Low PIP4K2B Expression in Human Breast Tumors Correlates with Reduced Patient Survival: A Role for PIP4K2B in the Regulation of E-Cadherin Expression

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Abstract
Phosphatidylinositol-5-phosphate (PtdIns5P) 4-kinase β (PIP4K2B) directly regulates the levels of two important phosphoinositide second messengers, PtdIns5P and phosphatidylinositol-(4,5)-bisphosphate [PtdIns(4,5)P2]. PIP4K2B has been linked to the regulation of gene transcription, to TP53 and AKT activation, and to the regulation of cellular reactive oxygen accumulation. However, its role in human tumor development and on patient survival is not known. Here, we have interrogated the expression of PIP4K2B in a cohort (489) of patients with breast tumor using immunohistochemical staining and by a meta-analysis of gene expression profiles from 2,999 breast tumors, both with associated clinical outcomes. Low PIP4K2B expression was associated with increased tumor size, high Nottingham histological grade, Ki67 expression, and distant metastasis, whereas high PIP4K2B expression strongly associated with ERBB2 expression. Kaplan–Meier curves showed that both high and low PIP4K2B expression correlated with poorer patient survival compared with intermediate expression. In normal (MCF10A) and tumor (MCF7) breast epithelial cell lines, mimicking low PIP4K2B expression, using short hairpin RNA interference-mediated knockdown, led to a decrease in transcription and expression of the tumor suppressor protein E-cadherin (CDH1). In MCF10A cells, knockdown of PIP4K2B enhanced TGF-β–induced epithelial to mesenchymal transition (EMT), a process required during the development of metastasis. Analysis of gene expression datasets confirmed the association between low PIP4K2B and low CDH1 expression. Decreased CDH1 expression and enhancement of TGF-β–induced EMT by reduced PIP4K2B expression might, in part, explain the association between low PIP4K2B expression and poor patient survival. Cancer Res; 73(23): 6913–25. © 2013 AACR.

Introduction
Alterations in components of various signaling pathways can lead to the generation of neoplasms derived from breast epithelium, which, through the acquisition of further somatic mutations and epigenetic changes, develop into malignant tumor that can metastasize, invade, and colonize new tissues, eventually leading to patient demise. The acquisition of invasive characteristics is often associated with a decrease in the level of the tumor suppressor CDH1 and a subsequent decrease in cell–cell adhesion, increase in cell motility, and degradation of the stromal matrix. CDH1 is an epithelial Ca²⁺-dependent adhesion molecule that facilitates cell–cell contact through homophilic interactions with adjacent CDH1 molecules expressed on neighboring cells (1, 2). Loss of CDH1 contributes to invasiveness, and the development of metastasis (3) and can occur through mutation, gene deletion, silencing of its promoter, and by proteolytic degradation. CDH1 loss facilitates and is also a hallmark of EMT (4). EMT frequently occurs during normal development, and is coordinated by transcriptional regulators such as the Snail and Twist family, Goosecoid, and members of the ZFH family (zinc-finger homeodomain, ZEB1 and ZEB2; ref. 5). Characteristics of EMT are normally associated with metastatic cells (6, 7). Extracellular factors such as TGF-β, which suppresses early tumor development, can also induce EMT at later stages to enhance tumor metastasis.

