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Phospholipase D1 Inhibition Linked to Upregulation of ICAT Blocks Colorectal Cancer Growth Hyperactivated by Wnt/β-Catenin and PI3K/Akt Signaling

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Abstract

Purpose: Dysregulated expression of PLD1 has emerged as a hallmark feature of colorectal cancer, which remains a major cause of mortality worldwide. Aberrant activation of Wnt/β-catenin signaling is a critical event in the development of colorectal cancer. Here, we investigated molecular crosstalk between the Wnt/β-catenin and PI3K/Akt pathways via inhibitor of β-catenin and T-cell factor (ICAT), a negative regulator of Wnt/β-catenin signaling. We also explored the effect of PLD1 inhibition on growth of colorectal cancer hyperactivated by Wnt/β-catenin and PI3K/Akt signaling.

Experimental Design: Expression of ICAT via targeting of PLD1 was assessed *in vivo* in $Apc^{Min/+}$ mice, an AOM/DSS model, and *in vitro* using various colorectal cancer cells. The relationship between ICAT/PLD1 expression and prognostic survival value of 153 colorectal cancer patients was examined. The therapeutic efficacy of PLD1 inhibitor was determined using a patient-derived xenograft model carrying *APC* and *PI3K* mutations.

Results: PLD1 promoted the Wnt/β-catenin signaling pathway by selectively downregulating ICAT via the PI3K/Akt-TopBP1-E2F1 signaling pathways. Low PLD1 expression and high ICAT expression were significantly associated with increased survival in colorectal cancer patients and vice versa. Furthermore, PLD1 inhibition suppressed growth of colorectal cancer activated by the Wnt/β-catenin and PI3K signaling pathways.

Conclusions: These results suggest that PLD1 linked to ICAT mediates molecular crosstalk between the Wnt/β-catenin and PI3K/Akt pathways and thus could be proposed as a novel colorectal cancer prognostic biomarker. These results may assist in the clinical development of a PLD1 inhibitor for treatment of colorectal cancer patients carrying *APC* and *PI3KCA* mutations. PLD1, a nodal modifier, acts as a potential therapeutic target for the treatment of colorectal cancer hyperactivated by the Wnt/β-catenin and PI3K/Akt signaling pathways. *Clin Cancer Res; 23(23); 7340–50. ©2017 AACR.*

Introduction

In colorectal cancer, the Wnt/β-catenin and PI3K/Akt signaling pathways are frequently dysregulated due to activating mutations in *APC* and the p110α subunit of *PI3K*. Thus, oncogenic pathway activation has become an attractive therapeutic target for drug development. (1, 2). Wnt/β-catenin oncogenic signaling confers resistance to apoptosis promoted by PI3K or Akt-inhibitory drugs (3, 4). Integration of these two important signaling pathways enhances cancerous growth in tumors and confers resistance against targeted therapies. Thus, it is crucial to elucidate the molecular mechanisms responsible for drug resistance and identify new predictive biomarkers of the drug response that could improve selection of patients sensitive to treatment. However, the molecular mechanism responsible for crosstalk between the Wnt/β-catenin and PI3K/Akt pathways in cancer remains elusive. Abnormal activation of the Wnt/β-catenin pathway by mutation of *APC* is responsible for the initiation and progression of almost all colorectal cancers (5). As a result, accumulated β-catenin in the nucleus binds to T-cell factor (TCF) transcription factor and induces expression of Wnt target genes, which play key roles in tumor progression (6). Recently, we reported that *phospholipase D* (*PLD*) isozymes (*PLD1/PLD2*) comprise the transcriptional target gene of β-catenin/TCF (7–9). In addition, PLD1 enhances Wnt signaling by increasing the β-catenin/TCF-4 interaction, revealing bidirectional crosstalk between the PLD1 and Wnt/β-catenin pathways (7–9). PLD hydrolyzes phospholipids to generate phosphatidic acid (PA) in response to mitogens and growth factors. PLD or its product PA is known to regulate a variety of homeostatic cellular functions such as cell proliferation, survival,

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

To date, hyperactivation of the Wnt/β-catenin and PI3K/Akt signaling pathways has limited clinical benefit mostly due to unknown resistance mechanisms and the lack of predictive biomarkers of the drug response. We also provide evidence for a rational stratification of patients hyperactivated by the Wnt/β-catenin and PI3K/Akt signaling pathways using PLD1 and ICAT as predictive targets of the drug response. Such refined molecular selection of patients could represent a significant improvement in response to treatment as well as an important step forward in advancing colorectal cancer therapy.

membrane trafficking, vesicular transport, cytoskeletal re-organization, and migration (10). Upregulation of PLD, which promotes cell proliferation and suppresses default apoptotic programs that prevent cancer, is known to be a critical aspect of tumorigenesis in various cancers (10–12). Here, we studied crosstalk between the Wnt/β-catenin and PI3K/Akt pathways in colon cancer based on their role in tumorigenesis. We observed that PLD1 promoted Wnt/β-catenin signaling by selectively downregulating inhibitor of β-catenin and T-cell factor (ICAT), a negative regulator of Wnt signaling, through the PI3K/Akt-TopBP1-E2F1 signaling axis. High expression level of PLD1 coupled with low expression of ICAT was significantly correlated with lower overall survival of colorectal cancer patients and vice versa, suggesting the potential value of PLD1 and ICAT as therapeutic targets of colorectal cancer. Moreover, PLD1 inhibition linked to upregulation of ICAT suppressed tumor growth in a patient-derived xenograft model harboring *APC* and *PI3KCA* mutations. Overall, our results suggest that PLD1 inhibition exhibits potent anticancer activity in colorectal cancer hyperactivated by the Wnt/β-catenin and PI3K/Akt signaling pathways.

Materials and Methods

Cell lines

Human cancer cell lines were purchased from the American Type Culture Collection, NCI Division of Cancer Treatment and Diagnosis, or Korea Cell Line Bank. Detailed cell line characterization and maintenance conditions are described in the Supplementary Methods. Mouse embryonic fibroblasts (MEF) from wild-type (WT), *Akt1*$^{-/-}$, and *Akt2*$^{-/-}$ mice were kindly provided by S.S. Bae (Pusan National University). MEFs from *Topbp1*$^{+/+}$ and *Topbp1*$^{-/-}$ mice were kindly provided by S.D. Hwang (Seoul National University). MEFs from *Rb1*$^{+/+}$, *Rb1*$^{-/-}$, *E2f1*$^{+/+}$, and *E2f1*$^{-/-}$ mice were kindly provided by H.W. Lee (Yonsei University).

Mice

Apc$^{min/+}$*Pld1*$^{-/-}$ mice were generated as previously described (13). *Apc*$^{min/+}$*Pld1*$^{-/-}$ mice and age-matched *Apc*$^{min/+}$*Pld1*$^{+/+}$ or WT littermate controls were used. An azoxymethane (AOM)/dextran sodium sulfate (DSS)–induced mice were treated with 10 mg/kg of PLD1 inhibitor as previously described (13). Animal studies were approved by the Institutional Animal Care Committee of Pusan National University.

Patient-derived xenograft models

Fresh colorectal tumors were obtained from consenting patients at Asan Medical Center in accordance with protocols approved by the Institutional Review Board (IRB). We excluded the patients who had neoadjuvant chemotherapy. For detailed development of patient-derived xenograft (PDX) models, see the Supplementary Materials and Methods.

Histology and immunohistochemistry

Human colon tumor and adjacent normal tissues were obtained from Pusan National University Hospital, a member of the National Biobank of Korea, with informed consent according to IRB-approved protocols. For immunohistochemistry (IHC) analysis, tissue microarray slides were purchased from SuperBioChips (CDA2, CDA3, and CD4) and US-Biomax (CC05-01-002). Each array includes more than 178 cases of normal, reactive, premalignant, and malignant tissues of the colon (various grades and stages). For detailed IHC, see the Supplementary Materials and Methods.

