Inositol polyphosphate 4-phosphatase II regulates PI3K/Akt signaling and is lost in human basal-like breast cancers

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Inositol polyphosphate 4-phosphatase-II (INPP4B) is a regulator of the phosphoinositide 3-kinase (PI3K) signaling pathway and is implicated as a tumor suppressor in epithelial carcinomas. *INPP4B* loss of heterozygosity (LOH) is detected in some human breast cancers; however, the expression of INPP4B protein in breast cancer subtypes and the normal breast is unknown. We report here that INPP4B is expressed in nonproliferative estrogen receptor (ER)-positive cells in the normal breast, and in ER-positive, but not negative, breast cancer cell lines. INPP4B knockdown in ER-positive breast cancer cells increased Akt activation, cell proliferation, and xenograft tumor growth. Conversely, reconstitution of INPP4B expression in ER-negative, INPP4B-null human breast cancer cells reduced Akt activation and anchorage-independent growth. INPP4B protein expression was frequently lost in primary human breast carcinomas, associated with high clinical grade and tumor size and loss of hormone receptors and was lost most commonly in aggressive basal-like breast carcinomas. INPP4B protein loss was also frequently observed in phosphatase and tensin homolog (PTEN)-null tumors. These studies provide evidence that INPP4B functions as a tumor suppressor by negatively regulating normal and malignant mammary epithelial cell proliferation through regulation of the PI3K/Akt signaling pathway, and that loss of INPP4B protein is a marker of aggressive basal-like breast carcinomas.

phosphatidylinositol 3,4-bisphosphate

The phosphoinositide 3-kinase (PI3K) signaling pathway promotes cell proliferation and survival. In response to extracellular stimuli, activation of PI3K results in the transient production of the phosphoinositides, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, at the plasma membrane. Both phosphoinositides can bind and activate multiple downstream effectors, most importantly the protooncogene, Akt, and both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are necessary for full Akt activation in vivo (1–3). Deregulated PI3K/Akt activation promotes oncogenesis and has been described in many human cancers (4). Recently, the PI3K signaling pathway has been identified as a putative therapeutic target in a range of human malignancies and there are currently a number of phase I–II clinical trials in progress investigating the efficacy of PI3K pathway inhibitors in the treatment of human cancers (4).

PtdIns(3,4,5)P_3 is negatively regulated via dephosphorylation by the 3-phosphatase and tumor suppressor, phosphatase and tensin homolog (PTEN), to form PtdIns(4,5)P_2, or by 5-phosphatases, which generate PtdIns(3,4)P_2 (1). In turn, PtdIns(3,4)P_2 is hydrolyzed by two inositol polyphosphate 4-phosphatases (type I and type II) (INPP4A and B, respectively) (5, 6). INPP4A expression protects neurons from excitotoxic cell death (7, 8) and this enzyme is also a negative regulator of Akt phosphorylation, cell proliferation, and orthotopic tumor formation in mice (9, 10). However, to date analysis of human tumors has revealed little evidence that INPP4A functions as a tumor suppressor. In contrast, the related INPP4B may function as a tumor suppressor in epithelial carcinomas. Short hairpin RNA (shRNA)-targeting of *INPP4B* leads to cell transformation in human mammary epithelial cells (HMECs) (11) and INPP4B knockdown in HMECs promotes Akt activation and anchorage-independent growth (12). Deletion of the *INPP4B* chromosome region occurs in some primary human breast cancers (13) and loss of heterozygosity (LOH) of *INPP4B* is frequently observed in BRCA1 mutant and hormone receptor-negative breast cancers (12). Furthermore, loss of INPP4B protein expression in breast and ovarian cancer is associated with decreased patient survival (12). To date, however, the expression of INPP4B protein in normal breast and in human breast cancer subtypes, relative to clinicopathologic variables, remains to be determined.

Invasive breast carcinomas can be categorized into distinct subtypes, luminal A, luminal B, HER2 positive and basal-like, on the basis of expression of various clinocopathologic markers (14–16). Luminal A and luminal B breast cancer subtypes express estrogen receptor (ER) and/or progesterone receptor (PgR), whereas HER2-positive and basal-like subtypes are hormone receptor negative and are in general more aggressive and confer a worse prognosis compared with luminal-type breast carcinomas (15, 16). The cellular mechanisms underlying these observations, however, are poorly defined and current research is focused on identifying new biomarkers to improve breast cancer classification and treatment.

