Phosphoinositide phosphatases are important regulators of signaling pathways relevant to both diabetes and cancer. A 3'-phosphoinositid phosphatase, phosphatase homologous to tensin (PTEN), is both a tumor suppressor and a negative regulator of insulin action. A 5'-phosphoinositid phosphatase, SH2-containing 5'-inositol phosphatase (SHIP2), regulates insulin signaling and its genetic knockout prevents high-fat diet-induced obesity in mice. SHIP2 also regulates cytoskeleton remodeling and receptor endocytosis. This and the fact that both PTEN and SHIP2 act on the same substrate suggest a potential role for SHIP2 in cancer. Here we report that, in direct contrast to PTEN, SHIP2 protein expression is elevated in a number of breast cancer cell lines. RNA interference-mediated silencing of SHIP2 in MDA-231 cells suppresses epidermal growth factor receptor (EGFR) levels by means of enhanced receptor degradation. Furthermore, endogenous SHIP2 in MDA-231 breast cancer cells supports in vitro cell proliferation, increases cellular sensitivity to drugs targeting the EGFR and supports cancer development and metastasis in nude mice. In addition, significantly high proportions (44%; \(P = 0.0001\)) of clinical specimens of breast cancer tissues in comparison with non-cancerous breast tissues contain elevated expression of SHIP2 protein. Taken together, our results demonstrate that SHIP2 is a clinically relevant novel anticancer target that links perturbed metabolism to cancer development.

Materials and methods

Materials

All cell lines were from American Type Culture Collection (Manassas, VA) except HME-50 cells which were from Dr Jerry Shay, University of Texas Southwestern Medical Center, Dallas, TX. Rabbit polyclonal antibodies against human SHIP2 (amino acids 1105–1213) and EGFR (22,23) were generous gifts from Stuart J.Decker (University of Michigan, Ann Arbor, MI). SHIP2 antibody was raised against the C-terminus region of SHIP2 and has been previously employed for specific detection of SHIP2 protein in immuno-fluorescence, immunoblot and immunoprecipitation assays (17,23). Retroviral vectors with cloned short hairpin RNA (shRNA) for human SHIP2 gene (GenBank: NP_001567) and for enhanced green fluorescent protein (EGFP) were obtained from Open Biosystems (Huntsville, AL). A recombinant human wild-type SHIP2 [with C-terminus FLAG tag (17)] was rendered RNAi resistant by introducing six silent mutations in the 22-nucleotide target sequence (Bio-Means, Sugar Land, TX). Monoclonal antibodies against phosphotyrosines (4G10) and vinculin were from Upstate Biotechnologies (Charlottesville, VA) and c-Cbl antibody was from Transduction Labs (Huntsville, AL). Antibodies specific for PTEN were from Cell Signaling Technologies (Danvers, MA). Monoclonal b-actin antibody was from Sigma-Aldrich (Saint Louis, MO). A cell quantification assay kit was from Promega (Madison, WI). PD 153035 was from Tocris Biosciences (Ellisville, MO) and AG 1478 and Hsp-90 inhibitor 17-AAG were from LC laboratories (Woburn, MA). Target retrieval reagent (pH 6.0) was from DakoCytomation (Carpinteria, CA), biotinyl tyramide signal amplification kit was from Perkin Elmer (Wellesley, MA) and aminoelethyl carbamazole was from Zymed (Carlsbad, CA). Paraflin-embedded, formalin-fixed
breast cancer and normal tissues were from the archives of Indiana University School of Medicine (Indianapolis, IN), cooperative human tissue network or from Imgenex (San Diego, CA).

**Cell culture**

Mammary carcinoma cell lines were cultured in modified Eagle medium [with t-glutamine/10% fetal bovine serum (FBS)], HeLa cells in Dulbecco’s modified Eagle’s medium/10% FBS and MCF-10A cells in Dulbecco’s modified Eagle’s medium/F-12 with 10 ng/ml EGF, 0.5 mg/ml hydrocortisone, 5 mg/ml insulin and 5% FBS. MDA-231 cells also received 10-3 M insulin. HME-50 cells were maintained in mammary epithelial cell growth medium (Cambrex/ BioWhittaker, East Rutherford, NJ). Cell counting was carried out using a hemacytometer in the presence of trypan blue.

For monolayer clonogenic assays, cells were plated at 500 cells per 100 mm dish or 150 cells per 60 mm dish and cultured in complete medium (with 10% FBS) for 13–15 days. Colonies were stained directly with SureStain (Fisher Scientific, Hampton, NH). In some clonogenic assays, instead of staining the colonies, cells were harvested by trypsinization and counted in a hemacytometer. 4′,6-diamidino-2-phenylindole staining was carried out as described previously (17,19).