Phosphoinositides are signaling lipids whose levels are regulated by an array of kinases and phosphatases (8). PtdIns(4,5)P2 is a substrate for both receptor-stimulated phospholipase C (PLC) as well as members of the PtdIns-3-kinase family, and it also acts as a signaling moiety in itself by recruiting and regulating the activity of specific interacting proteins (8). In breast tumors, PtdIns(3,4,5)P3 signaling is enhanced by gain-of-function mutations in PIK3CA (30%) and loss-of-function or deletion-negative regulators such as...
PTEN and INPP4A (9, 10). Upregulation of the phosphoinositide 3-kinase (PI3K) pathway is associated with resistance to therapeutic ERBB2 inhibitors (11) and PI3K inhibitors sensitize triple-negative breast tumor cells to PARP inhibition (12). In addition, increased PI3K signaling decreases CDH1 mRNA and protein levels (13). Further, PtdIns(4,5)P₂ signaling can impact on breast cancer. Two families of lipid kinases generate PtdIns(4,5)P₂. Three isoforms of PI4Ks, γ2, and δ, and IC, regulate the bulk synthesis of PtdIns(4,5)P₂ and modulate membrane trafficking, cytokoskeletal dynamics, and focal adhesion turnover (14). PI4Ks splice variant, PI4Kγ2, interacts with talin, localizes to focal adhesions, and regulates integrin activation (15, 16). Moreover, PI4Kγ2 regulates trafficking and expression of CDH1 (17), and high expression in breast tumors is associated with poor clinical prognosis (18). In addition, loss of the Breast cancer metastasis suppressor 1 (BRMS1) is associated with reduced survival of subsets of patients with breast tumor (19), increased metastasis (20), and interestingly increases PIP5K expression and PtdIns(4,5)P₂ and, in vivo, they regulate cellular pools of both PtdIns(4,5)P₂ and PtdIns(4,5)P₃. The three isoforms of PI4K, 2A (24, 25), 2B (26), and 2C, have distinct subcellular localizations; PI4K2A is predominantly cytosolic (27). PI4K2B localizes in the plasma membrane, the cytoplasm, and in nuclear speckles (27); and PI4K2C localizes to endomembranes (28). PI4K2B regulates AKT activation (29, 30), reactive oxygen accumulation (31), and, in the nucleus, controls PtdIns5P levels (22), which can regulate gene transcription (32, 33). In addition, PI4K2B is required for vitamin-D₃-induced CDH1 transcription (34). PI4K2B is highly expressed in several breast cancer cell lines such as UACC-812, BT474, and T47D, and the gene encoding PIP4K2B, highly expressed in several breast cancer cell lines such as MCF10A cells. Viral particles were generated in HEK293FT cells using pLKO-based vectors and plasmids encoding GAG-Pol and VSVG (4:2:1, respectively). Cells were transduced in the presence of polybrene (5 μg/mL) and selected using puromycin (2 μg/mL).

Patients and tumor samples
The tissue microarray (TMA) used included tumor cores from 489 consecutive breast cancer cases consisting mainly of ductal and lobular tumors diagnosed at the Department of Pathology, Malmö University Hospital, Sweden between 1988 and 1992 (36).

Immunocytochemistry
For immunocytochemistry, HEK293 cells were harvested, washed in PBS, and fixed for 4 hours in 4% paraformaldehyde. Cell pellets were dehydrated in a graded ethanol series and embedded in paraffin. The TMA slides were deparaffinized, rehydrated, and microwave-treated in a target-retrieval solution citrate buffer (10 mmol/L, pH 6.0). Sections were incubated with the indicated antibodies and were visualized using 3,3’-diaminobenzidine. The PIP4K2B antibody was used at a ratio of 1:500, whereas the anti-ERBB2 antibody was used according to the manufacturer’s instructions (Pathway CB-USA, 760–2694).

The PIP4K2B staining intensities were subdivided into six categories (groups 0, 1, 2, 3, 4, and 5), and, for further statistical analysis, we combined groups 0 and 1, groups 2 and 3, and groups 4 and 5 to generate three intensity groupings. ERBB2 staining was scored semiquantitatively by the intensity and percentage of staining: 0 and 1+, negative; 2+, equivocal; and 3+, as positive. Evaluation was performed by two independent observers (one a pathologist, with the pathologist’s score superseding the other observer’s at consolidation. Conflicting observations were low (<5%) for all evaluations made. All immunohistochemical evaluations were performed blind, and randomized without prior knowledge of tumor characteristics.