Primary cell culture from human colorectal cancer tissue

Experiments were performed after receiving patient-informed consent and approval from the IRB of Asan Medical Center. All procedures performed in studies involving human participants were conducted in accordance with International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS). Human colon samples were excised from 8 patients during surgery and were obtained through the Asan Bio Resource Center. A small piece of tumor tissue was removed and cultured to establish patient-derived primary cancer cell lines. Briefly, tumor tissues were minced with a scissor and subsequently digested using 1 mg/mL of type IV collagenase (Sigma) in DMEM/F12 for 90 minutes at 37°C. After incubation, tissues were washed with medium containing 10% FBS. We used two culture methods as follows: (a) to favor adhesion and growth of epithelial tumor cells, Renal Epithelial cell Basal Media (REBM; Lonza) containing hEGF, hydrocortisone, epinephrine, insulin, transferrin, GA-1000, FBS, and a Triiodothyronine-Single Quots Kit were used to culture primary colorectal cancer cells plated on collagen type 1 dishes in a humidified incubator with a 5% CO_2 atmosphere at 37°C; and (b) in coated Matrigel, we used REBM (without FBS) with 1 X N2, 1 X B27, 10 μmol/L Rock inhibitor, and 10 μmol/L gastrin as colorectal cancer primary cell culture media. Colorectal cancer (#945, #927, #942, #921, #821, #799, #982, and #934) patient-derived cells (PDC) were successfully isolated and cultured. For colorectal cancer PDCs, clinical characteristics of the 8 colorectal cancer patients with the corresponding original tumors are summarized in Supplementary Table S1.

Ion Torrent PGM sequencing and data processing

DNA samples from original tumor tissues and PDXs were obtained from PE blocks after a histopathology review. Cancer gene mutation profiling was performed using an Ion AmpliSeq Library Kit 2.0 and an Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies), which amplifies 207 amplicons covering approximately 2,800 COSMIC mutations in 50 oncogenes and tumor-suppressor genes, such as KRAS, PIK3CA, BRAF, TP53, and APC. Sequencing coverage of ×200 was used as the minimum requirement to verify the authenticity of sequence variants. We used IonTorrent Software for automated data analysis. All processes were conducted by TheragenETEX Bio Institute. Genetic profiles of

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Table 1. Genetic profiles of the original tumors, PDXs, and PDCs of CRC patients

		Genetic alteration				
Case number	**Type**	**APC**	**PIK3CA**	**KRAS**	**EGFR**	**TP53**
CRC #945	Original tumor	WT	WT	G12D	WT	R273H
	PDC	WT	WT	G12D	WT	R273H
CRC #927	Original tumor	WT	WT	G12D	WT	WT
	PDC	WT	WT	G12D	WT	WT
	PDX	WT	WT	G12D	WT	WT
CRC #942	Original tumor	WT	WT	A146T	WT	WT
	PDC	WT	WT	A146T	WT	WT
CRC #921	Original tumor	WT	E545K	G12D	WT	WT
	PDC	WT	E545K	G12D	WT	WT
	PDX	WT	E545K	G12D	WT	WT
CRC #821	Original tumor	WT	H1047R	WT	WT	R248Q
	PDC	WT	H1047R	WT	WT	R248Q
CRC #799	Original tumor	T1556fs*3	WT	G12D	WT	WT
	PDC	T1556fs*3	WT	G12D	WT	WT
	PDX	T1556fs*3	WT	G12D	WT	WT
CRC #982	Original tumor	WT	WT	WT	WT	R175H
	PDC	WT	WT	WT	WT	R175H
CRC #934	Original tumor	E1309fs*4	WT	WT	WT	WT
	PDC	E1309fs*4	WT	WT	WT	WT

Abbreviation: CRC, colorectal cancer.

the original tumors, PDXs, and PDCs of colorectal cancer patients are summarized in Table 1.

Statistical analysis

Data represent the mean ± SD. Data were analyzed by the Student *t* test, and $P < 0.05$ was considered to be statistically significant. The Spearman rank test was used to determine the correlation between *PLD1* and *ICAT* gene expressions in human cancer cells. Fitted survival function was analyzed by multivariate Cox proportional hazard model.

Results

PLD1 inhibition suppresses Wnt/β-catenin signaling via upregulation of ICAT and increases drug sensitivity in PDCs harboring PIK3CA and APC mutations

Our previous work demonstrated the role of PLD in the acceleration of Wnt signaling via enhanced association of β-catenin with TCF-4 (7–9). However, the mechanism by which PLD1 positively regulates Wnt activity is unclear. We examined whether or not PLD affects interactions between β-catenin and negative regulators of Wnt signaling, which bind to β-catenin and interactively inhibit binding of β-catenin to TCF. As previously reported (7, 8), PLD1 inhibitor (VU0155069) reduced binding of β-catenin to TCF4 (Fig. 1A). Of particular interest, PLD1 inhibition increased association of ICAT with β-catenin as well as expression of ICAT in human colorectal cancer cells in which β-catenin–TCF4-mediated transcription is aberrantly activated due to mutations in β-*catenin* (HCT116) and *APC* (SW480, DLD1; ref. 15). However, PLD1 inhibition did not affect expression of other negative regulators of Wnt signaling (Chibby, Plakoglobin, FoxO1, FoxO3, and PPARγ; refs. 16–19) or their interactions with β-catenin, suggesting that ICAT is a selective target of PLD1-mediated regulation of the Wnt/β-catenin signaling pathway (Fig. 1A). ICAT is known to competitively bind to β-catenin for inhibition of β-catenin/TCF-4 complex formation (20). PLD1 inhibitor and two different siRNAs targeting PLD1 enhanced mRNA expression of ICAT in several colorectal cancer cells (Fig. 1B; Supplementary Fig. S1A), suggesting that PLD activity is involved in suppressing the *de novo* expression of ICAT at the transcriptional level. Pharmacologic inhibition and genetic knockdown of PLD1 suppressed Wnt3a-induced binding of TCF4 with β-catenin but increased interaction of ICAT with β-catenin, followed by increased expression of Wnt target genes such as *c-myc* in HEK293 cells with nonactivated Wnt signaling (Fig. 1C). Moreover, both treatment with PA, the product of PLD activity, and ectopic expression of PLD1 reduced expression of ICAT in KM12 and SNU-C5 colorectal cancer cells with nonactivated Wnt signaling (Supplementary Fig. S1B). As a control, overexpression of ICAT reduces the association of TCF-4 with β-catenin as well as expression of Wnt target genes such as PLD1 and *c-myc* (Supplementary Fig. S1C). Similarly, ectopic expression of ICAT reduced Wnt3a-induced association of β-catenin with TCF4 as well as expression of Wnt target genes (Supplementary Fig. S1D). Conversely, knockdown of ICAT increased formation of the β-catenin/TCF-4 complex, whereas RNAi-mediated interference of ICAT in PLD1-depleted cells significantly restored formation of the complex compared with that in only PLD1-depleted cells (Supplementary Fig. S1E). Consequently, depletion of ICAT increased Wnt3a-induced TCF-4 transactivation and recovered expression of TOPflash reporter reduced by PLD1 knockdown (Supplementary Fig. S1F). In addition, depletion of both ICAT and PLD1 significantly restored TCF-4 transactivation compared with that in PLD1-depleted HCT116 cells (Supplementary Fig. S1G). Collectively, these results indicate that PLD1 promotes Wnt/β-catenin signaling by negatively regulating ICAT expression. Recently, PDC models have been suggested as an alternative preclinical model (21) to be used as a prediction tool for preclinical drug sensitivity. We examined whether or not PLD1 inhibitor shows a clinical response in a PDC model of selected colorectal cancer patients with genetic features. Using the Ion Torrent PGM system, we evaluated whether or not PDCs could maintain the genetic features of their parent tumors using eight pairs of PDCs and primary tumors from colorectal cancer patients. Frequently detected somatic mutations were identified in primary tumors and PDCs (Table 1). Mutation profiles were as follows: TP53 (three cases, 37.5%), KRAS (five cases, 63%), PIK3CA (two cases, 25%), and APC (two cases, 25%). All of the somatic mutations detected in the primary tumor were concordantly detected in PDC models. PLD inhibitor significantly reduced the viabilities of four