**Generation of RNA stable clones**

A retroviral packaging cell line, phoenix, was transfected with vectors harboring cloned shRNA for human SHIP2 gene or EGF (for use as control) by calcium phosphate method of transfection (Stratagene, La Jolla, CA). Forty-eight hours after transfection, virus-rich supernatant was collected and used to infect MDA-231 cells. Single-cell clones were derived from pools of puromycin-resistant cells with SHIP2 shRNA or EGF shRNA and analyzed for SHIP2 silencing by western blottting. SHIP2 silencing was stable for at least 10–12 (8–10 weeks) passages from the time of their isolation at the single colony stage.

**Western blot analyses**

Preparation of whole-cell lysates in a Triton-X100 lysis buffer as well as protein analyses by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot assays were performed as described earlier (18).

**Cell cycle analyses**

(i) Staining of whole cells with propidium iodide—harvested cells were fixed in 75% ice-cold absolute ethanol and stained with propidium iodide (50 "g/ml) resistant cells with SHIP2 shRNA or EGFP shRNA and analyzed for SHIP2 silencing by western blotting. SHIP2 silencing was stable for at least 10–12 (8–10 weeks) passages from the time of their isolation at the single colony stage.

**Drug response assays**

Cells were incubated with 1% FBS containing medium for 3 h followed by treatment with indicated inhibitor compounds at 0.5 mM concentration [all were at 1:1000 dilution of 0.5 mM stock solution in dimethyl sulfoxide] for 3 days. The vehicle dimethyl sulfoxide was used at 1:1000 as control. After 3 days, cells were fixed with 4% paraformaldehyde in PBS (10 min) followed by permeabilization with 0.2% Triton X-100 in PBS for 30 min. Cells were then stained with 4′,6-diamidino-2-phenylindole staining was carried out as described previously (17,19).

and any discrepancies were resolved through discussion and re-examination. Puromycin-resistant cells harboring shRNA were isolated by digesting tumor tissues with collagenase (300 U/ml) and hyaluronidase (100 U/ml) for 5 h at 37°C.

**Immunohistochemistry**

Tissue sections were stained with SHIP2 antibody (23) using a standard protocol as follows: deparaffinization; target retrieval (pH 6.0) at 95°C for 10 min; sequential blocking steps with 3% hydrogen peroxide (10 min), avidin–biotin (Vector Laboratories, Burlingame, CA, 15 min) and the blocking solution (30 min) and incubation with the primary antibody (polyclonal SHIP2 or normal rabbit serum) at 4°C overnight, biotinylated goat anti-rabbit IgG for 1 h followed by streptavidin-conjugated peroxidase for 30 min. Signal, amplified using biotinyl tyramide signal amplification kit, was detected with aminothiol carbamazole and counterstained with hematoxylin. Staining was scored independently by two individuals and confirmed by a board-certified pathologist. SHIP2 expression was scored on a scale of 0 (absence) to 4+ (intense and extensive) and for the purpose of statistical analysis, SHIP2 status was classified into three groups as low (1+), median (2+) and high (3+) to evaluate the link between SHIP2 expression and cancer. Anti-SHIP2 (against the C-termi

**Statistical analyses**

Two-sided Fisher’s exact test was used to evaluate the significance of SHIP2 expression and cancer in the immunohistochemistry studies. A P value of <0.05 is considered significant. For other comparisons, t-test was used to derive a two-tailed P value.

**Results**

SHIP2 is expressed at high levels in breast cancer cell lines

SHIP2 messenger RNA is ubiquitously expressed and its protein levels are thought to be regulated at the post-transcriptional level (26). Therefore, using a previously characterized polyclonal SHIP2 antibody (23), we examined SHIP2 protein expression in normal and cancer cell lines of the breast by western analyses. As previously observed in HeLa cervical carcinoma cells (17), SHIP2 protein is easily detectable in MDA-231, SKBR-3, MDA-468, MDA-436, MCF-7 and ZR-75 breast cancer cells (Figure 1A). In contrast, SHIP2 levels in non-transformed HME-50 and MCF-10A cells are many folds lower (ranged from ~6- to 18-fold for HME-50 and from ~2- to 5-fold for MCF-10A, when normalized to β-actin levels) than those of cancer cells (Figure 1A).