Meta-analysis of gene expression data from primary breast tumors and breast cell lines
Raw Cel files from 17 Affymetrix U133A/plus two primary breast tumor and three cell line gene expression datasets were downloaded from the National Center for Biotechnology Information Omnibus (NCBI GEO; GSE12276, GSE21653, GSE3744, GSE5460, GSE2109, GSE1561, GSE17907, GSE2990, GSE7390, GSE11121, GSE16716, GSE2034, GSE1456, GSE6532, GSE3494, GSE10890, GSE12777), ArrayExpress (E-TABM-194), or cBio (geral-00143) repositories, summarized with Ensembl alternative Chip Definition Files (37), and normalized with RMA (38), before integrating using ComBat to remove dataset-specific bias as previously described (39). The intrinsic molecular subtypes were assigned for each dataset separately based upon the highest correlation to those defined in an article by Sorlie and colleagues (40, 41). Centered average linkage clustering of the integrated tumor datasets was performed using the Cluster and TreeView programs. Survival curves related to PIP4K2B expression were generated without prior knowledge
of the data generated in the immunohistochemical analysis (IHC) study.

**Cell viability/proliferation assay**

MCF7 cells were plated (3,000 cells/well) in 96-well plates and viability/proliferation was monitored 1, 3, and 5 days later using Alamar Blue reagent (Invitrogen).

**Anchorage-dependent and -independent clonogenic growth assays**

MCF7 cells were plated at low density (1,000 cells/10-cm diameter plate), grown for 10 days, and colonies were stained with crystal violet (0.5% w/v in PBS–0.5% formaldehyde v/v) and quantified with ImageJ. For colony density analysis, colonies were also stained with ethidium bromide (Sigma), and fluorescence and phase-contrast images were quantified with ImageJ. Data were presented as the number of colonies divided by colony area. Anchorage-independent growth was monitored in soft agar. Images were obtained using an Axiocamera camera, and colonies were quantified with ImageJ.

**Western immunoblot analysis**

Protein expression was analyzed by standard Western blotting procedures using the following antibodies: PIP4K2BP6 (generated against the peptide CNLLSFPRFFGP), ERBB2 (Labvision Neomarkers), CDH1 (BD), actin (Chemicon International), fibronectin (FN1; BD), vimentin (VIM; Novo Castro), β-catenin (Cell Signaling Technology), and tubulin (Sigma).

**Luciferase assay**

Cells transfected with a vector encoding Renilla luciferase and pGL3-E-cadherin-luciferase were lysed, and luciferase was measured using the Dual-GLO Luciferase System (Promega).

**Real-time PCR analysis**

RNA was isolated with an RNeasy Plus Mini Kit and QIAshredder spin columns (Qiagen). cDNA was generated using a high-capacity reverse transcription kit (Applied Biosystems). qPCR assays were performed in a MicroAmp optical 384-well reaction plate (Applied Biosystems) and analyzed using an Applied Biosystems 7900HT Sequence Detection System (SDS). Ribosomal protein L32 was used as a loading control.

**Results**

**PIPK2B antibody characterization for IHC analysis**

PIPK2B expression was assessed using an specific antibody (PIPK2Bp6; ref. 27) generated against the peptide sequence NLLSFPRFFGP, which is absent in both the 2A and 2C isoforms (Fig. 1A). PIP4K2BP6 recognized purified GST-PIPK2B, but not equivalent, amounts of GST-PIPK2A assessed by ELISA (Fig. 1B) or by Western blotting (Fig. 1C) although 1 µg of purified PIP4K2A was loaded on the gels. PIP4K2BP6 recognized a 51-kD protein in total MCF10A lysates, which was absent after PIP4K2B knockdown (Supplementary Fig. S1A). PIP4K2BP6 was then used to stain HEK293 cells fixed, embedded, and sectioned in a similar manner to tissue sections. To generate control for the staining, we suppressed or overexpressed PIP4K2B in cells and also used a preimmune serum. PIP4K2BP6 strongly stained wild-type HEK293 cells compared with the preimmune serum (Fig. 1D, panels 1 and 2), and the staining was diminished upon knockdown of endogenous PIP4K2B (Fig. 1D, panel 3 and inset) and increased on overexpression of PIP4K2B (Fig. 1D, panel 4). We next stained sections of normal and tumor breast tissue. PIP4K2B was strongly expressed in the luminal epithelial cells of normal breast ducts and acini and was predominantly localized at the plasma membrane. Little staining was observed in the myoepithelial cell layer (Fig. 1E, panel 1). In tumors, heterogeneous staining was observed: strong plasma membrane staining was often lost, and cytosolic and nuclear staining became more apparent (Fig. 1F, panel 1). Specific immunodepletion of the antisera (Supplementary Fig. S1B) decreased the staining of both normal and tumor tissues (Fig. 1E and F, panel 2). Heterogeneous cellular localization of PIP4K2B observed in tumor tissue was also observed in several breast cancer cell lines (Supplementary Fig. S2). These data strongly support the use of the PIP4K2BP6 antibody to interrogate the expression of PIP4K2B in TMA.