In this study, we identify INPP4B as a previously undescribed marker of hormone receptor-positive breast cancers that functions to control both normal breast and malignant ER-positive breast cancer cell proliferation through regulation of PI3K signaling. Loss of INPP4B protein expression occurs most frequently in aggressive hormone receptor-negative basal-like breast carcinomas, associated with high tumor grade and size, and is frequently associated with loss of the tumor suppressor PTEN. These studies identify loss of INPP4B protein as a previously undescribed molecular marker for basal-like breast cancers and

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provide evidence for the cooperative promotion of oncogenesis through the PI3K/Akt signaling pathway.

Results

Expression of INPP4B Protein in Normal Human Breast. We generated monoclonal and polyclonal antibodies (Abs) to purified 6xHis-tagged human INPP4B, which both detected endogenous INPP4B in ER-positive MCF-7 breast cancer cells and purified recombinant His-INPP4B, but not recombinant INPP4A (Fig. S1). Using these Abs, we assessed INPP4B protein expression in normal human mammary tissue sections (Fig. 1 and Fig. S2). In epithelial cells comprising the mammary ducts, INPP4B expression was restricted to luminal epithelial cells and appeared absent in surrounding myoepithelial cells (Fig. 1*A*). In control studies, no immunoreactivity was detected using mouse IgG as a negative control (Fig. S2*A*). Interestingly, in the secretory lobular units, INPP4B was present in only ~20% of cells, with many cells showing no staining (Fig. 1*A*). The heterogeneous expression pattern of INPP4B in these structures may indicate a specific role for INPP4B in mammary cell differentiation and function.

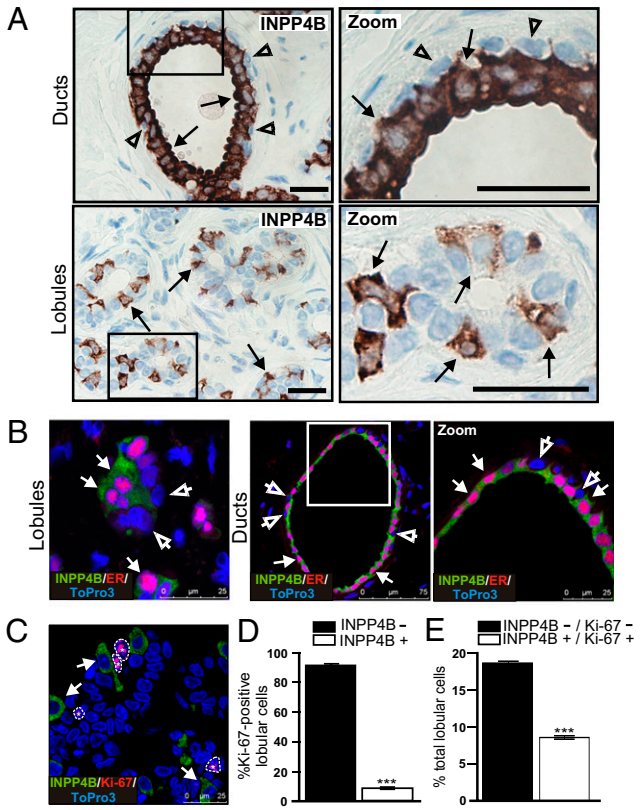

Fig. 1. INPP4B is expressed in nonproliferative ER-positive cells in normal human breast. (*A*) Normal human breast sections were immunostained using an INPP4B-specific monoclonal antibody (3D5). (Scale bars, 50 μm.) (*B*) Immunofluorescent images of normal breast sections costained for INPP4B (green), ER (red), and the nuclear marker, ToPro3 iodide (blue) (see also Fig. S2). Coexpression of INPP4B and ER is indicated by arrows. Arrowheads show cells negative for both INPP4B and ER. (Scale bars, 25 μm, 75 μm.) (*C*) Immunofluorescent costaining of normal human breast lobules for INPP4B (green), *Ki*-67 (red), and ToPro3 iodide (blue) (Fig. S2). Arrows show INPP4B-positive cells. Outlines and asterisks mark *Ki*-67–positive nuclei. (Scale bar, 25 μm.) More than 100 *Ki*-67–positive lobular cells/section were assessed for INPP4B expression, and the mean number of cells that were INPP4B negative (closed bars) or INPP4B positive (open bars) ± SEM from three normal breasts is shown in *D*. More than 1,000 lobular cells/section were assessed for *Ki*-67 and INPP4B expression and the mean number of INPP4B-positive lobular cells that were *Ki*-67 negative (closed bars) or *Ki*-67 positive (open bars) ± SEM from two normal breasts is shown in *E*. ***$P < 0.002$.