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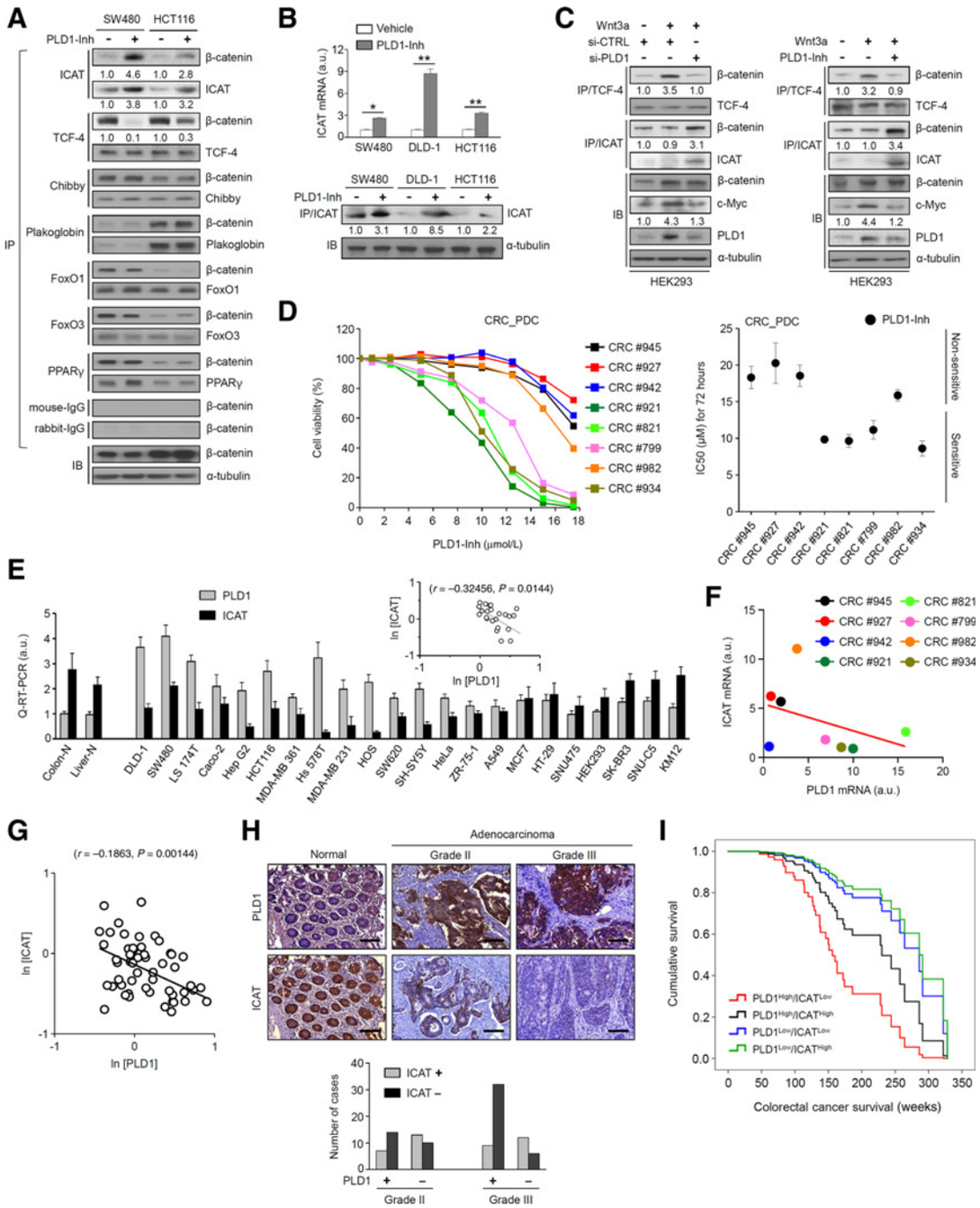

Figure 1.

PLD1 inhibition suppresses Wnt/β-catenin signaling via upregulation of ICAT and increases drug sensitivity in PDCs harboring PIK3CA and APC mutations. **A,** Colorectal cancer (CRC) cells were treated with 10 μmol/L PLD1 inhibitor (PLD1-Inh, VU0155069) for 24 hours, and the lysates were immunoprecipitated and immunoblotted using the indicated antibodies. **B,** Cells were treated with PLD1 inhibitor, and ICAT expression was analyzed by qRT-PCR and immunoblotting. A paired *t* test was used. Results are shown as mean ± SEM and are representative of at least three independent experiments. *, $P < 0.05$ and **, $P < 0.01$. **C,** HEK293 cells were transfected with siRNA for PLD1 (left) or treated with PLD1 inhibitor (right), followed by treatment with Wnt3a (150 ng/mL) for 24 hours. The lysates were immunoprecipitated and immunoblotted using the indicated antibodies. **D,** Effect of PLD1 inhibitor at various concentrations for 72 hours on cell viability of eight PDCs (left). IC_{50} values of all PDCs were calculated (right). **E,** PLD1 expression is inversely correlated with ICAT expression in various cancer cells. Using qRT-PCR, expression levels of PLD1 and ICAT from various cancer cells were analyzed relative to the amounts found in human normal colon or liver tissues, in which PLD1 expression was normalized to 1. Values represent the mean ± SD of four independent experiments. Inset, log values of PLD1 and ICAT expression were plotted against each other with the regression curve and its confidence interval. Spearman correlation coefficient (*r*) is provided with its degree of significance (*p*). **F,** In eight PDCs, there was an inverse correlation of PLD1 with ICAT mRNA expression. **G,** In 55 tumor tissues from colorectal cancer patients, there was an inverse correlation of PLD1 with ICAT mRNA expression. Spearman correlation coefficient (*r*) is provided with statistical significance. The black line represents the best-fit curves. **H,** IHC in tumor and adjacent normal tissues from colorectal cancer patients. **I,** Fitted survival function for the groups of PLD1/ICAT, using multivariate Cox proportional hazard model ($n = 153$). H, high expression; L, low expression.