**Low PIP4K2B expression correlates with adverse patient survival**

PIPK2B expression varied dramatically across a TMA containing 489 advanced breast tumor samples (Fig. 2A, panel 1–3). We categorized PIP4K2B expression into three groups with 1 representing the lowest and 3 the highest intensity (Fig. 2B). As the PIP4K2B gene can be coamplified with the neighboring ERBB2 gene (35), we also assessed the expression of the ERBB2 protein. Figure 2C shows a tumor sample that expressed high levels of both PIP4K2B and ERBB2. ERBB2 staining was strong at the membrane whereas PIP4K2B appeared more cytosolic. Statistical analysis showed a strong correlation between high ERBB2 expression and high PIP4K2B expression (Table 1), which confirms and extends previous studies (35). We did not observe a significant correlation of high PIP4K2B or high ERBB2 with poor patient survival (Fig. 3A).

Low PIP4K2B expression showed a significant correlation with increased Nottingham histological grade (NHG; \(P = 0.007\)), tumor size (\(P = 0.021\), and expression of Ki67 (\(P = 0.011\), all with the Pearson \(\chi^2\) test; Table 1). PIP4K2B expression did not correlate with estrogen receptor (ER) or progesterone receptor (PR) status, age, or lymph node positivity or with histologic subtype. Interestingly, low PIP4K2B expression correlated with increased distant metastasis during follow-up (\(P = 0.040\), Pearson \(\chi^2\) test). Together, these data suggest that low PIP4K2B expression correlates with a more malignant grade of tumor and with worse clinicopathologic parameters.

In accordance, a Kaplan–Meier survival curve showed that low PIP4K2B expression correlated with decreased patient survival (log-rank chi-squared = 6.753, \(P = 0.034\), defined by time from diagnosis until local, regional, or distant metastasis or breast cancer-specific death (Fig. 3A).
Meta-analysis of gene expression profiles in breast tumors confirms an association between poor patient outcome and PIP4K2B expression

In order to independently verify the IHC results, we carried out a meta-analysis of gene expression profiles derived from 17 data sets representing 2,999 breast tumor samples. Raw gene expression profiles were downloaded and normalized together as described earlier (Materials and Methods section). PIP4K2B expression was then divided into three groups (Fig. 3B). As observed in the IHC analysis, high PIP4K2B expression strongly correlated with high ERBB2 expression (Fig. 3B inset). The suggestion that they are coamplified in the majority of tumors is clearly illustrated by plotting the expression of genes surrounding the ERBB2/PIP4K2B region of chromosome 17 and clustering the tumors (Supplementary Fig. 3A). PIP4K2B is most highly expressed when all the genes between its genomic position and the genomic position of ERBB2 are also overexpressed. Coamplification of PIP4K2B and ERBB2 was confirmed by examining this region in a high-resolution comparative genomic hybridization dataset (Supplementary Fig. S3B; ref. 42). Kaplan–Meier survival curves showed that both high and low expression of PIP4K2B is associated with significantly worse prognosis than an intermediate level confirming and extending our IHC studies (Fig. 3C). PIP4K2B expression is significantly lower in invasive ductal carcinoma (IDC) compared with normal breast tissue (Supplementary Fig. S4A), and the lowest PIP4K2B expression is associated with the more aggressive basal subtype compared with the other molecular subtypes (Supplementary Fig. S4B; ref. 41). As expected, the average PIP4K2B expression was highest in the ERBB2 subtype.
Finally, in a panel of widely used breast tumor cells, the average PIP4K2B expression was significantly lower in the more aggressive basal subtype cells, with the lowest expression being found in the highly aggressive "claudin low" subtype (Supplementary Fig. S5B). Western blot analysis confirmed that PIP4K2B expression was low in MCF7 tumor cells compared with the noncancer cell breast cell line MCF10A and that PIP4K2B expression was highly upregulated in the ERBB2 overexpressing cell line ZR-75 (Supplementary Fig. S5C).