In the normal breast, ER is expressed in only 15–30% of epithelial cells and inversely correlates with cell proliferation (17, 18). Costaining normal breast sections with ER and INPP4B Abs revealed that INPP4B was expressed in ER-positive but not ER-negative cells (Fig. 1*B* and Fig. S2*B*). Colocalization of INPP4B and the proliferation marker, *Ki*-67 in breast lobules revealed that ~5–10% of lobular cells were *Ki*-67 positive and of these the majority (>90%) did not show INPP4B staining (Fig. 1 *C*–*D*). Of the INPP4B-positive cells (20% of lobular cells), the majority did not exhibit *Ki*-67 staining (Fig. 1*E*). Therefore INPP4B may suppress ER-positive lobular cell proliferation in the normal breast.

Characterization of INPP4B Protein Expression in Human Breast Cancer Cell Lines. We next assessed INPP4B protein expression in a panel of human breast cancer cell lines. INPP4B protein was expressed in ER-positive (MCF-7, T47D, and BT-474) but not ER-negative (MDA MB 231, Hs578T, and BT-549) cell lines (Fig. 2*A*). Quantitative real-time reverse-transcription PCR (qRT-PCR) analysis confirmed *INPP4B* mRNA levels were lower in all ER-negative relative to ER-positive cells, although a low level of *INPP4B* mRNA was detected in ER-negative cell lines (Fig. 2*B*). Furthermore, metaanalysis of human breast cancer datasets made publicly available in Oncomine (19, 20) revealed a positive association between *INPP4B* mRNA and the hormone receptors, ER and PgR, in human breast cancers in 11 and 3 independent studies, respectively (Fig. S3 *A* and *B*).

Control studies evaluated whether the related INPP4A is also expressed in breast cancer cell lines using previously characterized INPP4A Abs (21). Endogenous INPP4A was detected in mouse brain lysates but not in breast cancer cell lines (Fig. S3 *C* and *D*). Collectively this data indicates INPP4B is the only PtdIns $(3,4)P_2$ 4-phosphatase expressed in breast cancer cells and suggests a correlation between INPP4B and hormone receptor status in human breast cancer.

Involvement of INPP4B in ER-Positive Breast Cancer Cell Signaling, Proliferation, and Tumor Formation. To characterize INPP4B function in human breast cancer cells, shRNA-mediated INPP4B protein knockdown was undertaken in a variant of the ER-positive MCF-7 human breast cancer cell line, MCF-7-luc-F5, which stably expresses luciferase enzyme allowing detection of cells in vivo. MCF-7-luc-F5 cells exhibited similar INPP4B protein levels to the parental line (Fig S4*A*). In cell populations individually expressing two distinct *INPP4B*-directed shRNAs (INPP4B KD[1] and [2]) (Fig. S4*B*), INPP4B protein was decreased by ~70% and 60% compared with vector controls (Fig. 3*A*) and similar decreases were observed in *INPP4B* mRNA (Fig. S4*C*). The protooncogene Akt is activated by phosphorylation on serine and threonine residues. Significantly, in serum-starved cells,

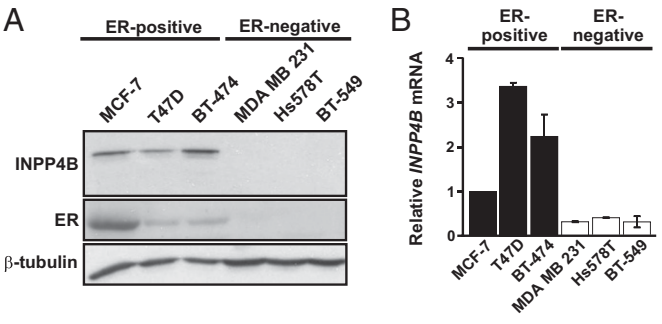

Fig. 2. INPP4B protein and mRNA expression in a panel of human breast cancer cells lines. (*A*) Human breast cancer cell line lysates were immunoblotted for INPP4B, ER, and β-tubulin protein expression, revealing INPP4B protein is expressed in ER-positive, but not ER-negative, cell lines. (*B*) qRT-PCR analysis of *INPP4B* mRNA, relative to *GAPDH*, in ER-positive versus ER-negative human breast cancer cell lines. The graph shows the mean *INPP4B* mRNA expression ± SEM from two independent experiments.

