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Cancer Research

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Tumor and Stem Cell Biology

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Phospholipase D1 Drives a Positive Feedback Loop to Reinforce the Wnt/β-Catenin/TCF Signaling Axis

Dong Woo Kang[1], Soung-Hoon Lee[2], Jeong Whan Yoon[3], Won-Sang Park[3], Kang-Yell Choi[2], and Do Sik Min[1]

Abstract

Activation of the Wnt signaling pathway occurs frequently in human cancers, but an understanding of the targets and regulation of this important pathway remains incomplete. In this study, we report that phospholipase D (PLD), a cell survival mediator that is upregulated in cancer, is an important target of the Wnt signaling pathway that functions in a positive feedback loop to reinforce pathway output. PLD1 expression and activity was enhanced by treatment with Wnt3a and glycogen synthase kinase-3 inhibitors, and the Wnt pathway–regulated transcription factors β-catenin and TCF-4 were required for this effect. Three functional TCF-4–binding sites were identified within the PLD1 promoter. Interestingly, suppressing PLD1 blocked the ability of β-catenin to transcriptionally activate PLD1 and other Wnt target genes by preventing β-catenin/TCF-4 complex formation. Conversely, tactics to elevate intracellular levels of phosphatidic acid, the product of PLD1 enzyme activity, enhanced β-catenin/TCF-4 complex formation as well as β-catenin–dependent TCF transcriptional activity. In cell-based assays, PLD1 was necessary for the anchorage-independent growth driven by Wnt/β-catenin signaling, whereas β-catenin/TCF-4 was necessary for the anchorage-independent growth driven by PLD1 activation. Taken together, our findings define a function for PLD1 in a positive feedback loop of Wnt/β-catenin/TCF-4 signaling that provides new mechanistic insights into cancer, with implications of novel strategies to disrupt Wnt signaling in cancer. *Cancer Res; 70(10); 4233–42. ©2010 AACR.*

Introduction

Phospholipase D (PLD), which has been implicated in survival signals, is elevated in many human cancers (1). PLD hydrolyzes membrane phosphatidylcholine (PC) to generate phosphatidic acid (PA) and choline in response to a variety of signals, including growth factors and mitogens (2). PA itself is an intracellular second messenger involved in many physiologic events, such as mitogenesis and inflammation (3). Aberrant PA signaling has been observed in several disease states (4). Elevated PLD activity and overexpression results in cellular transformation and has been implicated in multiple human cancers, including colorectal (5, 6), breast (7), renal (8), gastric carcinoma (9), and thyroid cancers (10).

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Genes encoding two mammalian PLD isozymes, designated PLD1 and PLD2, have been cloned. Stable cells overexpressing PLD1 and PLD2 in nude mice have shown anchorage-independent growth, upregulation of matrix metalloproteinase (MMP), and tumorigenesis (11–13). We recently reported on the significant cooverexpression of PLD isozymes with β-catenin in human colorectal cancers and induced the expression of PLD1 by tumor promoter phorbol 12-myristate 13-acetate (PMA) for the enhancement of the activity of MMP (5).

The Wnt signaling pathway regulates Wnt-responsive genes through the stabilization of the cytoplasmic component β-catenin, which subsequently enters the nucleus and complexes with the T-cell factor (TCF)/lymphoid-enhancing factor family of transcription factors, whose activity is promoted by their association with β-catenin. β-Catenin is an important component of the Wnt signaling pathway and is involved in diverse cellular processes, including cell adhesion, growth, differentiation, and transcription of Wnt-responsive genes (14, 15). In the absence of the Wnt signal, β-catenin is phosphorylated by glycogen synthase kinase-3 (GSK3β) in a multiprotein degradation complex, leading to its ubiquitination and degradation in the proteosome. In the presence of Wnt binding to the cell surface receptor, or after the mutation of the degradation complex, components consisting of adenomatous polyposis coli (APC), axin, or β-catenin phosphorylation and degradation of β-catenin are blocked. Mutations of Wnt signaling components are a major cause of colorectal cancer and other tumor

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types (16, 17). Deregulation of β-catenin leads to the formation of β-catenin-TCF complexes and altered expression of TCF target genes, which can then contribute to the development of cancer (18, 19). Therefore, regulation of β-catenin stability and, consequently, β-catenin/TCF–mediated transactivation are critical to Wnt signaling during development and tumorigenesis.

Proposed Wnt/TCF target genes in cancer cells include known oncogenes, such as *c-MYC* (20), *Cyclin D1* (21), *MMP-7* (22), *WISP-1* (23), *survivin* (24), *cyclooxygenase-2* (25), and *nitric oxide synthase* (*NOS2*; 26). Thus, the identification of new transcriptional targets of the Wnt/β-catenin signaling pathway and factors associated with promotion of Wnt/β-catenin activity could aid in the advancement of our understanding of the role of the canonical pathway in oncogenesis. Involvement of PLD in the Wnt/β-catenin signaling pathway has not yet been elucidated. Based on our recent evidence showing statistical correlation of PLD level with that of β-catenin in colon cancer (5), we investigated the role of PLD in the Wnt signaling pathway. The present results implicate PLD1 as a novel target of Wnt3a, one of the Wnts-mediated canonical signaling pathway that promotes Wnt/β-catenin activity, which in turn enhances anchorage-independent growth and invasion in concert with Wnt/β-catenin signaling. Identification of a Wnt-β-catenin-TCF–regulated PLD1 axis may provide new mechanistic insights into development of cancer.

Materials and Methods

Cell lines and reagents. HEK293, SW480, SNU-C5, HCT116, MDA-MB 361, MDA-MB 231, SK-BR3, HOS, and SNU475 cells were obtained from the American Type Culture Collection. Recombinant Wnt3a was purchased from R&D Systems. 6-Bromoindirubin-3-oxime (BIO) was purchased from Calbiochem. Lithium chloride (LiCl), dioctanoyl PA, 1-butanol, 3-butanol, and 1-propranolol were purchased from Sigma. PLD inhibitors were kindly provided by Drs. H. Alex Brown and Craig Lindsley (Vanderbilt University, Nashville, TN).