These data confirm and extend our IHC studies and show that both high and low PIP4K2B expression associate with decreased patient survival compared with intermediate expression.

**PIP4K2B knock down in MCF7 cells affects colony formation in anchorage-dependent clonogenicity**

To understand if the association of low PIP4K2B expression with poor patient survival might be functional, using two separate lentiviral-driven shRNAs we knocked down PIP4K2B in MCF7 cells and examined their growth properties. Western blot analysis demonstrated that both shRNA constructs strongly reduced the level of PIP4K2B protein (Fig. 4A). No significant differences between PIP4K2B knockdown cells and control cells were observed in growth rates (Fig. 4B), cell-cycle distribution (Fig. 4C), or anchorage-independent clonogenic growth (Fig. 4D). PIP4K2B knockdown, however, reduced the number of colonies observed in anchorage-dependent growth compared with control cells (Fig. 4E). Microscopic analysis showed that PIP4K2B knockdown colonies appeared less tightly packed than the control colonies. To quantify this, cell nuclei were stained with ethidium bromide and the cell number and the colony area was assessed using ImageJ. The number of cells per colony area was reduced in PIP4K2B knockdown cells compared with control cells (Fig. 4F). This decrease might reflect changes in cell–cell contact-mediated growth inhibition, surface area–cell number maintenance mechanisms, or may reflect an increase in cell migratory behavior.

**Lentiviral-mediated knockdown of PIP4K2B leads to a decrease in CDH1 expression**

PtdIns(4,5)P2 levels regulate trafficking of E-cadherin (17), and RNAi-mediated depletion of PIP4K2B decreases vitamin D3–induced expression of CDH1 (34). As the loss of cell–cell contacts and increased migration are often associated with loss of CDH1 (3, 9) and PIP4K2B knockdown cells appeared less tightly packed, we investigated whether PIP4K2B knockdown changed the levels of CDH1. Knockdown of PIP4K2B in MCF7 cells reduced total CDH1 staining and staining at cell–cell junctions (Fig. 5A). In the nontumorigenic breast cell line MCF10A, PIP4K2B knockdown also led to a reduction in CDH1 levels. shPIP4K2B #1 reduced PIP4K2B more efficiently than shPIP4K2B #2, correlating with the observed difference in inhibition of CDH1 expression (Fig.
5B). PIP4K2B can regulate AKT activation, which can impact CDH1 expression. However, AKT activation and the phosphorylation of its downstream target AKT1S1 (PRAS40) were not significantly different in PIP4K2B knockdown cells, although AKT activation decreased during the time course of the experiment (Fig. 5B). Total AKT levels were not decreased during the 4-day period in control cells (Supplementary Fig. S6A). The decrease in CDH1 expression induced by PIP4K2B knockdown was not suppressed by pretreatment with the proteasome inhibitor MG132, suggesting that ubiquitin-mediated proteolytic cleavage of CDH1 is not increased by PIP4K2B knockdown (Fig. 5C), although MG132 increased general polyubiquination (Supplementary Fig. S6B). Quantitative RT-PCR (qRT-PCR) analysis demonstrated that PIP4K2B knockdown decreased the levels of CDH1 mRNA (Fig. 5D) and also decreased CDH1 promoter activity assessed using the CDH1 gene promoter coupled to the synthesis of luciferase (Fig. 5E). These data show that PIP4K2B regulates the transcription of CDH1. We next assessed whether the knockdown of PIP4K2B impacted on the levels of cellular phosphoinositides. We observed a small but significant decrease in the levels of PtdIns(4,5)P2. No significant changes in the mass of PtdIns5P were observed (Supplementary Fig. S7) as measured using a specific mass assay (43, 44).

### Lentiviral-mediated knockdown of PIP4K2B induces EMT characteristics in MCF10A cells

Deregulation and loss of CDH1 expression facilitates and is a hallmark of EMT. Therefore, we assessed whether PIP4K2B knockdown induced the EMT program or enhanced TGF-β–induced EMT. PIP4K2B was knocked down in MCF10A cells, the cells were maintained untreated or treated with TGF-β for 72 hours, and cell lysates were analyzed for markers of EMT.