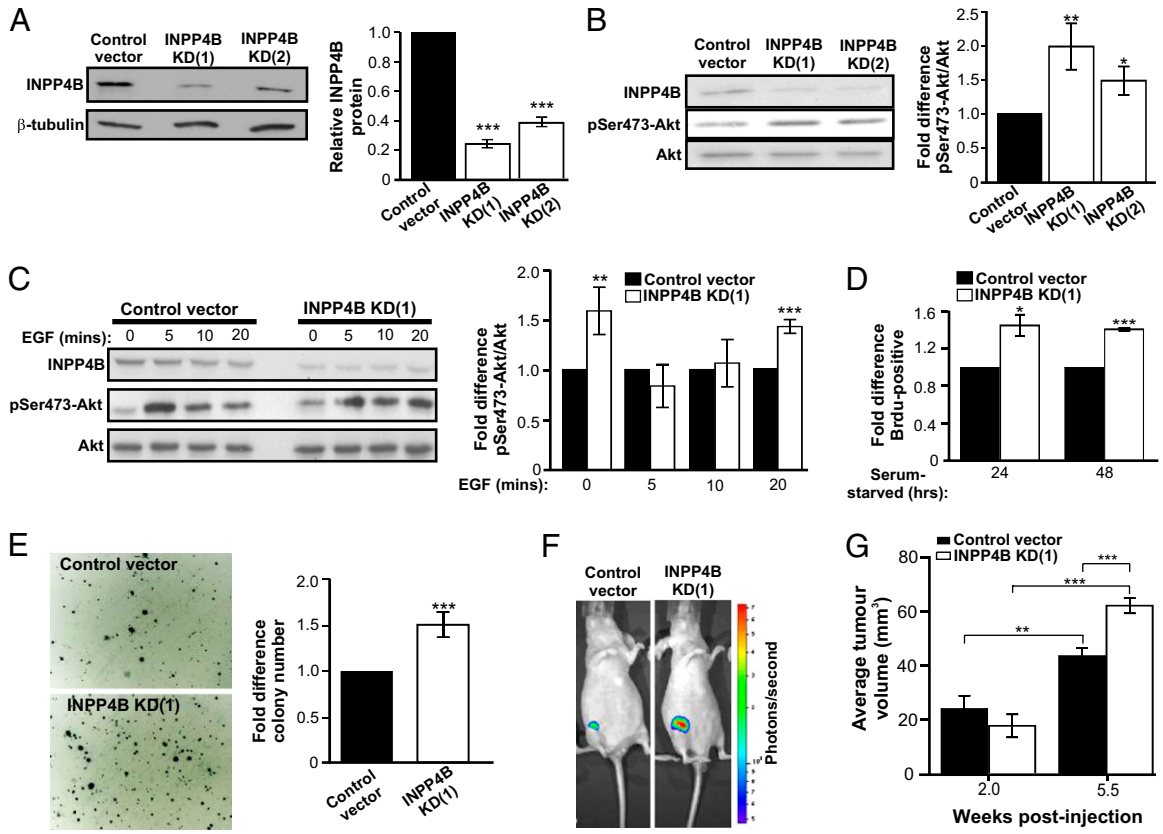

Fig. 3. Decreased INPP4B expression enhances Akt phosphorylation and tumorigenic potential. (*A*) INPP4B protein knockdown in two MCF-7-luc-F5 cell populations expressing unique INPP4B-specific shRNAs (INPP4B KD[1] and [2]) was confirmed by immunoblotting. The mean INPP4B protein levels relative to vector control ± SEM from three independent experiments is shown. (*B* and *C*) Immunoblot analysis of INPP4B, pSer473-Akt, and total Akt in cells serum-starved for 24 h (*B*) or stimulated with EGF (C), demonstrating enhanced pSer473-Akt in INPP4B knockdown cells. The mean fold change in pSer473-Akt/total Akt relative to control ± SEM from six (*B*) or three (*C*) independent experiments is shown. (*D*) INPP4B knockdown and control cells were serum starved for 24 and 48 h and assessed for BrdU incorporation as a marker of proliferation. The mean fold increase in BrdU-positive cells relative to control ± SEM from three independent experiments is shown. (*E*) INPP4B knockdown or control cells were grown in soft agar. The number of colonies/well was determined and the mean fold increase in colony number relative to control ± SEM from two independent experiments performed in triplicate is shown. (*F* and *G*) MCF-7-luc-F5 cells expressing control vector or INPP4B-specific shRNAS were injected into the mammary fat pads of Balbc nu/nu mice in the presence of estrogen and tumor growth was analyzed by bioluminescence and caliper measurement. Bioluminescent imaging of xenograft tumors in vivo (*F*) and mean tumor volumes at 2 and 5.5 wk postinjection (*G*) indicate INPP4B knockdown cells form larger tumors in vivo. The mean tumor volumes ± SEM of five animals/group is shown. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

basal phosphorylated Akt (pSer473-Akt) was increased by 1.5- to 2-fold in INPP4B knockdown cells, conditions under which Akt activation is normally minimal (Fig. 3*B*). Furthermore INPP4B knockdown in MCF-7-luc-F5 cells resulted in increased pSer473-Akt/Akt following 20 min EGF stimulation, relative to vector controls (Fig. 3*C*).