SHIP2 is tyrosine phosphorylated by EGF in breast cancer cells

As SHIP2 is tyrosine phosphorylated by various growth factors including EGF (23), and given that SHIP2 in HeLa cells regulates ligand-induced EGRF turnover via interactions with c-Cbl ubiquitin ligase (19), we examined whether SHIP2 in breast cancer cells is involved in EGF signaling pathway. Four of the five SHIP2 over-expressing cells also express high levels of EGFR (Figure 1B), suggesting a possible link between SHIP2 levels and EGFR over-expression. SHIP2 is tyrosine phosphorylated by EGF and is constitutively associated with c-Cbl in MDA-231 breast cancer cells (Figure 1C). EGF after 15 min of exposure (not after 5 min) appeared to decrease the SHIP2-Cbl association. Similar SHIP2 tyrosyl phosphorylation and SHIP2-Cbl association were also detected in MDA-468 and SKBR-3 breast cancer cells (data not shown). This suggests that SHIP2 in breast cancer cells, as in HeLa cells, may play a role in the regulation of EGFR turnover via Cbl-mediated down-regulation pathway (19).

SHIP2 silencing suppresses EGF-induced signaling and decreases EGFR levels

In order to understand the role of endogenously over-expressed SHIP2 in cancer cells, we employed the RNA interference approach of gene silencing. As studies utilizing the exogenously over-expressed SHIP2
with control cells harboring EGFP shRNA (Figure 2A). Since our studies suggested a potential role for SHIP2 in EGFR functioning in breast cancer cells (Figure 1B and C) and due to the fact that SHIP2 regulates EGFR endocytosis and ligand-induced degradation in HeLa cells (19), we questioned whether SHIP2 silencing in MDA-231 cells affected the turnover of the EGFR. SHIP2 shRNAcontaining MDA-231 cells showed substantial reduction in the levels of EGFR (Figure 2B, supplementary Figure 1A and B is available at Carcinogenesis Online). When treated with EGF, SHIP2 RNAi cells displayed further reduction in their EGFR levels. The extent of EGFR degradation was higher (44 and 57%) in SHIP2 RNAi cells when compared with control shEGFP cells (33 and 18%) after 1 h of 50 ng/ml or 3 h of 5 ng/ml EGF treatment, respectively (Figure 2B). As proteosome function is critical for initiation of EGFR internalization (27), treatment with proteasomal inhibitor MG132 (25 μM) increased EGFR levels in the untreated SHIP2 RNAi cells and prevented EGF-induced receptor degradation (Figure 2C, supplementary Figure 1B is available at Carcinogenesis Online). This confirms that decreased EGFR levels are due to enhanced internalization and subsequent proteolysis.

**SHIP2 re-expression restores EGFR levels**

Exogenous expression of wild-type SHIP2 that has been rendered resistant to RNAi (by introducing mismatches in the form of silence mutations at six of the nucleotides in the target sequence) was then used to confirm the direct role of SHIP2 function in regulating the EGFR levels. Detection of this version of wild-type SHIP2 is facilitated by the presence of FLAG epitope on the C-terminus. Two days after transfection, re-expression of wild-type SHIP2 modestly elevated EGFR levels by ~20% but substantially decreased EGF-induced degradation (Figure 3A). Furthermore, EGFR levels were increased by at least 2-fold as SHIP2 silencing effect diminished upon long-term culturing (>20 passages) of SHIP2 shRNA clones, demonstrating that EGFR levels are determined by the magnitude of SHIP2 expression (Figure 3B).

**Silencing of SHIP2 gene expression in MDA-231 cells is anti-proliferative**

Given the profound effects on EGFR levels by SHIP2, we closely monitored the cancer traits of these cells. Stable SHIP2 RNAi cells were slow growing and displayed significantly decreased cell numbers with no appreciable cell death as measured by standard cell counting (supplementary Figure 2 is available at Carcinogenesis Online) and a tetrazolium (MTT) compound-based cell quantification assay (Figure 4A). This apparent inhibition of cell proliferation of SHIP2 RNAi cells, as compared with EGFP RNAi cells, significantly correlated with lower proportion of cells in S phase (~25% less) with concomitant increase in the number of cells in G1 phase (Figure 4B). BrdU (10 μM) labeling confirmed decreased DNA synthesis upon SHIP2 RNAi as SHIP2 shRNA clones contain 30–35% fewer BrdU-positive cells subsequent to 1 h labeling (supplementary Figure 3 is available at Carcinogenesis Online). After a 24 hour chase following a 30 min pulse labeling with BrdU, there were ~55% more of BrdU-positive cells in the G1-phase of SHIP2 RNAi cells than that of control RNAi cells, indicating a slower cell cycle progression upon SHIP2 silencing (supplementary Table 1 is available at Carcinogenesis Online).