Plasmid, transient transfection, and reporter gene assay. Transfection and luciferase assays were performed as previously described (5). For the measurement of TCF activity, TOPflash and FOPflash luciferase plasmids were transfected into cells and luciferase activities were measured. TCF activity was calculated as fold of activity (TOP/FOP Luc). The human PLD1 promoter (pGL4-PLD1-Luc), linked to the luciferase reporter genes, has been previously described (5). Mutations of TCF-4–binding elements on the PLD1 promoter were generated using the Quick Change Site-Directed Mutagenesis kit (Stratagene; Supplementary Table S1).

PLD activity assay. PLD activity was assessed by measurement of the formation of [^{3}H] phosphatidylbutanol, the product of PLD-mediated transphosphatidylation, in the presence of 1-butanol, as previously described (27).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) experiments were performed as previously described (28), with minor modifications. ChIP-enriched DNA was quantified by quantitative real-time PCR (q-PCR) using primers that were specific for the promoter region (Supplementary Table S2). Data are expressed as the percentage of input DNA.

Quantitative real-time PCR. cDNA was synthesized from total RNA extracted with Trizol (Invitrogen). q-PCR was conducted with ~50 ng of cDNA using the Quantitect SYBR green PCR kit (Qiagen) and a Rotor-Gene RG-3000A apparatus (Corbett Research). All data were normalized with the *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) gene expression values. For q-PCR primer sequences, see Supplementary Table S3.

Immunoprecipitation and Western blotting. As previously described, lysates from cells or colorectal cancer tissues were analyzed by immunoprecipitation and/or immunoblot (27). Enhanced chemiluminescence was used for the detection of the signal. The antibodies used were as follows: anti–α-tubulin, anti–TCF-4, anti-NOS2, and anti–c-Myc (all from Santa Cruz Biotechnology); anti–β-catenin and anti-survivin (BD Biosciences); anti–phospho-GSK3β; anti–phospho-Akt; and anti-Akt (Cell Signaling Technology). A polyclonal anti-PLD antibody that recognizes both PLD1 and PLD2 was generated as previously described (29). Approval was obtained from the Institutional Review Board of The Catholic University of Korea, College of Medicine.

Small interfering RNA. A 19-nucleotide sequence small interfering RNA (siRNA) corresponding to the human PLD1 sequence (1,571–1,591, AAGGUGGGACGACAAUGAGCA) was purchased from Dharmacon Research.

Invasion assay. The assay used a Transwell apparatus (Corning) consisting of upper and lower chambers, which were separated by an 8.0-μm pore size membrane. Before addition of cells, the upper chambers were coated with a 1:5 dilution of Matrigel (BD Biosciences). Following introduction of cells, the Transwell apparatus was incubated for 5 hours and the cells were stimulated with Wnt3a for 24 hours. The extent of invasion, which was defined as movement of cells from the upper chamber to the lower chamber, was expressed as an average number of cells per microscopic field.

Anchorage-independent growth assay. Anchorage-independent growth was examined in soft agar. Fifty microliters of base agar matrix (CytoSelect 96-Well *In vitro* Tumor Sensitivity Assay kit; Cell Biolabs) were dispensed into each well of a 96-well plate. When the agar had solidified on the bottom surface of each well, 75 μL of a cell suspension/soft agar matrix containing 3×10^3 cells were layered on top of the agar. This was followed by the addition of 50 μL of 2× complete medium with Wnt3a and/or inhibitors. After 10 days of incubation, the agar matrix was solubilized and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was added to each well. The absorbance produced by the formation of insoluble formazan product by viable cells was recorded at 570 nm.

Statistical analysis. Data represent the mean SD. Data were analyzed using the Student's t test; $P < 0.05$ was considered statistically significant.

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Results

Wnt signaling induces expression of PLD1. HEK293 cells with a wild-type genetic status for APC and β-catenin were treated with Wnt signaling molecules to examine the question of whether Wnt signaling could increase PLD expression. Analysis by Western blot showed that treatment with purified recombinant Wnt3a enhanced the expression of both PLD1 and β-catenin in a time-dependent manner (Fig. 1A). Although results from immunoprecipitation and Western blot also showed slightly increased expression of PLD2 by Wnt3a (data not shown), we focused on the study of the regulation of PLD1 expression. Moreover, Wnt3a increased PLD1 expression in a time-dependent manner, as analyzed by q-PCR (Fig. 1A). We next asked whether the blockade of GSK3β might affect PLD1 expression using LiCl and BIO, which are established agonists that are known to mimic the Wnt-signaling pathway, leading to the activation and stabilization of β-catenin (30, 31). LiCl and BIO exhibited markedly elevated PLD1 protein levels (Fig. 1B). Wnt3a and Wnt mimetics increased the protein level of nuclear β-catenin, as well as the expression of *c-Myc* and *NOS2*, which are target genes of Wnt signaling. Moreover, Wnt mimetics also increased PLD1 expression, as determined by q-PCR (Fig. 1B). Wnt3a, LiCl, and BIO significantly enhanced PLD1 promoter activity (Fig. 1C). Wnt signaling was associated with a significant increase in gene expression from a TCF/lymphoid-enhancing factor-specific luciferase reporter