Akt activation promotes cell proliferation. INPP4B knockdown cells exhibited enhanced (1.4-fold) cell proliferation in response to serum-starvation (24–48 h), conditions under which cells normally become quiescent (Fig. 3*D*). The ability of cells to form colonies in soft agar and tumors in nude mice is a feature of transformed cells. INPP4B knockdown in MCF-7-luc-F5 cells enhanced anchorage-independent cell growth (Fig. 3*E*). To assess INPP4B regulation of ER-positive breast cancer growth in vivo, INPP4B knockdown or control MCF-7-luc-F5 cells were injected into the abdominal mammary fat pads of athymic nude mice and palpable tumors were measured over 6 wk. Seven of the eight mice injected with INPP4B knockdown cells developed palpable tumors that grew over the course of the experiment, compared with four out of eight mice injected with vector-control cells. Furthermore, INPP4B knockdown tumors exhibited a 3.2-fold increase in tumor size over time, compared with a 1.9-fold increase in vector controls (Fig. 3 *F* and *G*).

Effect of INPP4B Reconstitution on ER-Negative Breast Cancer Cell Growth and Akt Phosphorylation. We next evaluated the effects of INPP4B protein reconstitution in INPP4B-null, ER-negative MDA MB 231 breast cancer cells. MDA MB 231 cells are highly proliferative and exhibit anchorage-independent growth in soft agar. Expression of GFP-INPP4B reduced colony formation in soft agar by 35% (Fig. 4*A*) and suppressed EGF stimulated phosphorylation of Akt at Ser473 (pSer473-Akt) and Thr308 (pThr308-Akt) (Fig. 4 *B–D*), relative to GFP controls. Therefore in ER-negative breast cancer cell lines, which have lost endogenous INPP4B, reconstitution of this enzyme is sufficient to reduce EGF-stimulated Akt activation and anchorage-independent cell growth.

Expression of INPP4B Protein in Primary Human Breast Carcinoma Subtypes. We next assessed the expression of INPP4B protein in clinical human breast carcinomas. In tissue lysates from 22 primary human breast cancers, INPP4B protein was detected in all of the ER-positive tumors, but few of the ER-negative tumors (Fig. 5*A*). INPP4B protein expression was then evaluated in 107 primary breast carcinoma sections, collected as part of the Melbourne Collaborative Cohort Study (MCCS) (22, 23), by IHC. Notably, INPP4B protein was not detected in 22/107 (21%) of all breast cancers analyzed, but adjacent normal epithelium exhibited INPP4B immunoreactivity in every case (Fig. 5*B*). In INPP4B-positive tumors, protein expression was exclusively cytoplasmic

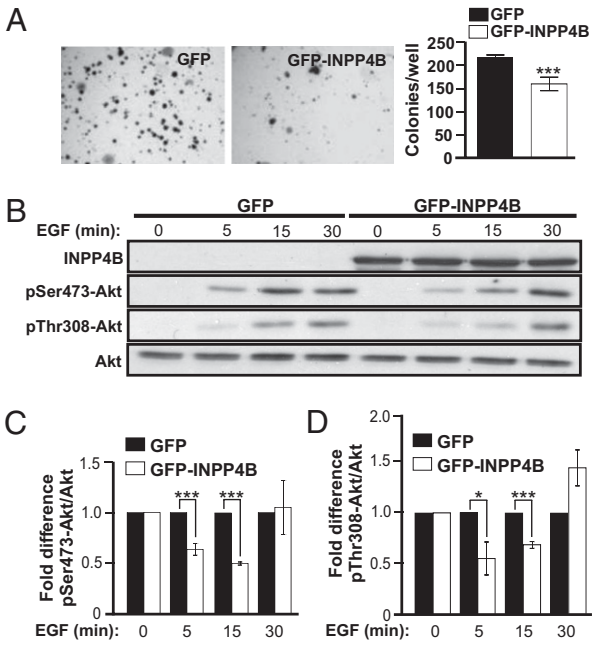

Fig. 4. INPP4B protein reconstitution decreases Akt phosphorylation and cell proliferation. (*A*) INPP4B-null MDA MB 231 cells expressing GFP alone or GFP-tagged INPP4B were suspended in 0.3% agar and colonies allowed to grow for 3 wk. The number of colonies/well was then determined and the mean number of colonies/well ± SEM from two independent experiments performed in triplicate is shown. (*B*) Immunoblot analysis of INPP4B, pSer473-Akt, pThr308-Akt, and total Akt in GFP or GFP-INPP4B–expressing cells in response to EGF. The mean fold difference in pSer473-Akt (*C*) and pThr308-Akt (*D*) normalized to total Akt relative to controls ± SEM from three independent experiments is shown. $*P = 0.05$, $***P < 0.005$.