We then examined the effect of SHIP2 knockdown on monolayer clonogenic ability of the cells. Although SHIP2 silencing did not significantly decrease the number of the colonies formed (supplementary Figure 4 is available at Carcinogenesis Online), it strikingly reduced the size of these colonies (Figure 4C). These smaller colonies formed by SHIP2 knockdown cells contained significantly fewer cells (40–50% that of control colonies), confirming that silencing of endogenous SHIP2 decreases cell proliferation (supplementary Figure 5 is available at Carcinogenesis Online).

**Therapeutic implication of SHIP2-induced alterations in EGFR levels**

Changes in EGFR levels and signaling upon SHIP2 silencing prompted us to question whether SHIP2 modifies the sensitivity of cancer
cells to EGFR inhibitors. MDA-231 cells with SHIP2 gene silencing showed a substantial increase (up to 60%) in the apoptotic response to EGFR inhibitors PD153035 (28) and AG1478 (29) at 0.5 μM, a concentration that produced only a marginal response (20%) in control cells (Figure 4D). 17-AAG, an Hsp-90 inhibitor that degrades multiple signaling molecules including EGFR (30), was equally effective regardless of SHIP2 expression levels. These results demonstrate the specificity of over-expressed SHIP2-induced effect on EGFR signaling and further establish the therapeutic implication of SHIP2 silencing.

Inhibition of in vivo tumor growth and spontaneous lung metastasis upon SHIP2 silencing

The importance of SHIP2 function for tumor growth was evaluated using orthotopic implantation approach in nude (nu/nu) mice. When injected into the mammary fat pads of nude mice (n = 14 per clone), tumors from SHIP2 RNAi cells appeared relatively slowly; every mouse in the control group developed tumors (mean size = 222 mg) at 6 weeks after injection as compared with one mouse (size = 75 mg) that received SHIP2 RNAi cells. Tumors that eventually grew from SHIP2 RNAi cells were significantly smaller (Figure 5A). SHIP2-silenced cells displayed nearly 3 week growth delay (T–C of 20.6 days at 750 mg evaluation size) and had a dramatically decreased extent (with modestly decreased incidence) of spontaneous lung metastases (Figure 5B). A majority of mice with tumors from control cells (8/14) displayed extensive lung metastases involving >25% of the lung area, whereas mice carrying tumors from SHIP2 RNAi cells contained either no detectable masses (2/13) or micromasses in a vast majority (8/13) (Figure 5C; one animal in the SHIP2 group died during the extended observation period explained below precluding proper collection of the tissues).

To test whether reduction in metastatic load is a function of smaller tumor size formed by SHIP2-silenced cells, four mice carrying small tumor masses (average size = 467 ± 91 mg) from this group were observed for an additional 5 week period (beyond the end of the study at 11th week). The tumor size in these animals at the end of this period (average size = 4267 ± 446 mg) was comparable with the control group at 11th week. Despite their large tumor size, two of the four animals showed mere micrometastases while the other two formed massive metastases (Figure 5C; denoted by *). While tumor size and/or duration of cancer growth undoubtedly influence the extent of metastasis, SHIP2 silencing alone (independent of tumor size) appear to reduce metastasis in two of the four animals. Puromycin-resistant cells isolated from the aforementioned tumor xenografts displaying SHIP2 suppression also contained significantly lower levels of EGFR.