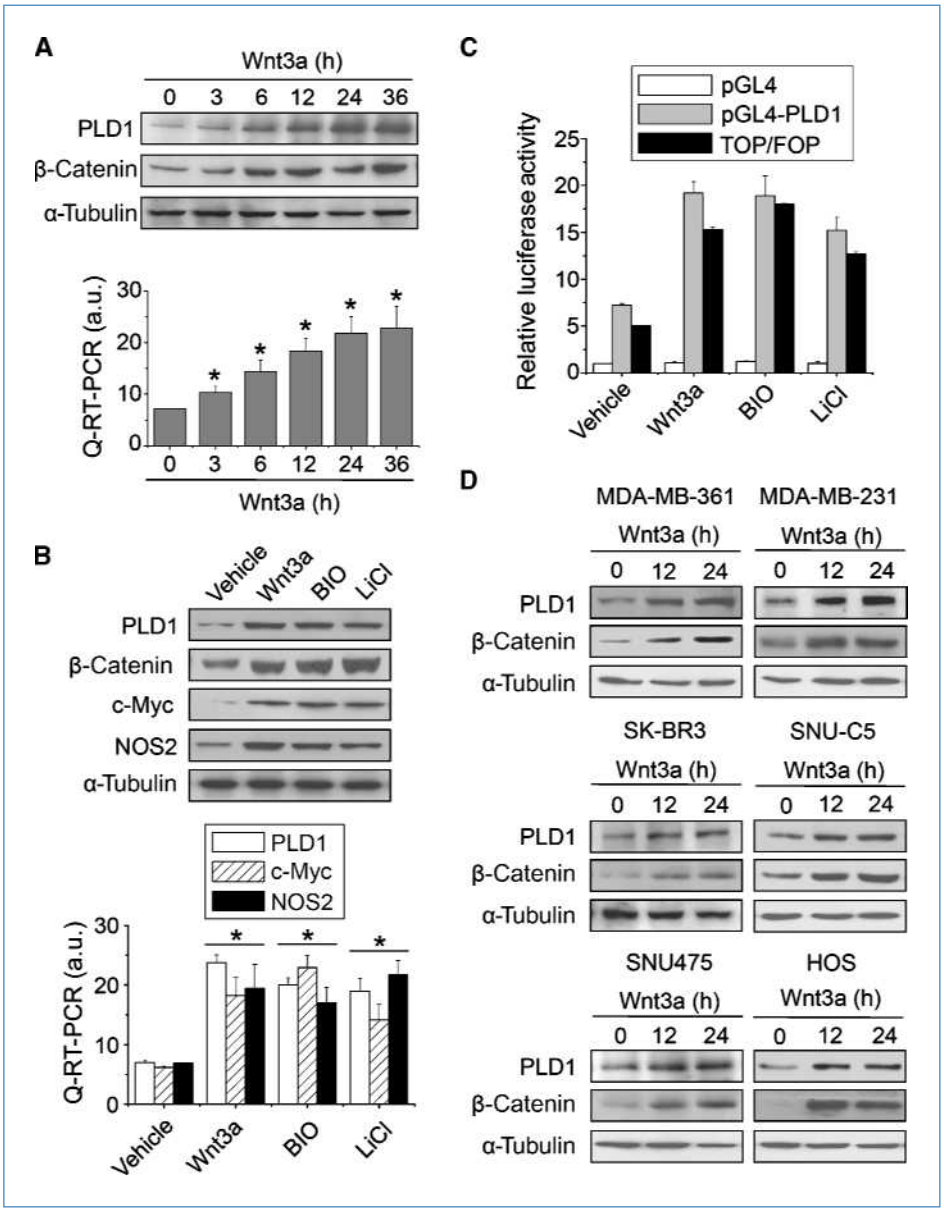

Figure 1. PLD1 expression is upregulated by Wnt3a and GSK3β inhibitors. A, purified Wnt3a (150 ng/mL) was supplied to HEK293 cells for the indicated times; lysates were immunoblotted with the indicated antibodies (top). PLD1 expression was also analyzed by q-PCR (bottom). *, $P < 0.05$ compared with nontreatment. A.u., arbitary unit. B, cells were stimulated with Wnt3a (150 ng/mL), BIO (1 μmol/L), or LiCl (20 mmol/L) for 24 h; lysates were immunoblotted with the indicated antibodies (top). β-Catenin was analyzed by Western blotting using nuclear lysates. Gene expression was also analyzed by q-PCR (bottom). *, $P < 0.05$ versus vehicle. C, promoter reporter plasmids were transfected and treated with Wnt3a, LiCl, or BIO for 12 h; luciferase activity was then determined. Columns, mean of five independent experiments; bars, SD. D, Wnt3a was used for treatment of the cells for the indicated times. Lysates were immunoblotted with the indicated antibodies. Data are representative of three independent experiments.

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plasmid (TOP/FOP Luc) that was used as a control. Observed upregulation of PLD1 expression by Wnt signaling strongly implicates a central role for inhibition of GSK3β and the canonical Wnt signaling pathway in Wnt-mediated effects. In addition, induction of the PLD1 protein was observed in various cancer cells, including SNU-C5 colon cancer cells; SNU475 hepatocellular carcinoma cells; SK-BR3, MDA-MB-231, and MDA-MB-361 breast cancer cells; and HOS osteosarcoma cells (Fig. 1D). Cancer cells in which the status of the Wnt pathway was normal or marginally weaker were chosen for the observation of PLD1 induction in response to Wnt signaling. MDA-MB-361, MDA-MB-231, and SK-BR3 breast cancer cells are genetically wild-type for APC and β-catenin (32, 33). Although the genetic status of APC and β-catenin in SNU-C5, HOS, and SNU 475 cancer cells is unknown, low basal TCF transcriptional activity has been reported. Collectively, these observations supported the suggestion that Wnt3a-induced PLD1 expression is a general phenomenon that is detected in various cells. Taken together, these results implicate the transcriptional regulation of PLD1 induction as a novel target of Wnt signaling.

β-Catenin and TCF-4 bind to the PLD1 promoter and enhance PLD1 expression. TCF/β-catenin complexes induce Wnt-responsive genes that have been implicated in various types of cancer (34, 35). Thus, we examined the question of whether β-catenin or TCF-4 increased PLD1 expression. As shown in Fig. 2A, ectopic expression of TCF-4 and a stable β-catenin mutant (S37A β-catenin) enhanced the protein levels of PLD1, as well as Wnt target genes, such as c-Myc, NOS2, and survivin in HEK293 cells (Fig. 2A, left). Moreover, transfection with a dominant-negative TCF-4 construct lacking the β-catenin binding region (ΔN30 TCF-4)