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PDCs (PDC #921, #821, #799, and #934) relative to four other PDCs (PDC #945, #927, #942, and #982; Fig. 1D). Interestingly, PLD inhibitor showed drug sensitivity in PDCs harboring PIK3CA and APC mutations, independent of KRAS or TP53 mutation (Fig. 1D). We further examined the relationship between expression levels of PLD1 and ICAT in various human cancer cells. An overall statistically significant correlation was detected between increased PLD1 expression and repression of ICAT or *vice versa* (Spearman correlation coefficient: $r = -0.62$, $P < 0.05$), as analyzed by qRT-PCR (Fig. 1E), suggesting the biological relevance of mutual expression between PLD1 and ICAT. Although the eight colorectal cancer PDCs represent a small cohort, expression levels of PLD1 and ICAT showed a strong inverse correlation of 87.5% (Fig. 1F). Moreover, gene expression levels of *PLD1* and *ICAT* showed a strong negative correlation in 55 human colon tumor tissues, as analyzed by qRT-PCR (Fig. 1G). Furthermore, immunohistochemical analysis of colorectal cancer tissues exhibited elevated expression of PLD1 and reduced expression of ICAT compared with those in normal tissues (Fig. 1H). Further analysis of the prognostic value of combining PLD1 and ICAT expression in colorectal cancer was conducted. The tumors were interpreted as low (L) or high (H) when immunopositive cells represented <10% or >10% of the cancer cells, respectively. Factors of multivariate variables, the basic features and the protein group using Cox's proportional hazard model, show that survival difference between PLD1/ICAT expression groups is in a statistical significance, but not associated with gender, organ, tumor stage, and age (Fig. 1I; Supplementary Table S2). These data support an intimate association of PLD1 expression with levels of ICAT genes in survival of colorectal cancer patients and suggest the potential value of PLD1 and ICAT as prognostic biomarkers and therapeutic targets of colorectal cancer. Collectively, these results indicate that PLD1 inhibition suppresses Wnt/β-catenin signaling via upregulation of ICAT and increases drug sensitivity in PDCs harboring PIK3CA and APC mutations.

Targeting of PLD1 upregulates ICAT in intestinal tumorigenic mouse model

Recently, we reported that targeting of *PLD1* attenuates spontaneous and colitis-associated intestinal tumorigenesis in $Apc^{Min/+}$ and AOM/DSS mouse models, respectively (13). $Apc^{Min/+}$ mice contain a germline mutation in the *Apc* gene that results in activation of the Wnt/β-catenin pathway and spontaneous development of numerous adenomatous polyps in the intestine (22). Loss of PLD1 in $Apc^{Min/+}$ mice greatly increased the ICAT level in an adenoma at 16 weeks of age relative to control mice, as analyzed by IHC, Western blotting, and qRT-PCR (Fig. 2A–C). As a control, expression of Wnt target genes such as *PPARδ* and *c-myc* was reduced upon inhibition of PLD1 in $Apc^{Min/+}$ mice (Fig. 2C). PLD1 inhibitor-treated $Apc^{Min/+}$ mice (10 mg/kg, 3 times a week for 4 weeks) also showed results comparable with those observed in response to PLD1 ablation (Fig. 2A–C). For the AOM/DSS model, mice were given a single intraperitoneal injection of mutagen AOM, after which they received drinking water containing 2%–3% DSS over several 5-day periods interspersed with periods in which they received normal water. As previously reported (13, 14), PLD1 inhibition reduced expression of PLD1 in $Apc^{Min/+}$ and AOM/DSS mice. Targeting of PLD1 in the AOM/DSS mouse model also generated results comparable with those observed in $Apc^{Min/+}$ mice (Fig. 2D–F). These results suggest *in vivo* relevance of down-regulation of ICAT by targeting of PLD1 in mouse intestinal tumors.

PLD1-PI3K/Akt pathways regulate ICAT expression via binding of E2F1 to ICAT promoter

To further investigate the signaling pathways responsible for PLD1-regulated ICAT repression, catalytically active (ca) constructs of various signaling molecules were transfected, and the effect of PLD1 inhibition on ICAT expression was examined. Ectopic expression of PI3K and Akt significantly reduced PLD1 inhibition–induced ICAT expression (Supplementary Fig. S2A). Treatment with PA increased serine 473-phosphorylation of Akt, a downstream target of PI3K, in SNU-C5 and KM12 cells (Supplementary Fig. S2B). PLD1 inhibition suppressed phosphorylation of Akt in SW480 and DLD1 cells, which show higher PLD1 activity and level, compared with those in SNU-C5 and KM12 cells (Supplementary Fig. S2C and S2D). PLD1 inhibition–induced ICAT upregulation was significantly reduced by transfection with caPI3K and Akt1 (Fig. 3A). Moreover, PA-mediated suppression of ICAT was recovered by treatment with a selective PI3K inhibitor (LY294002) as well as ectopic expression of dominant-negative (dn) PI3K and Akt1 (Supplementary Fig. S2E). Thus, it is suggested that PLD-mediated PI3K/Akt activation is responsible for negative regulation of ICAT expression. To further examine the regulation of PLD-mediated ICAT expression at the transcriptional level, we analyzed the ICAT promoter. We observed the presence of three putative E2F1-binding sites in the proximal region (−480/−204) of the ICAT promoter (Fig. 3B). E2F1 transcription factor has been identified as a negative regulator of β-catenin/TCF-dependent transcription (23). To examine whether or not E2F1 is responsible for ICAT expression, we cloned the promoter region of human ICAT (pGL4-ICAT; 820 bp) and generated its various deleted constructs (Fig. 3B). PLD1 inhibition and E2F1 increased the promoter activity of ICAT, which was suppressed by expression of caAkt1 and PLD1, respectively (Fig. 3B). However, deletion of three putative E2F1-binding sites (D4, D5) did not affect the luciferase activity of PLD1 inhibition or E2F1-induced ICAT promoter, suggesting involvement of E2F1 in PLD1-mediated ICAT regulation. Moreover, inhibition and depletion of PLD1 enhanced binding of E2F1 to the ICAT promoter, which was abolished by ca-Akt1 (Fig. 3C). As a negative control, the region (−205/+56) in which putative E2F1-binding sites are absent showed no response to E2F1. Depletion of E2F1 abolished PLD1 inhibition–induced ICAT expression (Fig. 3D). Moreover, PA and ca-Akt1 attenuated E2F1-induced ICAT expression (Supplementary Fig. S2F). These data indicate that ICAT acts a transcriptional target of E2F1, and PLD1-PI3K/Akt pathways regulate ICAT expression via binding of E2F1 to the ICAT promoter.

PLD1-PI3K/Akt-TopBP1-E2F1 signaling axis is involved in regulation of ICAT expression and β-catenin/TCF transactivation

It has been reported that the PI3K/Akt signaling pathway regulates E2F1 through the E2F1-interacting protein TopBP1 (topoisomerase IIβ−binding protein), which inhibits E2F1-dependent apoptosis (24). Phosphorylation of TopBP1 by Akt is crucial for TopBP1 to interact with and repress E2F1 (24). We observed that inhibition of PLD1 and PI3K abolished serine phosphorylation of TopBP1 as well as the interaction of TopBP1 with E2F1 (Fig. 4A). Moreover, caAkt1 and E2F1 deletion reduced the interaction of ICAT with β-catenin induced by PLD1