### Table 1. High PIP4K2B expression correlates with ERBB2 expression and low PIP4K2B expression associates with worse clinical parameter

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NOTE: Correlation between PIP4K2B staining and clinicopathologic and molecular parameters of advanced breast cancer. Significant P values are marked in bold.
Although PIP4K2B knockdown decreased the basal level of CDH1, it did not induce the expression of the mesenchymal markers FN1 or VIM (Fig. 6A), whereas TGF-β led to the expected decrease in CDH1 and increase in FN1 and VIM. Treatment of PIP4K2B-knockdown cells with TGF-β further decreased CDH1 levels and augmented the increase in the mesenchymal markers FN1 and VIM compared with TGF-β treatment of control cells. SNAI1 is a master transcriptional regulator of the EMT program, and is induced by TGF-β treatment. PIP4K2B knockdown alone did not induce SNAI1 levels but augmented TGF-β–induced SNAI1 levels (Fig. 6A).

TGF-β induces EMT through regulating gene transcription programs and, therefore, we assessed whether knockdown of PIP4K2B augmented TGF-β1–induced transcriptional regulation (Fig. 6B). PIP4K2B knockdown decreased basal levels of CDH1 mRNA, but did not increase VIM, FN1, and SNAI1 mRNA. In addition, TGF-β1 treatment reduced CDH1 mRNA and increased VIM, FN1, and SNAI1 expression. In accordance with the Western blot data, PIP4K2B knockdown augmented increases in TGF-β–induced VIM, FN1, and SNAI1 mRNA. PIP4K2B knockdown did not significantly change the expression of Slug (SNAI2) before or after treatment with TGF-β. These data suggest that although PIP4K2B regulates CDH1 expression, it does not induce the EMT program.

**Figure 3.** Low expression of PIP4K2B correlates with adverse parameters of tumor progression. A, Kaplan-Meier curve showing the effect of PIP4K2B protein expression levels on event-free survival as a function of time from diagnosis until local, regional, or distant metastasis or breast cancer-specific death. Low PIP4K2B expression group 1 correlated with poor patient outcome in contrast to the intermediate and high expression of PIP4K2B (groups 2 and 3), (log-rank chi-squared = 6.753, P = 0.034). B, distribution of PIP4K2B gene expression was assessed across 2,999 primary breast cancer tumors integrated from 17 studies. The data are presented as a frequency distribution of PIP4K2B expression. The samples were amalgamated into three groups based on PIP4K2B expression for further analysis: top, 10% (red); middle, 80% (green); and the lowest, 10% (blue). High expression of PIP4K2B (red) was clearly associated with high ERBB2 expression. C, cumulative recurrence-free outcome was plotted for low, middle, and high PIP4K2B expression as defined earlier.
Figure 4. PIP4K2B knockdown in MCF7 does not affect normal cell growth, cell-cycle distribution, or growth in soft agar, but decreases anchorage-dependent clonogenic growth. MCF7 cells were transduced with a control lentiviral vector or two different shRNA vectors targeting PIP4K2B (#1 and #2) and then analyzed as described in the following. A, cell lysates were subjected to Western blot analysis to detect PIP4K2B and actin. B, MCF7 cells were plated and the viability/proliferation was measured on days 1, 3, and 5 post plating using Alamar Blue. Data are representative of three experiments and represent the mean ± SD. C, cells were fixed and labeled with propidium iodide and analyzed by FACS. D, MCF7 cells were plated in soft agarose to assess anchorage-independent growth. The graph presents the numbers of three different sizes of colonies [ImageJ, arbitrary units (AU), from small (0.002) to big (0.05)] divided by the total number of colonies. Data are representative of two experiments performed in triplicate, and the data represent the mean ± SD. E, 1,000 MCF7 cells were plated in a 10-cm plate and grown for 10 days. Typical images of the colonies are shown, and quantification of colony number showed significant differences **, P < 0.01; *** P < 0.001, Student t test) between pLKO and PIP4K2B knockdown constructs (#1 and #2). F, colony density from E was evaluated by staining cell nuclei with ethidium bromide (EtBr) to assess cell number per colony, and phase-contrast images were used to determine the colony area. The data are presented as the number of cells per colony surface area (ImageJ, arbitrary units). **, P < 0.01; ***, P < 0.001 (Student t test).
consistent with a role for PIP4K2B in regulating the sensitivity of cells to TGF-β.