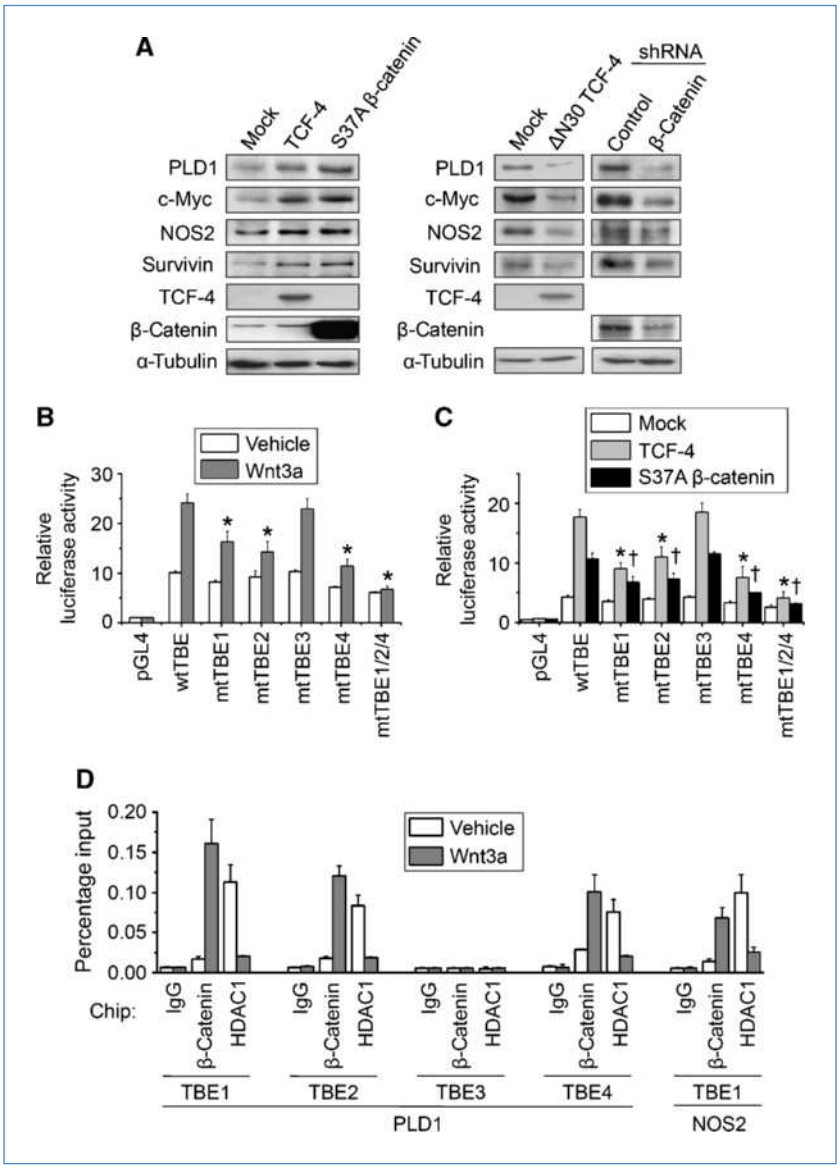

Figure 2. β-Catenin and TCF-4 bind specifically to the *PLD1* promoter and increase PLD1 expression. A, HEK293 cells were transfected with the indicated expression vectors; lysates were then immunoblotted with the indicated antibodies. B, cells were transfected with wild-type (wt) pGL4-PLD1, a one or triple TBE mutant form (mt) of pGL4-PLD1. Luciferase activity was determined 12 h after stimulation with Wnt3a. *, $P < 0.01$ versus Wnt3a. C, cells were cotransfected with the indicated expression vectors along with the wt or mt*PLD1* promoter. *, $P < 0.01$ versus S37A β-catenin; †, $P < 0.05$ versus TCF-4. D, ChIP assay was performed using preimmune IgG, anti–β-catenin, or anti-HDAC1 antibody and analyzed by q-PCR. ChIP analysis of the NOS2 promoter containing TBE as a positive control was performed. Columns, mean of three independent experiments; bars, SD.

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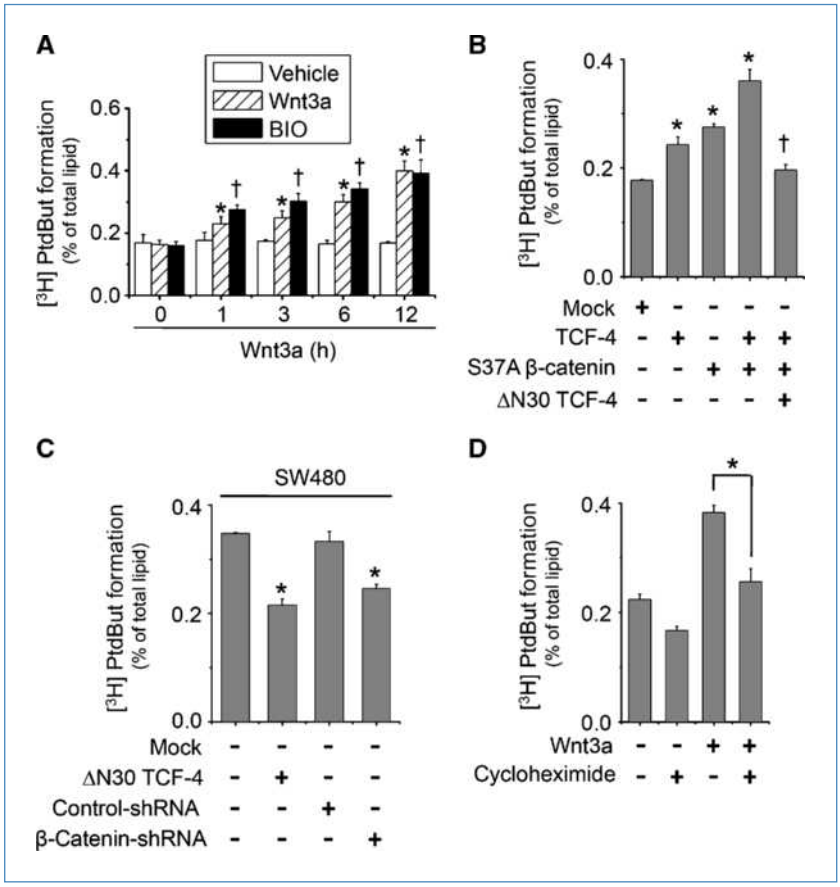

Figure 3. Wnt signaling stimulates PLD activity. A, HEK293 cells were labeled with [^{3}H] myristate for 12 h and treated with Wnt3a or BIO for the indicated times. *, $P < 0.01$; †, $P < 0.05$ versus vehicle. B, cells were transfected with the indicated expression vectors and PLD activity was measured. *, $P < 0.01$ versus mock; †, $P < 0.01$ versus β-catenin/TCF-4. C, SW480 cells were transfected with shRNA for control or β-catenin and PLD activity was measured. *, $P < 0.01$ versus control short hairpin RNA. D, HEK293 cells were pretreated with 10 μmol/L cycloheximide for 30 min and treated with Wnt3a. PLD activity was measured. *, $P < 0.01$ versus Wnt3a. Columns, mean of three independent experiments; bars, SD.

and depletion of β-catenin decreased the protein level of PLD1 and Wnt target genes in HEK293 cells (Fig. 2A, right). These phenomena were also observed in cells of HCT116, a Wnt-relevant colon cancer cell line with a deletion at residue S45 in β-catenin (Supplementary Fig. S1; ref. 36).