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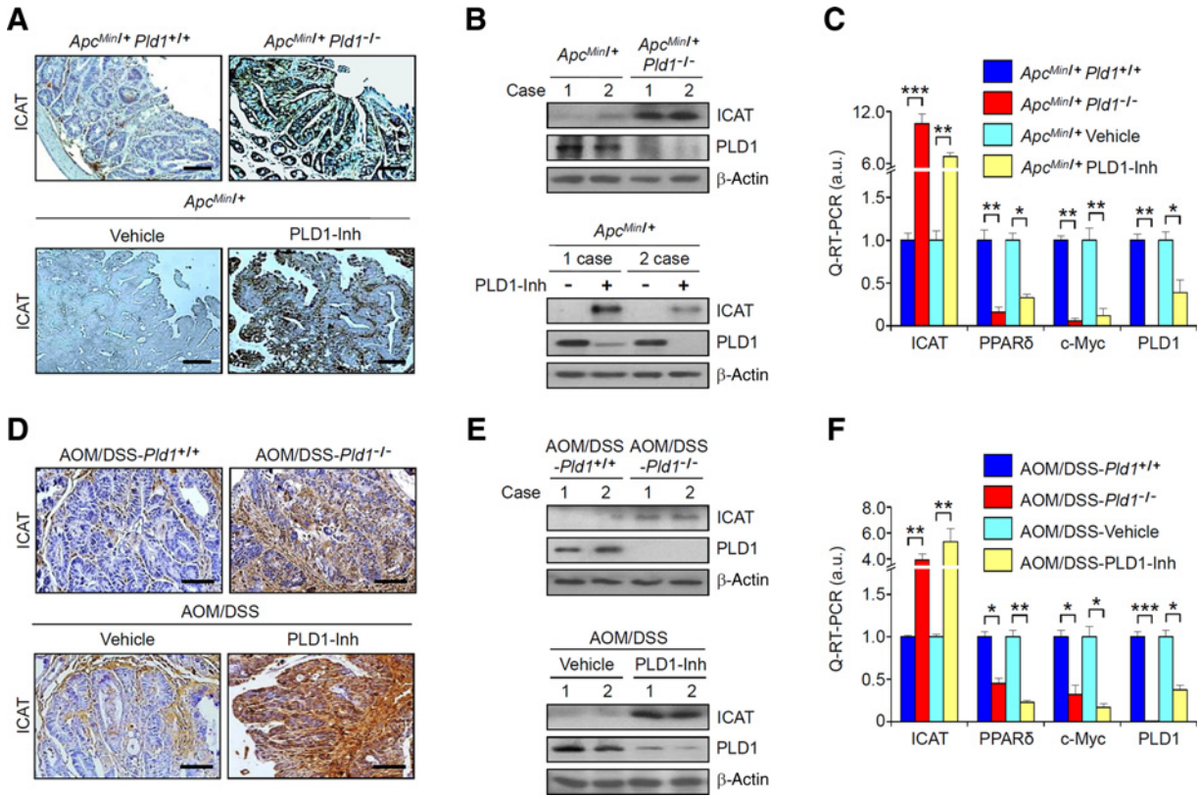

Figure 2.

Targeting of PLD1 increases expression of ICAT in intestinal tumorigenic mouse model. **A,** IHC for ICAT in tumor tissues of $Apc^{Min/+}$ and $Apc^{Min/+}/Pld1^{-/-}$ mice (top). Male $Apc^{Min/+}$ mice (12-week-old) were subjected to intraperitoneal injection with either vehicle or PLD1 inhibitor (10 mg/kg) 3 times a week for 4 weeks. $Apc^{Min/+}$/vehicle ($n = 8$) and $Apc^{Min/+}$/PLD1-Inh ($n = 10$) mice were sacrificed at 16 weeks. IHC for ICAT in tumor tissues of $Apc^{Min/+}$ treated with or without PLD1 inhibitor (bottom). Representative images were selected from at least four different fields. **B,** Effect of PLD1 targeting in $Apc^{Min/+}$ mice on expression of ICAT protein. **C,** QRT-PCR analysis of indicated mRNAs in tumor tissues of $Apc^{Min/+}$, $Apc^{Min/+}Pld1^{-/-}$, $Apc^{Min/+}$/vehicle, and $Apc^{Min/+}$/PLD1-Inh mice. **D,** IHC for ICAT in tumor tissues of non-AOM/DSS-$Pld1^{+/+}$ and AOM/DSS-$Pld1^{-/-}$ mice (top) or vehicle- and PLD1 inhibitor–treated AOM/DSS mice (bottom). **E,** Effect of PLD1 targeting in AOM/DSS on expression of ICAT protein. **F,** QRT-PCR analysis of indicated mRNAs in tumor tissues of the indicated AOM/DSS mice. A paired *t* test was used (**C**, **F**). Results are shown as mean ± SEM and are representative of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

inhibition, whereas it rescued binding of TCF4 with β-catenin reduced by PLD1 inhibition (Fig. 4B). To further examine whether or not PLD1 regulates ICAT expression via the PI3K-Akt-TopBP1-E2F1 signaling pathway, we used MEFs deficient in genes involved in signaling. *E2f1*-deficient MEFs were not responsive to ICAT expression regulated by PLD1 inhibition or ca-Akt1 (Fig. 4C). Suppression of E2F1-induced ICAT expression by PA was recovered by LY2942002 in WT and $Akt2^{-/-}$ MEFs but not in $Akt1^{-/-}$ and $Topbp1^{-/-}$ MEFs (Supplementary Fig. S3A and S3B), suggesting involvement of Akt1 and TopBP1 in the regulation of PLD1-mediated ICAT expression. E2F1 is a key downstream target of the retinoblastoma tumor suppressor (RB1). Interaction of RB1 with E2F1 inhibits E2F1 transcriptional activity, and hyperphosphorylation of RB1 by cyclin-dependent kinases causes release of RB1 from E2F inhibition, thereby allowing transcriptional activation. We further examined whether or not RB1 affects expression of ICAT. PLD1 inhibition increased ICAT expression in both $Rb1^{+/+}$ and $Rb1^{-/-}$ MEFs but downregulated the level of *Rb1* in $Rb1^{+/+}$ MEFs (Supplementary Fig. S3C). Overexpression of RB reduced expression of ICAT induced by depletion and inhibition of PLD1 (Supplementary Fig. S3D). In addition, PA suppressed exogenous E2F1-induced ICAT expression in *Rb1*-deficient MEFs as well as $Rb1^{+/+}$ MEFs, which was recovered by inhibition of PI3K (Supplementary Fig. S3E). These data suggest that PLD1 inhibition might increase ICAT expression by downregulating RB1 and inducing the free form of E2F1, which is regulated by the PI3K/Akt-TopBP1 pathway. Next, we examined whether or not the PI3K/Akt-TopBP1-E2F1 pathways are involved in PLD1-mediated transactivation of β-catenin/TCF4. Depletion of E2F1 and ICAT significantly recovered β-catenin/TCF4 transactivation reduced by inhibition of PLD1 or PI3K (Fig. 4D). Moreover, E2F1-mediated suppression of TCF4 transactivation was recovered by PA, caAkt1, and ICAT depletion (Fig. 4E). In addition, ectopic expression of E2F1 suppressed Wnt3a-induced TCF transactivation, which was recovered by PA and caAkt1 (Supplementary Fig. S3F). However, these phenomena were not observed in $Akt1^{-/-}$ or $Topbp1^{-/-}$ MEFs (Supplementary Fig. S3G and S3H). Furthermore, suppression of Wnt3a-induced TCF transactivation by PLD1 inhibition was recovered by expression of caAkt1 in $E2f1^{+/+}$ MEFs but not in *E2f1*-deficient MEFs (Fig. 4F), indicating the requirement of E2F1 in PLD1 inhibition–induced suppression of TCF transactivation. Next, we examined whether or not E2F1-induced ICAT expression is functionally required for E2F1-mediated inhibition of Wnt/β-catenin oncogenic signaling.