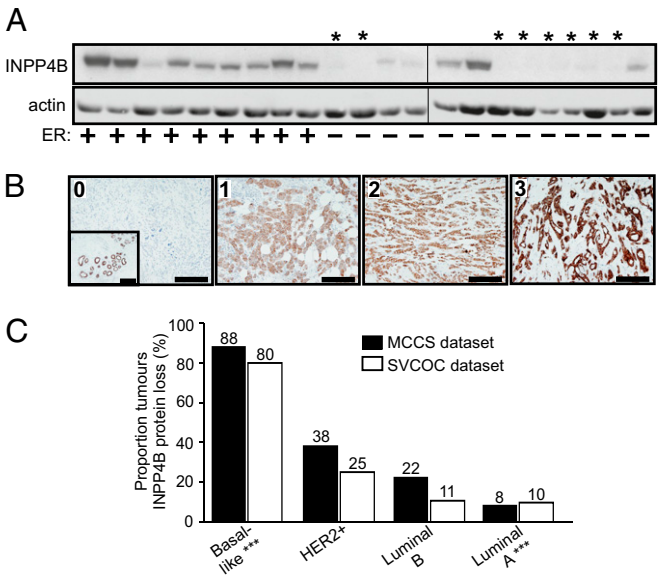

Fig. 5. INPP4B protein expression in primary human breast cancers. (*A*) Immunoblot analysis of INPP4B protein expression in a panel of ER-positive and ER-negative primary human breast cancer lysates. ER-negative tumors were predominantly INPP4B-negative (asterisks). (*B*) Representative IHC staining of INPP4B in primary human breast carcinomas, scored as: 0, no expression; 1, low; 2, moderate; and 3, strong. Inset image in tumor scored 0 for INPP4B shows INPP4B expression in adjacent normal tissue. (Scale bar, 200 μm.) (*C*) The frequency of INPP4B protein loss of expression in breast cancer subtypes from two independent human datasets [MCCS (black bars) and SVCOC (open bars)] was determined by IHC. Loss of INPP4B was positively correlated with the basal-like subtype and negatively correlated with the luminal A subtype in both datasets. $***P < 0.0001$.

and ranged from low, moderate, to high (staining score 1–3) (Fig. 5*B*). Significantly, INPP4B protein deficiency correlated with loss of both ER ($P < 0.0001$) and PgR expression ($P < 0.0001$) and positively correlated with the basal marker, CK5/6 ($P = 0.0014$), and approached significance for epidermal growth factor receptor (EGFR) expression ($P = 0.06$) (Table 1). Loss of INPP4B was also associated with high tumor grade ($P = 0.018$). There was no significant correlation between HER2 amplification and INPP4B expression ($P = 0.17$). Tumors were then classified into breast cancer subtypes as: luminal A (ER^+ and/or PgR^+, $HER2^-$), luminal B (ER^+ and/or PgR^+, $HER2^+$), $HER2^+$ (ER/PgR^-, $HER2^+$), or basal-like (ER/PgR/$HER2^-$, $CK5/6^+$, and/or $EGFR^+$), as previously described (24–26). Strikingly, loss of INPP4B expression was observed most frequently in the hormone receptor-negative subtypes, basal-like (7/8, i.e., 88%) and $HER2^+$ (3/8, i.e., 38%) and less frequently in the hormone receptor-positive subtypes, luminal B (2/9, i.e., 0.22%) and luminal A (6/72, i.e., 8%) (Fig. 5*C*).

These results were independently validated by assessing INPP4B immunostaining in a more extensive cohort of 267 invasive ductal carcinomas drawn from the St. Vincent's Campus Outcome Cohort (SVCOC) (25–27). INPP4B protein expression was lost in 71/267 (27%) of all tumors analyzed from this dataset, associated with loss of both ER and PgR expression ($P < 0.0001$) and positively correlated with CK5/6 ($P = 0.0003$), but not HER2 amplification ($P > 0.999$) (Table 1). Again, INPP4B expression was lost most frequently in basal-like carcinomas (24/30, i.e., 80%, $P < 0.0001$) and infrequently in luminal A carcinomas (16/158, i.e., 10%, $P < 0.0001$) (Fig. 5*C*). The luminal A subtype is the least aggressive tumor type, associated with the best patient prognosis (15, 16, 28, 29), whereas basal-like breast carcinomas are in general aggressive, exhibiting high histologic tumor grade and mitotic index (30). Consistent with this phenotype, loss of INPP4B expression positively correlated with high tumor grade ($P < 0.0001$) and size >20 mm ($P = 0.047$) and expression of proliferation markers, *Ki*-67 ($P = 0.003$) and cyclin E1 ($P = 0.0001$), and was inversely associated with the cell cycle inhibitor, p27 ($P = 0.0055$) (Table 1). Interestingly, there was no correlation between INPP4B loss and axillary lymph node involvement ($P = 0.89$), indicating that INPP4B may be a regulator of tumor cell proliferation and growth, but not metastasis.