To further examine the question of whether PLD1 is directly regulated by β-catenin/TCF, we analyzed the transcriptional control sequences of the *PLD1* gene for TCF protein binding sites. Four putative consensus TCF-DNA binding elements (TBE; designated as TBE1–4) were identified. TBE1 (TGCAAAG) and TBE3 (AATAAAG) are located −0.79 and −0.27 kb upstream, respectively, and contain an inverted match with the consensus CTTTG(A/T) (A/T) sequence for TCF-4 binding (20). TBE2 (CTTTGCT), which is located −0.51 kb upstream, nearly matched the consensus sequence. TBE4 (CTTTGTA), which is located −19 bases upstream, contained a perfect match. Conservation of TCF binding sites on the *PLD1* promoter has been shown across species (Supplementary Fig. S2). To examine the functional importance of the TBE motif in the regulation of *PLD1* gene transcription, site-directed mutations of TBE sites were generated in the *PLD1* promoter (Supplementary Table S1). Mutations in TBE1, TBE2, or TBE4 sites, but not in TBE-3, significantly decreased Wnt3a-induced *PLD1* promoter activity in HEK293 cells (Fig. 2B). Moreover, TCF-4– or β-catenin–induced *PLD1* promoter activities were similar to those of Wnt3a-induced *PLD1* promoter activity (Fig. 2C). These data implicate the *PLD1* promoter as a target of the β-catenin/TCF-4 complex through the consensus TBE sites.

In addition, ChIP assays were carried out in HEK293 cells for confirmation of *in vivo* binding of β-catenin/TCF-4 to the *PLD1* promoter. ChIP-enriched DNA was quantified by q-PCR using primers that were specific for the *PLD1* promoter region. As shown in Fig. 2D, Wnt3a enhanced the binding of β-catenin/TCF-4 to the TBE1, TBE2, and TBE4 sites of the *PLD1* promoter, but not to TBE3. These results were comparable with those of the promoter assay using mutagenesis. ChIP analysis of the *NOS2* promoter containing TBE as a positive control was performed. Wnt3a targets β-catenin to chromatin for the removal of the corepressor histone deacetylase 1 (HDAC1; ref. 37). As expected, Wnt3a significantly suppressed the binding of HDAC 1 to TBE1, TBE2, and TBE4 of the *PLD1* promoter. Taken together, these data implicate *PLD1* as a direct transcriptional target of β-catenin/TCF signaling *in vivo*.

Wnt signaling stimulates PLD activity. We examined the question of whether induction of PLD1 through Wnt signaling increases its enzymatic activity. As shown in Fig. 3A, HEK293 cells treated with Wnt3a and BIO showed increased PLD activity in a time-dependent manner. We observed an early increase in PLD activity resulting from treatment with Wnt3a and BIO (1 h). The mechanism(s) for early activation

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of PLD by Wnt signaling remains unclear and will require additional study. In addition, ectopic expression of β-catenin and/or TCF-4 stimulated PLD activity, whereas transfection of ΔN30 TCF-4 inhibited β-catenin/TCF-4–induced PLD activation (Fig. 3B). Moreover, consistent with constitutively active Wnt signaling (38), transfection with ΔN30 TCF-4 or short hairpin RNA for β-catenin resulted in reduced PLD activity in SW480 colorectal cancer cells expressing mutant APC deleted at the COOH terminus (Fig. 3C; ref. 39). Furthermore, we examined the direct regulatory effect of Wnt signaling on PLD activity. Use of the translation inhibitor cycloheximide significantly suppressed both basal level and Wnt3a-driven PLD activity in HEK293 cells (Fig. 3D). This observation was consistent with the suggestion that Wnt signaling–induced PLD1 upregulation increases enzymatic activity.

PLD activity is required for the promotion of β-catenin/TCF transcriptional activity. In light of our findings, we next asked whether Wnt signaling–induced PLD activation might modulate β-catenin–dependent TCF activity. Involvement of PLD activity was examined when 1-butanol was used to block PA production by PLD, by virtue of formation of phosphatidylbutanol through transphosphatidylation; 1-Butanol essentially abrogated β-catenin–induced TCF transcriptional activity, whereas an identical concentration of 3-butanol, an inactive analogue of PLD-mediated PA formation, did not have a significant effect on TCF activity (Fig. 4A). Selective PLD inhibitors have recently been developed (40, 41). Thus, we examined the effect of the PLD inhibitor on TCF activity. Interestingly, VU0155056, a dual PLD1 and PLD2 inhibitor (40), abolished β-catenin–induced TCF activity in a dose-dependent manner (Fig. 4A). Isoform-selective PLD inhibitors also suppressed β-catenin/TCF signaling (data not shown). Furthermore, we observed that pretreatment of 1-butanol or VU0155056, but not 3-butanol, significantly abrogated the basal level of TCF activity in the Wnt-relevant SW480 colon cancer cell line (Fig. 4B). Dependence on PLD activity was further confirmed by treatment of cell-permeable dioctanoyl-PA and by accumulation of endogenous PA by treatment of 1-propranolol, a PA phosphatase inhibitor (Fig. 4C). Moreover, PA rescued TCF activity suppressed by the PLD inhibitor or by the overexpression of GSK3β (Supplementary Fig. S3). Taken together, these results suggest that PLD activity is required for the promotion of β-catenin–dependent TCF transcriptional activity.