Reduced CDH1 expression is associated with more aggressive tumors, and might in part explain poorer survival of patients with tumors that have low PIP4K2B expression. We, therefore, interrogated CDH1 and PIP4K2B expression levels in the unpublished Bittner and colleagues breast tumor array and the published Curtis and colleagues METABRIC study (45) using Oncomine. The data were sorted with respect to PIP4K2B expression, and the CDH1 levels were determined in the top and bottom 10% of the PIP4K2B-expressing population. We observed a significant decrease in the expression levels of CDH1 in the low PIP4K2B-expressing tumors compared with the high PIP4K2B-expressing

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Figure 5. Lentiviral-mediated knockdown of PIP4K2B leads to a decrease in CDH1 expression. A, MCF7 cells transduced with control (1) or with shRNA constructs targeting PIP4K2B (2 and 3) were fixed and immunostained with antibodies to CDH1 and analyzed by fluorescence microscopy. Total immunofluorescence staining was determined and is graphically presented. Statistically, differences were determined using the Student’s t test between control and PIP4K2B knockdown cells (**, P < 0.01). B, MCF10A cells were transduced with control (pLK01) or with lentiviral-mediated shRNAs against PIP4K2B (#1 and #2). Cell lysates were immunoprobed with the indicated antibodies. C, control and PIP4K2B knockdown MCF10A cells were with MG132 as indicated. Cell lysates were analyzed by Western blot with the indicated antibodies. D, control and PIP4K2B knockdown MCF10A cells were assessed for the relative expression of PIP4K2B and CDH1 mRNA using qRT-PCR. The data are presented as fold-change compared with the control. E, MCF10A cells transduced with the indicated shRNAs were transfected with Renilla and E-cadherin-luc. A luciferase activity was measured, and the data are presented after correction for the expression of the control Renilla luciferase.
populations in both data sets ($P = 0.0039$ in the Bittner array and $P = 0.000437$ in the Curtis array; Fig. 6C). Moreover, a similar trend was observed in the tumor data sets used in this study. No significant changes were observed in SNAI1 or TWIST1, two upstream repressors of $CDH1$ expression.
Discussion

We describe the characterization and use of a specific PIP4K2B antibody to interrogate its expression in a TMA of breast tumor samples, enabling the correlation between PIP4K2B expression and clinicopathologic parameters. The protein expression studies and their outcomes were supported by an independent meta-analysis of gene expression profiles from 17 previously published studies representing 2,999 breast tumors. We found that both high and low expression of PIP4K2B associates with poor survival compared with intermediate expression. High PIP4K2B levels were strongly correlated with high ERBB2 expression, which is likely to explain the associated poor patient survival. We discovered that low expression of PIP4K2B induced by shRNA in normal and breast tumor cell lines leads to a decrease in the expression of the tumor suppressor CDH1 and enhances TGF-β–induced EMT.

High PIP4K2B expression strongly correlated with high ERBB2 expression, and genomic analysis of a published breast cancer dataset (42) confirmed that PIP4K2B can be coamplified with ERBB2 (35). The association between high PIP4K2B expression and decreased patient survival was only observed in the analysis of gene expression profiles and not in the TMA. It is unclear why the established association between expression of ERBB2 and poor patient survival was not evident in the TMA, but is likely due to different patient cohorts. Whether high expression of PIP4K2B affects patient survival independently of its association with high ERBB2 expression remains to be determined. PIP4K2B can associate with members of the EGFR receptor family (46) and, therefore, might play a role in regulating their activity during breast cancer development. Interestingly, Luoh and colleagues (35) observed one tumor with PIP4K2B, but not ERBB2, amplification. Although we did not specifically study gene amplification, 58 tumors in the TMA showed strong PIP4K2B expression in the absence of ERBB2 expression.