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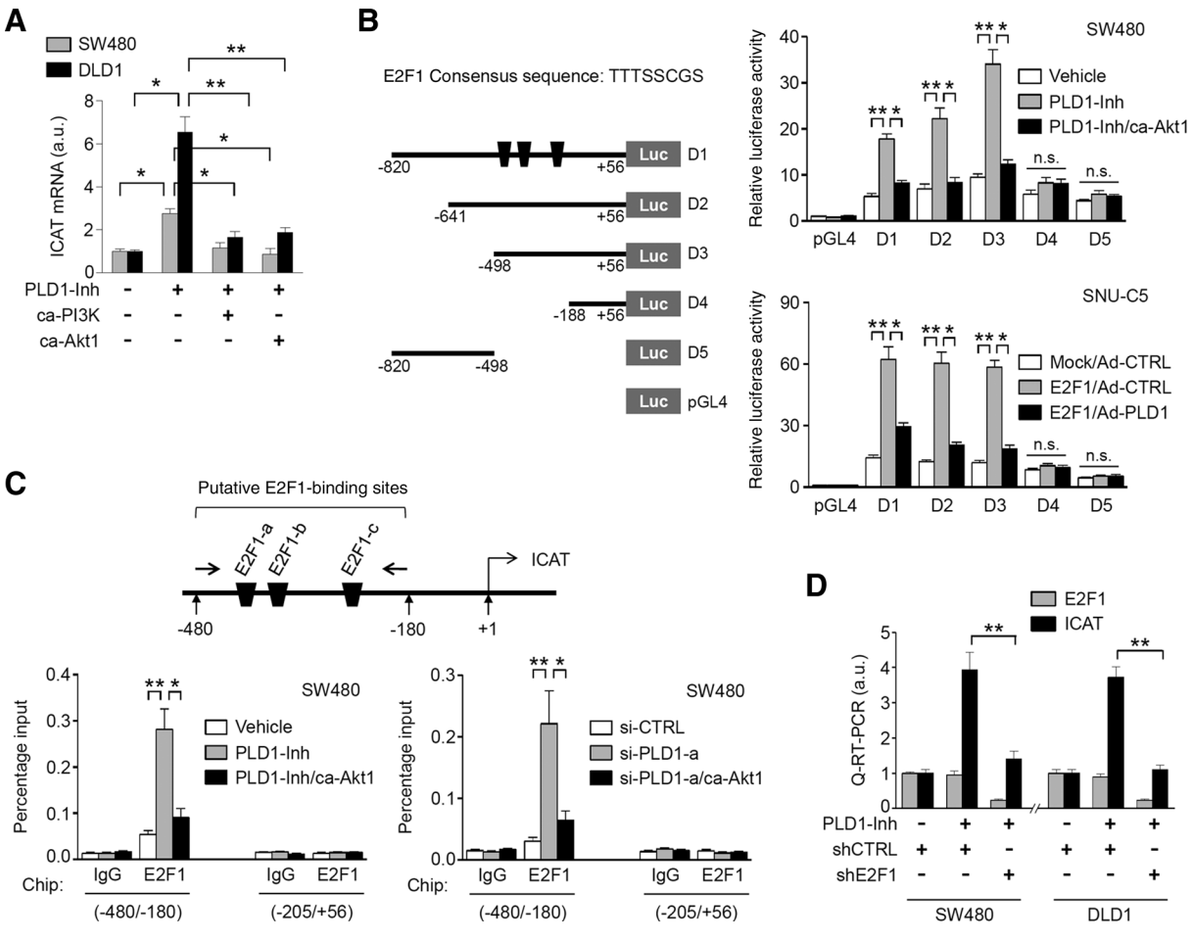

Figure 3.

PLD1-PI3K/Akt pathways regulate ICAT expression via binding of E2F1 to ICAT promoter. **A,** Effects of PI3K and Akt on PLD1 inhibition–induced ICAT expression. **B,** Schematic representation of putative E2F1-binding sites on ICAT promoter (left). Effect of Akt or PLD1 on PLD1 inhibitor or E2F1-induced luciferase assay of various ICAT promoters (right). **C,** ChIP assays for binding of E2F1 to the promoter of ICAT. Effect of Akt1 on binding of E2F1 to ICAT promoter, which was induced by inhibition and depletion of PLD1. **D,** Effect of E2F1 on PLD1 inhibition–induced ICAT expression. **A–D,** A paired *t* test was used. Results are shown as mean ± SEM and are representative of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; n.s., nonsignificant.

Wnt3a-induced anchorage-independent growth of NIH3T3 fibroblasts was reduced by inhibition and depletion of PLD1, whereas RNA interference–mediated silencing of E2F1 and ICAT recovered anchorage-independent growth suppressed by targeting of PLD1 (Fig. 4G; Supplementary Fig. S3I). Moreover, anchorage-independent growth and invasive capacity reduced by knockdown and inhibition of PLD1 in SW480 cells was significantly recovered by depletion of E2F1 and ICAT (Fig. 4H). These results suggest that ICAT negatively regulates anchorage-independent growth and invasion. Taken together, these results suggest that the PI3K/Akt-TopBP1-E2F1-ICAT signaling axis is involved in PLD1-mediated regulation of ICAT expression and β-catenin/TCF transactivation.

PLD1 inhibition linked to upregulation of ICAT suppresses tumor growth in xenograft models derived from patient colorectal cancer cells harboring *APC* and *PI3KCA* mutations

For clinical implications, we further performed functional studies using PDCs from selected colorectal cancer patients showing genetic features. We first examined the effect of PLD inhibition on invasion of PDCs. Treatment with PLD1 inhibitor significantly reduced invasive capacity in four PDCs carrying *PIK3CA* and APC mutations (PDC #921, #821, #799, #934), which was recovered by depletion of ICAT (Fig. 5A). However, PLD1 inhibition did not significantly affect PDCs harboring *KRAS* (#927) or *TP53* (#982) mutation. Moreover, PLD1 inhibition significantly suppressed colony-forming capacities in PDCs carrying *PIK3CA* and APC mutations but not in PDCs carrying *KRAS* or *TP53* mutation alone (Fig. 5B). These results suggest that PLD1 inhibition sensitizes PDC carrying *PIK3CA* and APC mutations, independent of *KRAS* or *TP53* mutation. Recent evidence suggests that PDX models can maintain certain pathologic and molecular features of the original disease (25).

To further investigate the preclinical effects of PLD1 inhibitor on tumor growth in a PDX model of colorectal cancer patients, NOD/SCID mice were subcutaneously implanted with three kinds of PDXs (#927, #921, and #799) and then treated with PLD1 inhibitor ($n = 6$) or vehicle ($n = 6$; 10 mg/kg, 5 times a week for 4 weeks). Treatment with PLD1 inhibitor significantly reduced tumor volume and growth in mice bearing #921 PDXs with

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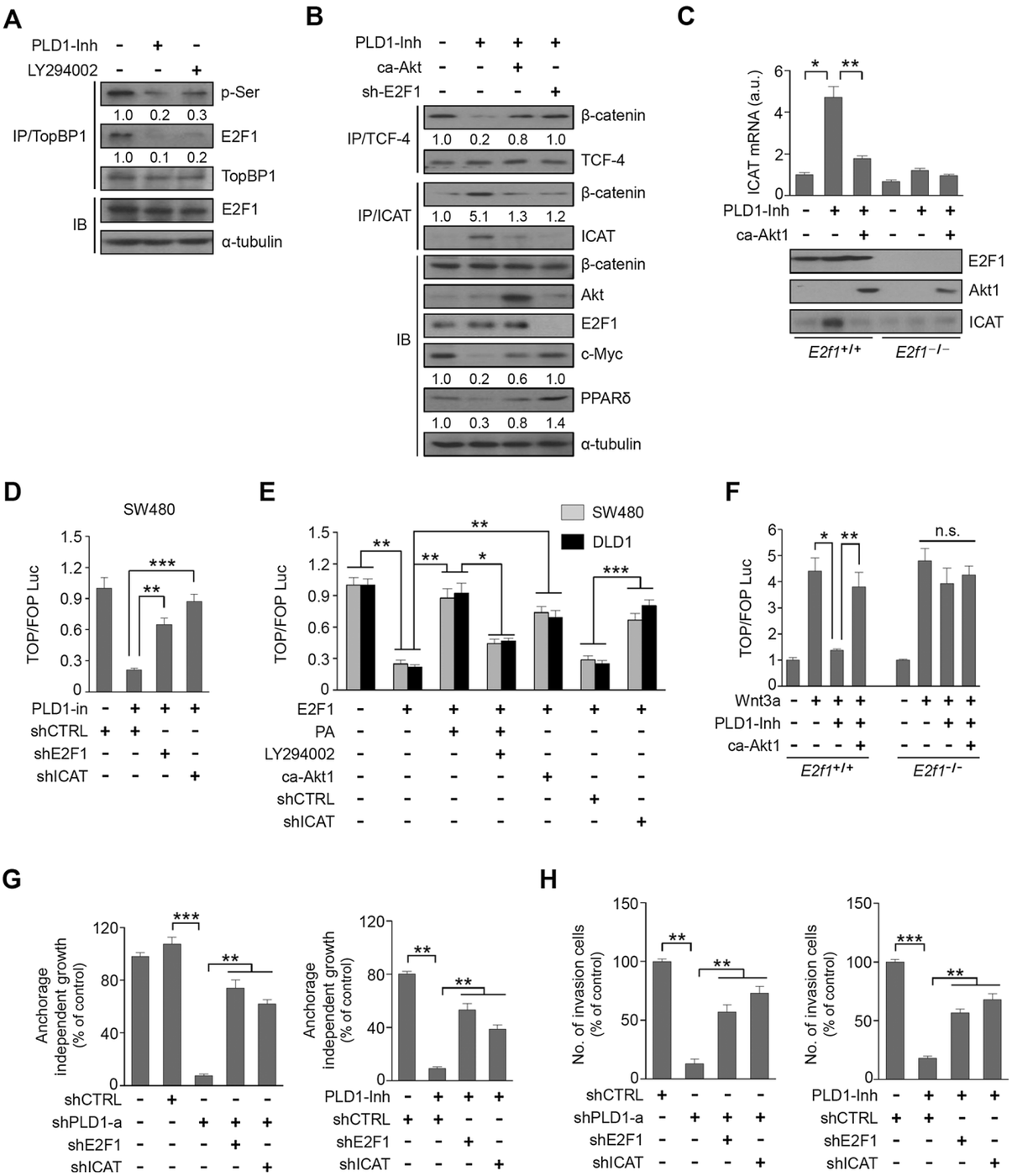