Wnt signaling–induced PLD upregulation increases the expression of its target gene by promotion of the association of β-catenin with TCF-4. Because the integrity of the β-catenin/TCF complex is required for proper transcriptional activity, we examined the possibility that Wnt-induced PLD activation could enhance the formation of the complex. Wnt3a increased the association of β-catenin with TCF-4, whereas pretreatment with 1-butanol, but not 3-butanol, significantly disrupted Wnt-induced β-catenin/TCF-4 association and expression of Wnt target genes without detectable modulation of β-catenin and TCF-4 levels (Fig. 5A). Additionally, PLD inhibitor suppressed the basal level of β-catenin/TCF-4 association in SW480 cells. Interestingly, the PLD inhibitor alone significantly abrogated the basal level of β-catenin/TCF-4 interaction and expression of its target gene (Fig. 5A), suggesting that PLD activity is required for complex formation and for expression of TCF-4 target genes. PA and propranolol increased interaction and target gene expression (Fig. 5B). Furthermore, depletion of PLD1 significantly suppressed Wnt-induced interaction and expression of its target gene (Fig. 5C). The effect of PLD1 siRNA on PLD1 expression is shown in Supplementary Fig. S4. PA also rescued the expression of Wnt target genes that had been suppressed by the overexpression of GSK3β or the PLD inhibitor (Fig. 5D). Taken together, these results suggest that Wnt signaling–induced PLD1 activity promotes β-catenin/TCF activity by increasing the association between β-catenin and TCF-4.

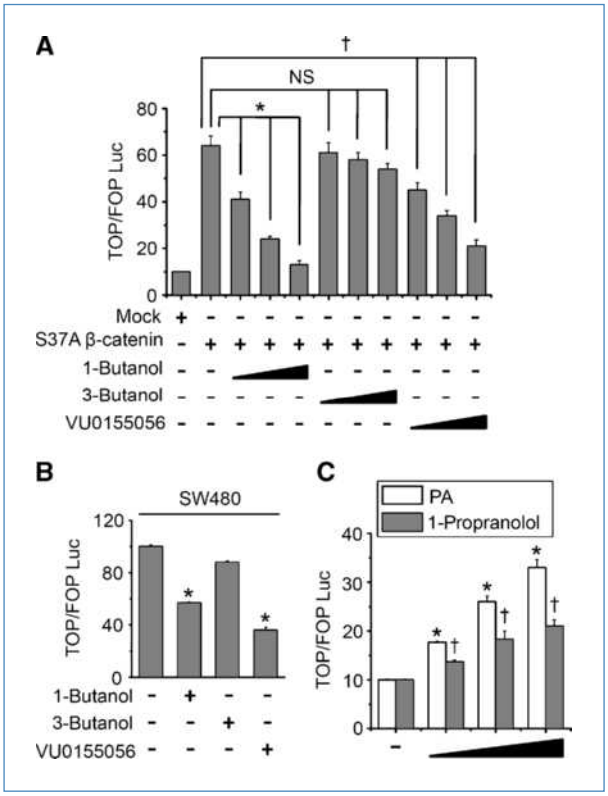

Figure 4. PLD activity is required for the promotion of β-catenin/TCF transcriptional activity. A, after cotransfection with TOP/FOP reporters and S37A β-catenin, HEK293 cells were treated with 1-butanol, 3-butanol (0.3%, 0.6%, or 0.9%), or PLD inhibitor (VU0155056; 2, 5, or 10 μmol/L) before the measurement of luciferase activity. *, $P < 0.01$; †, $P < 0.05$ versus S37A β-catenin. NS, nonsignificant. B, SW480 cells were transfected with TOP/FOP reporters and treated with 1- or 3-butanol (0.6%) or PLD inhibitor (5 μmol/L) for 24 h; luciferase activity was then measured. *, $P < 0.05$ versus vehicle. C, HEK293 cells were transfected with TOP/FOP reporters and then stimulated with PA (20, 50, and 100 μmol/L) and propranolol (10, 20, and 40 μmol/L); relative TCF activity was then determined. *, $P < 0.05$; †, $P < 0.05$ versus vehicle. Columns, mean of four independent experiments; bars, SD.

PLD1 promotes anchorage-independent growth and invasion in concert with the Wnt/β-catenin/TCF–dependent pathway. Aberrant Wnt/β-catenin signaling increases the

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expression of specific target genes that play critical roles in cell survival, migration, and invasion. As shown in Fig. 6A, Wnt3a-induced invasion of HEK293 cells was suppressed by the depletion of PLD1. PLD1 depletion inhibited Wnt3a-induced migration of HEK293 cells (Supplementary Fig. S5). Using an *in vitro* tumor sensitivity assay, we further investigated the question of whether PLD1 was required for Wnt/β-catenin–mediated tumorigenic effects. Treatment of NIH3T3 cells with Wnt3a increased anchorage-independent colony growth, whereas PLD inhibitor and PLD1 depletion significantly suppressed Wnt3a-driven anchorage-independent growth (Fig. 6B). Furthermore, β-catenin–induced anchorage-independent colony growth was abolished by the PLD inhibitor and PLD1 depletion (Supplementary Fig. S6). PLD inhibition and depletion of β-catenin or PLD1 also suppressed the anchorage-independent growth of SW480 cancer cells (Fig. 6B). Additionally, PLD1-induced anchorage-independent colony growth was abolished by dominant-negative TCF-4 in SW480 and NIH3T3 cells (Fig. 6C), implicating Wnt signaling as essential to the PLD1-induced tumorigenic effect. Moreover, results of Western blot analysis showed the correlation of expression patterns of PLD1 and β-catenin in colorectal tumors versus the adjacent normal mucosa (Fig. 6D), which provided evidence of the Wnt-β-catenin/PLD1–positive feedback loop *in vivo*. Taken together, these results were consistent with the suggestion that PLD1 contributes to the anchorage-independent tumorigenic effect in concert with the Wnt/β-catenin/TCF–mediated pathway; thus, there is a functional connection between Wnt and PLD1.