Association of INPP4B Protein Loss with Altered Parameters of the PI3K Pathway. The incidence of INPP4B loss of expression in breast cancers, as reported here, is comparable to those reported for *PIK3CA* mutations (7–40%) (26, 31–36) or loss of the tumor suppressor PTEN (13–37%) (26, 37–39). To date, most studies indicate that alterations to *PIK3CA* and PTEN in human cancers occur in a mutually exclusive manner (39–42). We evaluated whether loss of INPP4B was associated with alterations in other components of the PI3K pathway in 267 tumors from the SVCOC dataset that had been previously assessed for *PIK3CA* amplification/mutation, Akt hyperactivation, or PTEN loss (26). Notably, loss of INPP4B protein did not correlate with *PIK3CA* mutation/amplification ($P = 0.54$), indicating that these events are mutually exclusive in breast cancer (Table 1). In contrast, INPP4B protein expression was lost in 49% (36/73) of PTEN-null tumors, compared with 14% (25/185) of PTEN-expressing tumors, revealing a significant positive correlation between INPP4B deficiency and PTEN loss ($P < 0.0001$). Whereas high pAkt alone did not significantly correlate with INPP4B loss ($P = 0.493$), PTEN null tumors that showed high pAkt frequently exhibited INPP4B deficiency ($P < 0.0002$) (Table 1). Therefore, concomitant loss of both PTEN and INPP4B may contribute to Akt hyperactivation and the development of aggressive forms of breast cancer.

Table 1. Association analysis of INPP4B protein loss with clinicopathologic, cell cycle, and PI3K pathway variables in human breast cancers (MCCS and SVCOC datasets)

	MCCS			SVCOC			
Variable	Total (n = 107)	INPP4B loss (n = 22)	P value	Total (n = 267)	INPP4B loss (n = 71)	P value	Nature of association
Clinical							
Histological grade 3	34	12 (35%)	0.018	123	48 (39%)	<0.0001	Positive
Size (>20mm)	-	-	-	101	36 (33%)	0.047	Positive
LN status (positive)	-	-	-	119	31 (26%)	0.89	Nil
Receptors/Markers							
ER status (positive)	68	5 (7%)	<0.0001	178	19 (11%)	<0.0001	Negative
PgR status (positive)	56	3 (5%)	<0.0001	150	11 (7%)	<0.0001	Negative
HER2 amplification	26	8 (31%)	0.17	47	11 (23%)	>0.999	Nil
CK5/6 (positive)	6	5 (83%)	0.0014	62	28 (45%)	0.0003	Positive
EGFR (positive)	7	3 (43%)	0.06	-	-	-	Positive*
Cell Cycle							
Ki-67 (high)	-	-	-	123	40 (33%)	0.0003	Positive
Cyclin E1 (high)	-	-	-	66	29 (44%)	0.0001	Positive
p27 (high)	-	-	-	113	18 (16%)	0.0055	Negative
PI3K pathway							
PIK3CA amp/mut	-	-	-	26	8 (31%)	0.54	Nil
PTEN loss	-	-	-	73	36 (49%)	<0.0001	Positive
pAkt > mean	-	-	-	62	17 (23%)	0.49	Nil
PTEN loss/pAkt >mean	-	-	-	17	11 (65%)	0.0002	Positive

LN, axillary lymph node involvement; ER, estrogen receptor; PgR, progesterone receptor; CK, cytokeratin; EGFR, epidermal growth factor receptor 1; amp, amplified; mut, mutated; pAkt, phospho-Akt.
*Approaching significance.

Discussion

This study identifies INPP4B as a previously undescribed regulator of ER-positive normal and breast cancer cell proliferation and tumor growth. In the normal breast INPP4B is expressed predominantly in ER-positive cells that are nonproliferative. INPP4B protein knockdown in ER-positive breast cancer cells enhances baseline and EGF-dependent Akt phosphorylation, cell proliferation, anchorage independent cell growth, and xenograft tumor formation. Conversely, in INPP4B-null, ER-negative MDA MB 231 cells, reintroduction of this PtdIns(3,4)P_2 4-phosphatase inhibits EGF-dependent Akt signaling and suppresses cell growth in soft agar.