Figure 4.

PLD1-PI3K/Akt-TopBP1-E2F1 signaling axis is involved in regulation of ICAT expression and β-catenin/TCF transactivation. **A,** Effect of PLD1 or PI3K inhibition on phosphorylation of TopBP1 and interaction of TopBP1 with E2F1. **B,** SW480 cells were transfected with the indicated constructs and treated with PLD1 inhibitor. Lysates were immunoprecipitated and/or immunoblotted with the indicated antibodies. **C,** Effect of Akt1 on PLD1 inhibition–induced ICAT expression in *E2f1*$^{+/+}$ and *E2f1*$^{-/-}$ MEFs. **D,** Effect of depletion of E2F1 and ICAT on β-catenin/TCF4 transactivation suppressed by inhibition of PLD1. **E,** Effects of PA, caAkt1, and ICAT depletion on E2F1-mediated suppression of TCF4 transactivation. **F,** Effects of PLD1 inhibition and Akt1 on Wnt3a-induced TCF transactivation in *E2f1*$^{+/+}$ and *E2f1*$^{-/-}$ MEFs. **G,** Effects of E2F1 and ICAT on anchorage-independent growth suppressed by knockdown and inhibition of PLD1 in SW480 cells. **H,** Effects of E2F1 and ICAT on invasion reduced by targeting of PLD1. (**C–H**) A paired t test was used. Results are shown as mean ± SEM and are representative of at least three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and n.s., nonsignificant.

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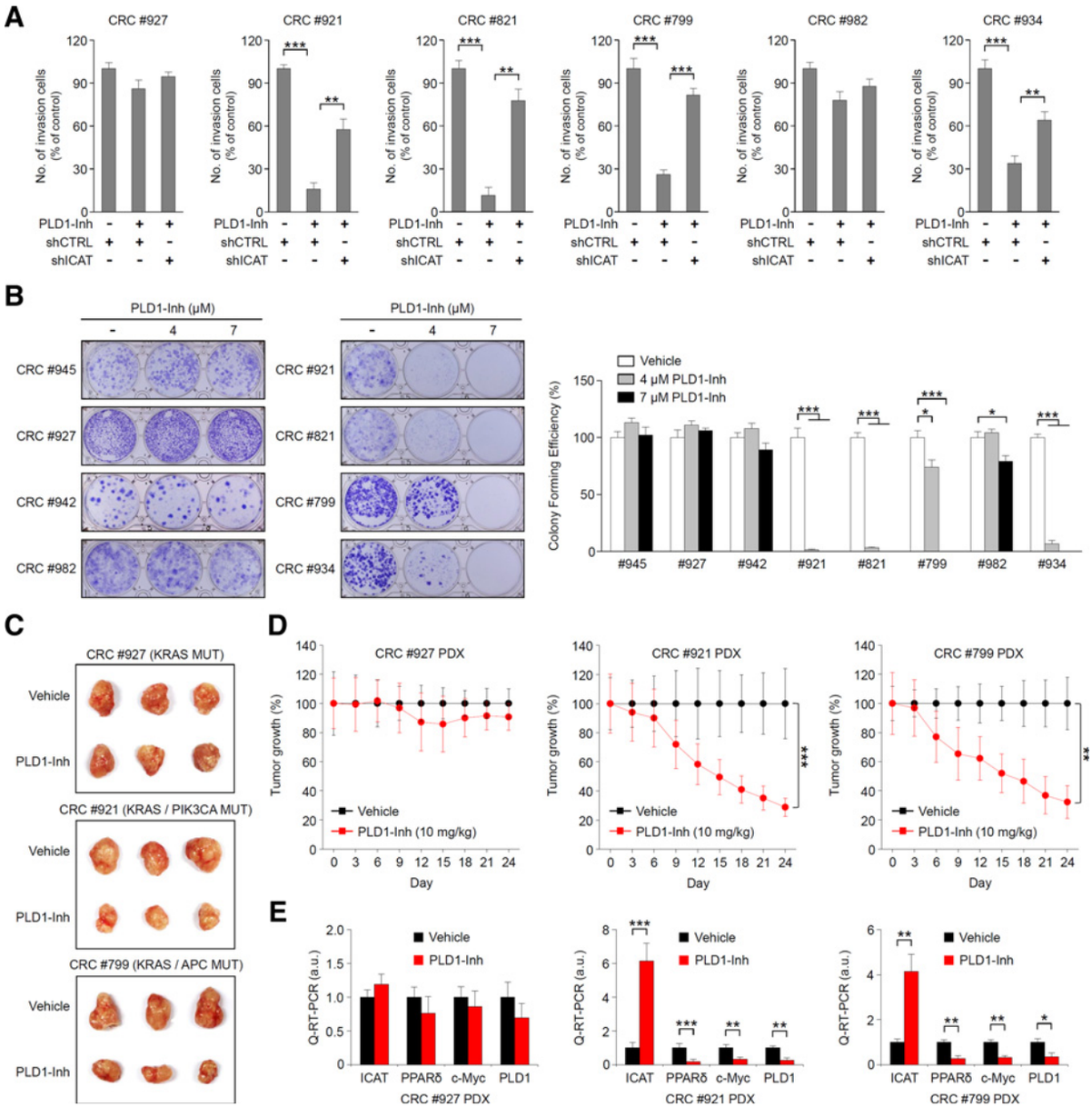

Figure 5.