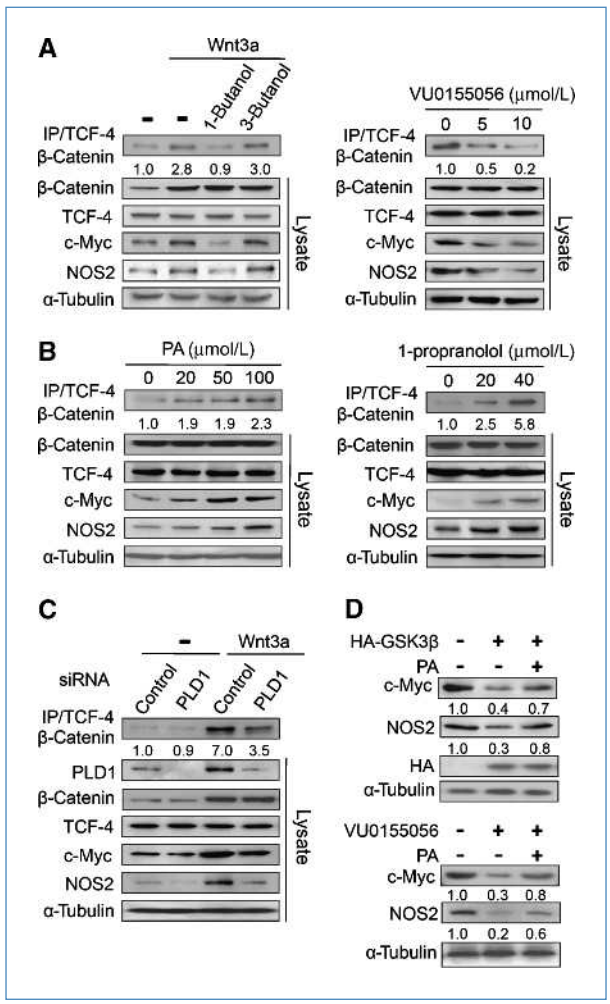

Figure 5. Wnt-induced PLD1 upregulation promotes the association of β-catenin with TCF-4 and induces expression of its target gene. A, HEK293 cells were pretreated with 1- or 3-butanol (0.6%) and stimulated with Wnt3a for 24 h (left). SW480 cells were treated with PLD inhibitor for 24 h (right). B, HEK293 cells were treated with the indicated concentration of PA (left) or 1-propranolol (right) for 24 h. C, HEK293 cells were transfected with siRNA for control or PLD1 and treated with or without Wnt3a for 24 h. Cell lysates (A–C) were immunoprecipitated with anti–TCF-4 antibody and immunoblotted by anti–β-catenin antibody. D, SW480 cells were transfected with GSK3β and stimulated with PA (100 μmol/L) for 24 h (top). Cells were pretreated with PLD inhibitor (5 μmol/L) and treated with PA (100 μmol/L) for 24 h (bottom). Interaction levels or protein expression were quantitated by densitometer analysis. Protein levels were analyzed by immunoblotting with the indicated antibodies. Data are representative of three independent experiments.

Discussion

Evidence to date indicates the predominance of activation of β-catenin/TCF–regulated target genes following Wnt pathway deregulation in the development and progression of cancer. The present study offers evidence that PLD1 drives a positive feedback loop to reinforce the Wnt/β-catenin/TCF signaling axis. We found that injection of LiCl into mice significantly increased the level of PLD1 protein in colon tissues (data not shown), suggesting its physiologic relevance *in vivo*. β-Catenin and TCF-4 elevated the expression and activity of PLD1. We were able to define three functional TCF binding sites within the PLD1 promoter, suggesting that the *PLD1* gene is a direct transcriptional target of β-catenin/TCF signaling. We found that the mRNA level and promoter activity of PLD1 were diminished by wild-type ectopic expression of APC, axin, and GSK3β, whereas expression of Dvl3 and inactive mutant APC as positive regulators of Wnt signaling increased expression of PLD1 (data not shown). Thus, it is suggested that canonical Wnt signaling molecules regulate the expression of PLD1.

Presently, Wnt signaling also increased the expression of PLD1 and PLD2. TCF binding elements exist in the promoter region of the *PLD2* gene and PLD2 also promotes Wnt signaling through TCF4 transcriptional activation (data not shown). Our present focus was on the role of PLD1 in Wnt signaling.

Other signaling cascades can also impinge on the expression of PLD. For instance, tumor promoter signals, such as PMA, selectively stimulate PLD1 expression through the NFκB signaling pathway (5). Ewing's sarcoma fusion protein selectively induces PLD2 expression by binding to the ETS domain of the PLD2 promoter (42). Therefore, several signaling cascades can contribute to the control of PLD gene expression, which might be used differentially in distinct types of tumors.

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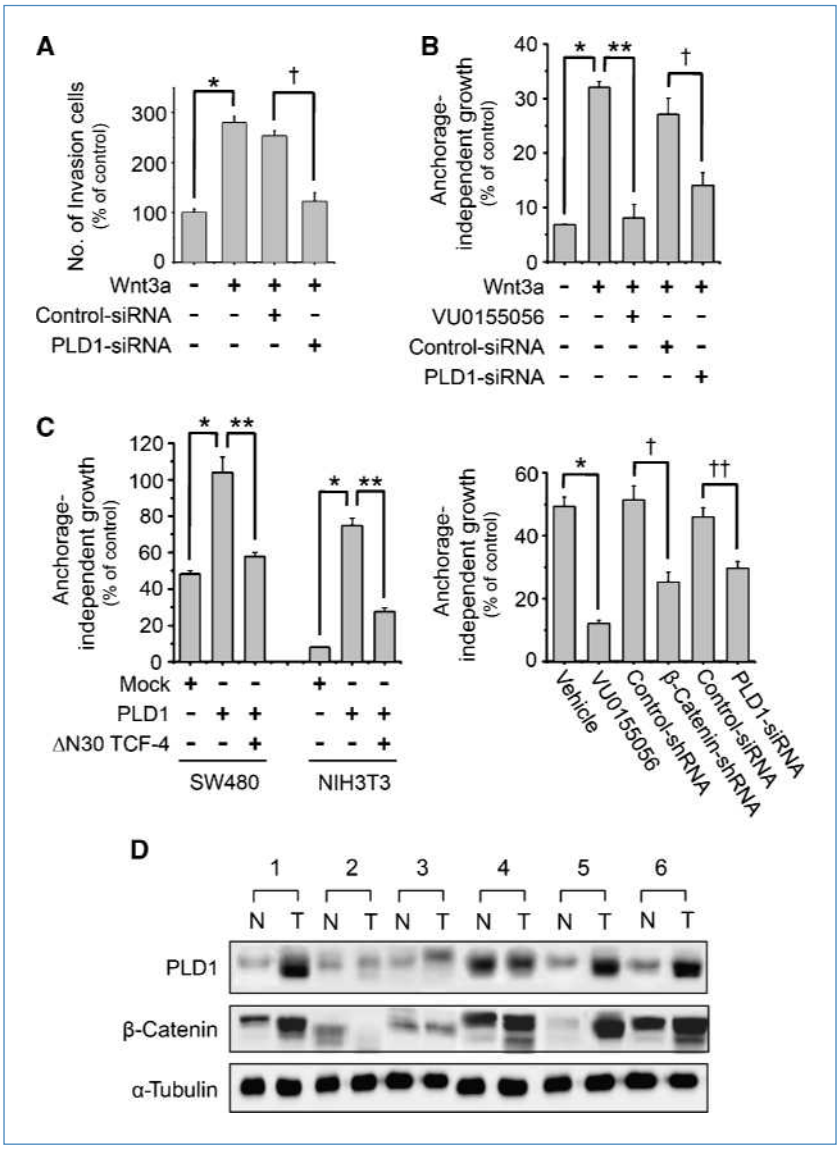