IHC analysis and analysis of gene expression profiles both showed that low PIP4K2B expression correlated with increased malignancy and poorer patient outcome related to a greater incidence of metastasis. In accordance, PIP4K2B levels were lower in tumors compared with normal breast tissue samples, and the lowest levels of PIP4K2B were associated with more aggressive tumors and tumor-derived cell lines. PIP4K2B knockdown had little effect on the growth rate of MCF7 cells under normal conditions or in soft agar, but decreased their growth in anchorage-dependent clonogenic assays contrary to what might be expected from the patient survival correlations. PIP4K2B regulates many pathways such as PKB signaling (29, 47), reactive oxygen accumulation (31), and p53 activation and apoptosis (33), and the balance of these pathways is likely to underlie the survival of cells clonogenic growth assays. How this might relate to overall patient survival, which is more closely related to the onset of metastasis, is unclear. However, PIP4K2B knockdown decreased the expression of CDH1, a tumor suppressor that is a critical regulator of cell–cell adhesion and whose loss can drive invasive carcinoma development in mice (3, 4, 48); moreover, it is associated with high-grade tumors and poor prognosis (49, 50).

How does PIP4K2B regulate CDH1 transcription? PIP4K2B could regulate the levels of both PtdIns(4,5)P2 and PtdIns5P in the nucleus or in the cytoplasm (27), which could impact directly or indirectly on CDH1 expression. In fact, we observed a significant decrease in total PtdIns(4,5)P2 levels, without any significant changes in PtdIns5P. Interestingly, decreased expression of PIP4K2B suppressed vitamin-D3–induced CDH1 transcription, which was suggested to occur through a decrease the synthesis of nuclear PtdIns(4,5)P2 (34). Whether our observed changes in PtdIns(4,5)P2 occur in the nucleus, cytoplasm, or both remains to be determined. As we did not observe an increase in PtdIns5P levels after knockdown of PIP4K2B, the decrease in PtdIns(4,5)P2 might be indirect. In addition, it should be noted that PIP4K2B homo- and heterodimerizes with PIP4K2A, and, as PIP4K2B has approximately 1,000 times less activity than PIP4K2A (27), it is difficult to predict exactly how changes in the expression of PIP4K2B might impact on subcellular pools of PtdIns5P/PtdIns(4,5)P2. The recent identification of PHD fingers as receptors for nuclear phosphoinositides (32, 33) and of nuclear proteins that interact with PtdIns(4,5)P2 (51) might provide plausible pathways for direct transcriptional regulation of CDH1 expression by nuclear phosphoinositides. Moreover, PIP4K2B has been implicated in EGF and TNF receptor function (26, 52); (46), AKT activity (29), ubiquitination (53), and p38-regulated pathways (22), all of which can also impact CDH1 regulation. In addition, it is possible that scaffolding functions of PIP4K2B unrelated to its PIP4K activity or noncoding regions within the mRNA-encoding PIP4K2B (54) could be involved in regulating CDH1 expression.

CDH1 expression can be transcriptionally regulated by repressors such as ZEB1, ZEB2, SNAI1 and SNAI2, and TWIST family members and by epigenetic and chromatin remodeling factors such as EZH2(5), all of which also induce EMT. Although knockdown of PIP4K2B alone reduced CDH1, it did not induce EMT, and is unlikely to regulate CDH1 expression through those transcriptional repressors. Understanding exactly how PIP4K2B regulates CDH1 expression remains a challenge. EMT is required for metastasis in vivo (55) and can be induced by a number of factors, including TGF-β1. TGF-β1 plays a complex role during tumor development, acting both as an early tumor suppressor and as a late oncogene. Although downregulation of PIP4K2B did not induce EMT, it did enhance TGF-β1–induced expression of mesenchymal markers, suggesting a possible function for PIP4K2B in regulating TGF-β sensitivity.

Our data are consistent with an association between PIP4K2B expression and patient survival in breast cancer, which might be related to PIP4K2B mediated-regulation of the expression of the tumor suppressor protein CDH1 and increased TGF-β–induced EMT.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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