10131035, Thermo) and the resulting single clones were analyzed for stable expression.

Negative control siRNA (siNeg) and siRNAs for PKC\(\gamma\) (Qiagen, Hilden, Germany) were used at final concentrations of 20 nM. Transient transfections were performed using polyethyleneimine, Lipofectamine 2000, or...
Lipofectamine RNAi max (Invitrogen), according to the manufacturer’s protocols. In every experiment, the total amount of transfected DNA or siRNA was adjusted using the relevant empty vector or negative control siRNA, respectively.

Luciferase assay
The luciferase reporter construct pPKCγ-Luc was constructed by amplifying the PRKCG proximal promoter region using the primers pPKCγ-F (5'-CAC AAG ATC TGA GAT TGG GTC AGA GAG AAA GGGA -3') and pPKCγ-R (5'-ACC AGC CAT GGC CCC AGAA -3'), and subcloned into the BglII–NcoI region of the pGL4.10-luc2 vector (Promega). pPKCγ-Luc mutants were constructed using the following primers: m1-F, 5'-ATC CTG TTT CCC CCA AGA AAG GCA -3'; m2-F, 5'-ATC CTG GAG GTG CCT TGC CCC T -3'; m3-F, 5'-ATG GTG CCG GAG CTG GAG CTC -3'; m4-F, 5'-GGG AGG AAT TTT GTC CCG TGTC TCT CCGG -3'; and m5-F, 5'-ATC CTG TGG GGG GCG GGG -3'. Cells were co-transfected with the pPKCγ luciferase reporter constructs and a pGL4.70 internal control plasmid (Promega) using Lipofectamine 2000 reagent (Invitrogen). The TOP/FOP FLASH assay was performed to evaluate T-cell factor/lymphocyte enhancer factor transcriptional activity. Luciferase activity was then measured using the Dual-luciferase Reporter Assay System (Promega), as described previously.

Immunohistochemistry
Human colon carcinoma tissue arrays with matched adjacent normal colon tissue were purchased from US Biomax (Rockville, MD, USA). The immunostaining was performed as described previously with anti-PKCγ antibody (H00005582, Abnova).

Immunocytochemistry
Immunofluorescence microscopy was performed as described previously using antibodies targeting E-cadherin (580061, BD Biosciences) and ARHGEF18 (EB06163, Everest Biotech). F-actin was stained with Acti-stain 488 phalloidin (Cytoskeleton Inc.). Images were obtained using a BZ-X700 microscope (Keyence) with sectioning and z stack modules.

RNA isolation, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)
RNA isolation, cDNA synthesis, and qRT-PCR were performed as described previously. To calculate the relative mRNA expression in the qRT-PCR analysis, standard curve lines for each primer set were plotted and then used for analysis. The primers used were PRKCG (PKCγ), F; GTTTAAGGAGCCCCATGCAG, R; CCCTCAGCATCCAGCATCAC, ACTB, F; GCCCTGACCCACGACAAT, R; GGAGGGCGCCGACTCG TCAT, GAPDH, F; AGCCCTCCCGTCCGACTCTCT, R; CCAGGCGCCCAATACGACCA, SNAI1, F; CTGC GGGAAGGCCTCTCT, R; CGCCTGGCACTGGTACTCTT.

Cell proliferation and scratch migration assays
Cell proliferation were assayed as described previously. For scratch migration assay, cells were seeded in 96-well plate at a density of 0.25 × 10^4 cells per well. After 6 days, cells were starved for 24 h and the cell layers were scratched with sterile pipette tip from top to bottom. Images at the same position were obtained using InCell Analyzer 2000 (GE Healthcare) before and after 19 h incubation.

Soft agar colony formation assay
The cells were layered in base agar (0.8%)-coated 6-well plates with room temperature agar (0.5%) in RPMI 1640 medium. The plates were then incubated at 37°C for 2–3 weeks, the number of colonies was counted, and images were obtained.

Immunoprecipitation
Cells were lysed in immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl at pH 7.5, 0.2% deoxycholate, and 0.5% Nonidet P-40) supplemented with a phosphatase inhibitor cocktail (Sigma-Aldrich) and a protease inhibitor cocktail (Roche, Mannheim, Germany). The lysate was incubated with anti-FLAG M2 monoclonal antibody (F1804, Sigma) or anti-Halo antibody (G928A, Promega) at 4°C overnight and then precipitated using protein G (GE Healthcare). Immunoprecipitates were washed four times with ice-cold PBS and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Silver staining and nano-LC–MS/MS

Silver staining was performed using a Silver Stain kit for MS (Apro Science, Tokushima, Japan), according to the manufacturer’s protocol. The tryptic peptides were fractionated using an UltiMate 3000 RSLCnano system (Thermo Fisher Scientific) and analyzed using an Orbitrap Elite (Thermo Fisher Scientific). Tandem mass spectra were extracted using Proteome Discoverer version 1.4. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.5.1), which was set up to search the NCBIprot_Homo_sapiens_20180308 database (1,228,116 entries), assuming the use of the digestion enzyme trypsin (Support Center for Advanced Medical Sciences, Tokushima University Graduate School of Biomedical Sciences).

Recombinant protein purification

HEK293 cells were transfected with FLAG or Halo-tagged expression vectors. After 2–3 days, FLAG-tagged protein-expressing cells were lysed in immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl at pH 7.5, 0.2% deoxycholate, and 0.5% Nonidet P-40) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany); anti-FLAG affinity agarose gel (A2220, Sigma) was added and the mixture was incubated overnight at 4°C. The gel was washed thoroughly with PBS and FLAG-tagged protein was eluted by adding FLAG peptide. The Halo-tagged protein was purified using HaloLink resin (G1914, Promega), according to the manufacturer’s protocol.

In vitro kinase assay

Purified substrate proteins (0.3–1 μg/mL) were loaded into ELISA plates (Iwaki) at 4°C overnight. After washing twice with Tris-buffered saline supplemented with 0.05% Tween 20 (TTBS), 50 μL of kinase buffer (150 mM NaCl, 20 mM HEPES at pH 8, 10 mM MgCl₂, 1 mM DTT, and 20 μM ATP, 0.5 μM or 1 mM CaCl₂) with 1 μM bryostatin 1 and 1 μg/mL PKC was added and the plate incubated at 30°C for 1 h. After washing twice with TTBS, anti-phosphorylated PKC substrate (1/1000) was added and the plate was incubated for 1 h; then, HRP-conjugated anti-rabbit IgG (1/1000) was added for 30 min, the plate was washed three times with TTBS, incubated with 50 μL of TMB Super Sensitive HRP Substrate (Surmodics) for 30 min, and the absorbance was read at 370 nm using a microplate reader.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Data were analyzed using the R statistical software package (version 4.0.3). The statistical analyses are described in the Figure legends. The tests used included Wilcoxon signed-rank test, Fisher exact test, two-tailed unpaired Mann–Whitney U-test, and two-tailed unpaired Student’s t test. Dunnett’s or Tukey’s test was used for post-hoc multiple comparisons. *p < 0.05 was considered to represent statistical significance (*p < 0.05; **p < 0.01; ***p < 0.001). Data are presented as the mean ± standard deviation (s.d.) (n ≥ 3 independent experimental replicates), unless otherwise stated.

Analysis of gene expression datasets

Using gene expression datasets (GSE39582) from human cancers that included information on survival, the patients were divided into high and low PKC expression groups. A Kaplan–Meier survival curve was constructed in Survfit (R package). p values were calculated using the log rank test in Survdiff (R package).

ADDITIONAL RESOURCES

Supplemental information contains four figures and three tables.