Figure 6. PLD1 promotes anchorage-independent growth and invasion in concert with the Wnt/β-catenin/TCF–dependent pathway. A, HEK293 cells were transfected with PLD1 or siRNA for control. The Transwell invasion assay was then performed after stimulation with Wnt3a for 24 h. *, $P < 0.05$ versus vehicle; †, $P < 0.05$ versus Wnt3a/control-siRNA. B, anchorage-independent growth was suppressed by PLD inhibitor and depletion of PLD1 or β-catenin. NIH3T3 cells were transfected with or without siRNA for PLD1 and suspended in agar matrix, and were left either treated or nontreated with VU0155056 (10 μmol/L) and/or Wnt3a (150 ng/mL; left). *, $P < 0.05$ versus Mock; **, $P < 0.05$ versus Wnt3a; †, $P < 0.05$ versus Wnt3a/control-siRNA. SW480 cells were treated with or without VU0155056 or transfected with the indicated siRNAs (right). An anchorage-independent growth assay was then performed. *, $P < 0.01$ versus vehicle; †, $P < 0.01$ versus control-shRNA; ††, $P < 0.05$ versus control-siRNA. C, PLD1 mediates anchorage-independent growth through Wnt signaling. SW480 and NIH3T3 cells were transfected with the indicated expression vectors; the anchorage-independent growth assay was then performed. *, $P < 0.05$ versus mock; **, $P < 0.05$ versus PLD1. Columns, mean of three independent experiments; bars, SD. D, expression of PLD1 and β-catenin in colorectal cancer tissues (T) and their corresponding normal colonic mucosa (N) was analyzed by Western blot.

Our data show the promotion of the β-catenin/TCF signaling pathway by increased formation of β-catenin/TCF-4 complex, followed by enhanced expression of TCF-4–dependent genes. PLD activity is involved in these processes.

It is likely that the PLD1 protein itself is not directly involved in regulation of the interaction. It is possible that PA might modulate the expression level of some proteins, which can associate with β-catenin or TCF, subsequently affecting β-catenin/TCF-4 activity. Several proteins, such as Chibby, Groucho, and inhibitor of β-catenin and TCF-4, interactively inhibit binding of β-catenin to TCF (43–45). Moreover, Wnt-induced PLD activation may induce a protein(s) that stimulates the formation of a transcriptionally active complex between TCF-4 and β-catenin. It has recently been reported that glycophosphatidylinositol-specific PLD can promote Wnt signaling by relieving retention of Wnt in the endoplasmic reticulum (46). Because the PC-specific PLD described in our study cannot hydrolyze glycophosphatidylinositol, it is not known whether PC-PLD would directly regulate Wnt signaling by relieving retention of Wnt in the endoplasmic reticulum. Future studies will be needed for the determination of the mechanism of PLD-modulated Wnt signaling or the interaction between TCF4 and β-catenin.

In light of rather limited data on the role of candidate TCF target genes in the cancer process, our previous findings showing that overexpression of PLD isozymes can induce neoplastic transformation, anchorage-independent growth, and tumor after injection into nude mice (11) are noteworthy. Elevated expression and activity of PLD have also been detected in various human tumors. We have recently reported on the statistical correlation of PLD and β-catenin levels in clinic samples, as analyzed by immunohistochemistry using tissue microarray (5). Cooverexpression of PLD and β-catenin was detected in 64 (52%) of 122 colorectal cancers

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(5). In this study, results from Western blot showed the correlation of PLD1 and β-catenin levels in colorectal cancer tissues, indicating the *in vivo* presence of the Wnt-β-catenin-PLD1 positive–feedback loop.

Many of the components of Wnt/β-catenin signaling that have been studied may serve as potential targets for use in the development of therapeutic agents. Our data suggest the promotion of tumorigenic effects by PLD1 in concert with the Wnt/β-catenin/TCF–mediated pathway, as shown in cell-based assays. Growing evidence in support of PLD involvement in cancer development further strengthens the tumorigenic contributions of β-catenin and, thereby, establishes the critical role of the Wnt/β-catenin/PLD pathway, with important implications in carcinogenesis. We have reported on the enhancement of expression of the *MMP-2* gene by increased DNA binding activity of NFκB and Sp1, which promotes glioma cell invasion (12). Thus, it is possible that promotion of the tumor phenotype by PLD1 is independent of TCF-induced genes.

The present study relates previously unconnected molecular pathways with important roles in tumor progression, namely the β-catenin/TCF signaling pathway and PLD-mediated pathway. Therefore, it is conceivable that therapeutic interventions targeting PLD1 may confer a clinical benefit in β-catenin–driven malignancies such as cancer. Further studies will undoubtedly enhance the understanding of the specific mechanisms by which increased expression of PLD contributes to the development and progression of cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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