Our study is unique in investigating and reporting a correlation between INPP4B protein expression and clinicopathologic parameters in human breast cancer. Gene array studies have predicted a correlation between *INPP4B* expression and hormone receptor status in human breast cancer (43, 44), and the studies presented here support this association at the protein level. The nature of the relationship between ER and INPP4B, however, remains elusive. Although PI3K can negatively regulate ER protein levels by decreasing ER protein stability (45), we noted that INPP4B protein knockdown in ER-positive MCF-7-luc-F5 cells, or reconstitution in ER/INPP4B-negative MDA MB 231 cells, did not affect ER protein expression (Fig. S5 *A* and *B*). Similarly we have demonstrated estrogen stimulation of MCF-7 cells, or treatment with ER antagonists does not affect the protein levels of INPP4B (Fig. S5*C*), despite gene array studies indicating *INPP4B* transcription may be enhanced by estrogen stimulation (46). Therefore the nature of the relationship between INPP4B and ER is likely to be complex, possibly involving signaling derived from stromal tissue (47).

We report that INPP4B protein expression is lost in 84% of human basal-like breast carcinomas, which are generally highly aggressive with poor clinical outcome and are frequently associated with breast cancer 1 (*BRCA1*) gene mutations (30). Basal-like carcinomas are defined as exhibiting basal cytokeratin and EGFR expression and low or absent expression of hormone receptors (30, 48). Our studies indicate that absence of INPP4B protein may be an additional marker of the basal-like carcinoma signature profile. LOH of *INPP4B* has recently been reported to occur in 55% of human ER/PgR/HER2 triple-negative breast cancers (12); however, this report did not assess expression of basal markers to discriminate between triple-negative and basal-like cancers or investigate INPP4B protein expression in breast cancer subtypes, as reported here. The discrepancy observed between the frequency of *INPP4B* LOH previously reported (55%) (12) and loss of INPP4B protein expression presented here (84%) in basal-like breast cancers suggests alternative mechanisms for INPP4B protein loss in addition to gene deletion. Interestingly, *INPP4B* transcription has recently been shown to be influenced by gene hypermethylation (47). Our studies suggest that suppression of INPP4B protein expression specifically in basal-like breast cancers may contribute to the aggressive nature of this breast cancer subtype.

Like INPP4B, expression of the tumor suppressor and 3-phosphatase, PTEN, is also frequently lost in basal-like carcinomas (26, 39). Notably, we report that concomitant loss of both INPP4B and PTEN proteins is commonly observed in human primary breast cancers. Concurrent PTEN and INPP4B absence may result in the accumulation of their respective phosphoinositide substrates, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, which together promote maximal Akt activation. We report that in human breast cancers loss of INPP4B is frequently associated with PTEN-null tumors exhibiting high pAkt, although loss of INPP4B alone did not show such an association with high pAkt. This is of interest as we, and others (12), have noted only a modest increase in Akt activation following INPP4B knockdown in response to cell stimulation (1.2–1.4-fold). Therefore INPP4B may not regulate the amplitude of Akt activation following cell stimulation but rather the duration of the signal. Consistent with this contention we noted elevated pAkt/Akt in serum-starved INPP4B-depleted cells, conditions under which Akt activation is normally minimal. Although recent studies report that knockdown of INPP4B and PTEN together in immortalized normal human mammary epithelial cell lines leads to cellular senescence (12), it is likely that in human cancers loss of INPP4B and PTEN occurs during tumor progression in combination with additional mutations. Indeed, knockdown of the tumor suppressor TP53 in INPP4B/PTEN-deficient HMECs rescues the senescence phenotype (12).

In summary, the studies reported here identify INPP4B as a putative tumor suppressor that functions to regulate PI3K signaling and ER-positive mammary cell proliferation. In addition, we provide evidence that loss of INPP4B is a unique marker of human basal-like carcinomas. This study reports the concurrent loss of two phosphoinositide phosphatases in human breast cancer and provides evidence for the cooperative promotion of oncogenesis through alterations to multiple components of the PI3K signaling pathway. There are currently no directed therapies for the treatment of human basal-like cancers and tumors exhibiting loss of INPP4B protein with or without concurrent PTEN loss may represent ideal candidates for treatment with PI3K pathway inhibitors.

Materials and Methods

Tumorigenicity in Mice. For xenograft tumor growth assays, all procedures involving mice were approved by the animal ethics committee at the Monash University School of Biomedical Sciences (SOBSB/B/2008/14) and were established and analyzed as described in *SI Materials and Methods*.

Patients, Breast Carcinoma Samples, and INPP4B Expression Analysis. Ethics approval was obtained from the Standing Committee on Ethics in Research Involving Humans, Monash University (CF08/1142–2008000564) for all human tissues and cognate clinicopathological data used in this study. Details of the human breast cancer cohorts and analysis of INPP4B are described in *SI Materials and Methods*.

Further details of materials and methods can be found in *SI Materials and Methods*.

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