PLD1 inhibition linked to upregulation of ICAT suppresses tumorigenic potential in PDCs and PDXs of colorectal cancer (CRC) patients harboring *APC* or *PI3KCA* mutation. **A,** Effect of ICAT depletion on invasion reduced by PLD1 inhibition in PDCs. **B,** Effect of PLD1 inhibitor on colony formation in the indicated PDCs. Histograms show colony formation efficiency relative to vehicle. Association between mutation status of the four indicated genes and colony-forming efficiency of eight PDCs treated with PLD1 inhibitor. Two-way ANOVA test was used. **C,** *In vivo* efficacy of PLD1 inhibitor in the indicated PDX model. Mice were treated daily with PLD1-Inh (10 mg/kg, $n = 6$) or vehicle ($n = 6$) for 24 days. **D,** Tumor growth (%) was analyzed relative to vehicle. Values represent the mean ± SD of four independent experiments. **E,** QRT-PCR analysis of *ICAT, PPARδ, c-Myc*, and *PLD1* mRNAs in tumor tissues of the indicated PDX mice. A paired *t* test was used. Results are shown as mean ± SEM and are representative of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

PIK3CA and *KRAS* mutations as well as #799 PDXs with *APC* and *KRAS* mutations relative to that of vehicle (Fig. 5C and D). However, PLD1 inhibition did not affect the size or growth of tumors in mice bearing #927 PDXs with *KRAS* mutation alone (Fig. 5C and D). Furthermore, PLD1 inhibition significantly increased expression of ICAT and reduced expression of Wnt target genes in tumor tissues from #921 and #799 PDXs compared with those of vehicle (Fig. 5E). On the other hand, PLD1 inhibition had marginal effects in tumor tissues from #927 PDXs (Fig. 5E). In addition, treatment with PLD1 inhibitor greatly suppressed phosphorylation of Akt and increased ICAT expression in tumor tissues from #921 PDXs with mutations in *PIK3CA* and *KRAS* as well as #799 PDXs with mutations in *APC* and *KRAS* compared with those of #927 PDXs with mutation in *KRAS* alone,

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as analyzed by IHC (Supplementary Fig. S4). The tumor tissues from #921 PDXs and #799 PDXs showed very high levels of phosphor-Akt and PLD1 relative to those of #927 PDX. As previously reported (13, 14), PLD1 inhibition reduced expression of PLD1 in tumor tissues from #921 PDXs and #799 PDXs (Supplementary Fig. S4). Collectively, these results suggest that PLD1 inhibition linked to upregulation of ICAT sensitizes colorectal cancer cells hyperactivated by the Wnt/β-catenin and PI3K/Akt signaling pathways.

Discussion

The Wnt/β-catenin and PI3K/Akt pathways play a central role in cancer, but the molecular mechanisms of their crosstalk in cancer have not been elucidated. Wnt/β-catenin pathways play important roles in the maintenance of cancer stem cells (26). One of the typical characteristics of cancer stem cells is resistance to various kinds of cancer treatment. Given that the β-catenin pathway is important for cancer stem cells, it would be interesting to examine whether or not the PI3K/Akt-β-catenin pathway contributes to resistance mechanisms to other molecular targeted drugs as well as other conventional chemotherapies or radiotherapies. Akt is part of the Ras-PI3K-PTEN-Akt-mTOR oncogenic pathway, which is frequently altered by activating mutations in the colon and many other cancers (27). We reported that PLD1 inactivation reduces the self-renewal capacity of colon cancer–initiating cells (13). Recently, it has been reported that Akt inhibition induces nuclear FOXO3a accumulation and, in concert with nuclear β-catenin, promotes expression of metastasis-related target genes and metastasis (3). Nuclear accumulation of both factors correlates with tumor budding in human colon carcinomas. Thus, it was suggested that β-catenin confers resistance to PI3K and Akt inhibitors in colorectal cancer. Here, we showed that PLD1 linked to ICAT, a negative regulator of Wnt/β-catenin signaling, mediates molecular crosstalk between the Wnt/β-catenin and PI3K/Akt pathways and thus could be proposed as a novel colorectal cancer prognostic biomarker. Several proteins are known to inhibit binding of β-catenin to TCF and subsequent target gene transactivation (16–20). PLD1 inhibition selectively enhanced expression of ICAT and the interaction of ICAT with β-catenin. Thus, it is suggested that ICAT is a selective molecular target of the PLD1-mediated Wnt/β-catenin signaling pathway. PLD1 promotes Wnt signaling by selectively downregulating ICAT via the PI3K/Akt-TopBP1-E2F1 signaling axis. Although prolonged suppression of PLD1 induces downregulation of β-catenin (13), short-term or transient suppression of PLD1 did not affect the level of β-catenin. Expression of ICAT was reported to be inversely correlated with the known transcriptional activity of β-catenin (28); ICAT is predominantly expressed in the villi of epithelial cells (where β-catenin is not active in Wnt-induced transcription) and is downregulated in crypt cells (where β-catenin is transcriptionally active). Of particular interest, expression of ICAT showed a statistically inverse correlation with that of PLD1 in various cancer cells and colorectal cancer tissues. Low PLD1 expression and high ICAT expression were significantly associated with increased survival in colorectal cancer patients, suggesting the biological significance of the PLD1-ICAT negative feedback loop. Furthermore, ICAT was shown to inhibit the growth of colorectal cancer cells with an APC or β-catenin mutation as well as hepatocellular carcinoma cells with an Axin mutation (15). High ICAT expression is associated with reduced survival in melanoma patients (29). Moreover, it has been reported that the *ICAT* gene is downregulated in high-grade glioma tissues compared with low-grade tissues and normal controls and that lower ICAT expression indicates a worse prognosis (30). In the present study, we demonstrated that ICAT negatively regulates oncogenic growth of colorectal cancer cells. The function of ICAT as an antagonistic regulator of the Wnt/β-catenin pathway suggests that it could be a tumor-suppressor gene (15). E2F1 functions as a tumor suppressor in colorectal cancer by negatively regulating Wnt/β-catenin activity (31, 32). E2F1 has been reported to negatively regulate Wnt/β-catenin activity (32). Here, we showed that ICAT as a transcriptional target of E2F1 acts a crucial node in the link between E2F1 and β-catenin signaling, and PLD1-PI3K/Akt pathways negatively regulate ICAT expression via suppression of E2F1 binding to ICAT promoter. Analysis of the signaling events involved in PLD1-mediated β-catenin/TCF-4 activity elucidated a previously unknown link between the β-catenin/TCF-4 and PI3K/Akt-TopBP1-E2F1-ICAT pathways. *APC* mutations are a risk factor for patients treated with PI3K/Akt pathway inhibitors (33). This would suggest that oncogenic activation of the Wnt/β-catenin pathway could be a mechanism of resistance to PI3K and Akt inhibitors. Hyperactivation of the Wnt/β-catenin and PI3K/Akt signaling pathways has limited clinical benefit mostly due to unknown resistance mechanisms and the lack of predictive biomarkers of the drug response. Interestingly, we observed that PLD1 inhibition significantly suppressed the growth of colorectal cancer cells harboring *APC* and *PI3KCA* mutations in patient-derived primary cultures and in corresponding xenograft tumors in mice. In the era of targeted therapy, mutation profiling of cancer has gained influence on therapeutic decisions. Compared with standard cell lines, the greatest advantage of PDC models is their ability to better predict a clinical tumor response. Relative to cell line–derived xenograft models, PDXs are the only models harboring bona fide cancer targets directly from the patient. These results might assist in the clinical development of a PLD1 inhibitor for treatment of colorectal cancer patients carrying *APC* and *PI3KCA* mutations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.W. Kang, S.J. Jang, Y.M. Kim, D.S. Min
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.H. Lee, Y.-S. Choi, K.-Y. Choi, D.S. Min
Writing, review, and/or revision of the manuscript: D.W. Kang, Y.M. Kim, D.S. Min
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.-A. Suh, S.J. Jang, Y.M. Kim, D.S. Min
Study supervision: D.W. Kang, Y.M. Kim, D.S. Min

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Dong Woo Kang, Bo Hui Lee, Young-Ah Suh, et al.

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