Fig. 2. SHIP2 regulates EGFR turnover. (A) Stable RNAi-mediated silencing of SHIP2 in MDA-231 breast cancer cells—anti-SHIP2 western blot of three shSHIP2 (S2, S3 and S8) and two control shEGFP (E2 and E7) clones of MDA-231 breast cancer cells. Relative SHIP2 expression values normalized to β-actin signal are shown below the blot. (Average silencing efficiencies estimated from multiple experiments (n = 5) are clone S3, 50 ± 2%; clone S8, 57 ± 3% and clone S2, 54 ± 6%). (B) SHIP2 silencing enhances EGF-induced EGFR degradation. EGFR western blot of the protein extracts prepared from multiple co-cultured MDA-231 shRNA clones (S—shSHIP2; equal proportions of S2, S3 and S8 clones at plating and E—shEGFP; equal proportions of E2 and E7 clones at plating) were treated with EGF (none, 5 or 50 ng/ml for 1 or 3 h) as indicated. The membrane was reprobed with β-actin as loading control and EGFR expression was normalized to β-actin levels. EGF-induced changes in EGFR levels in relation to the untreated sample of each clone (black bars) is shown (as %; decrease is indicated by ‘minus’ sign) on the top of the bars. Similar results were also obtained with individual clones (supplementary Figure 2A and B is available at Carcinogenesis Online). (C) Proteasome inhibition prevents SHIP2 RNAi-induced changes in EGFR levels. EGFR western blot of protein extracts from MDA-231 shSHIP2 cells (clone #3) that were pre-treated for 60 min with vehicle dimethyl sulfoxide or proteasome inhibitor MG132 (25 μM) followed by treatment with EGF in the presence of vehicle or the inhibitor. β-Actin western blot of the same membrane is shown as loading control along with normalized densitometric values for EGFR signal. Results from two independent experiments are shown for the S3 shSHIP2 clone. Supplementary Figure 2B shows results for control shEGFP clone and for S8 shSHIP2 clone.
in western blot analyses. The graph shows the quantification of data where SHIP2 expression in acinar and ductal epithelial cells (Figure 6A and B).

We then set out to investigate the translational importance of SHIP2 over-expression in the cancer cells. To this end, we compared a small subset of breast cancer cell lines and clinical specimens of cancer tissues to determine the relative expression of SHIP2 and EGFR levels. This comparison is critical in understanding the role of SHIP2 in cancer cell biology.

To examine the potential role of SHIP2 in cancer development and progression, we used a novel breast cancer cell line model, for the first time, showing SHIP2 positively influences cancer cell proliferation and tumor development. Previously, we demonstrated a novel role for SHIP2 in actin cytoskeleton remodeling and in receptor endocytosis (17–19). Subsequent reports describing the interactions of SHIP2 with filamin (31), vinexin (32), and c-Met (33) further support the observation that regulation by SHIP2 of EGFR turnover in cancer cells may have implications for tumor progression. We envisioned a role for SHIP2 in cancer cell biology as altered cell adhesion and spreading properties are important in cancer development and progression (20,34).

Discussion

Our study in a breast cancer cell line model, for the first time, shows SHIP2 positively influences cancer cell proliferation and tumor development. Previously, we demonstrated a novel role for SHIP2 in actin cytoskeleton remodeling and in receptor endocytosis (17–19). Subsequent reports describing the interactions of SHIP2 with filamin (31), vinexin (32), and c-Met (33) further support the observation that regulation by SHIP2 of EGFR turnover in cancer cells may have implications for tumor progression. We envisioned a role for SHIP2 in cancer cell biology as altered cell adhesion and spreading properties are important in cancer development and progression (20,34).

As SHIP2 over-expression in cancer cells is functional (8,35) and given that SHIP2 is expressed at high levels (17,35), raising questions about its function as a negative regulator of PI3K signaling in the context of cancer development and progression (20,34). Additionally, regulation of endocytosis by SHIP2, via changes in receptor internalization/ degradation, has potential implications to cancer. Here, we experimentally demonstrate a novel, positive role for SHIP2 in cancer cell biology and metastasis. Our observation that many cancer cell lines and clinical specimens of cancer tissues express elevated levels of SHIP2 is counter-intuitive to the assumption that SHIP2 actions on PI3P down-regulates PI3K/Akt signaling, a well-established onco-genic pathway. HeLa and K562 cancer cells are shown previously to express SHIP2 at high levels (17,35), raising questions about its function as a negative regulator of PI3K signaling in the context of cancer. Exogenous over-expression of SHIP2 in cancer cells has produced contradictory effects on Akt activation and cell cycle progression precluding a clear understanding of SHIP2 role in cancer (8,16).

As SHIP2 expressed in cancer cells is functional (8,35) and given that SHIP2 may function differently in adhesion signaling in contrast to its role in insulin signaling (e.g. regulation of SHIP2 by Src downstream of integrin but not insulin) (18), it is possible that upon over-expression SHIP2 may impact an effect on cancer development that is different from its regulation of insulin function in normal physiological situations. We show here that regulation by SHIP2 of EGFR turnover is one such critical aspect of cell biology that supports cancer development. The genetic make-up of the cell such as activated EGFR is one such critical aspect of cell biology that supports cancer development. Additionally, regulation of endocytosis by SHIP2, via changes in receptor internalization/degradation, has potential implications to cancer. Here, we experimentally demonstrate a novel, positive role for SHIP2 in cancer cell biology and metastasis. Our observation that many cancer cell lines and clinical specimens of cancer tissues express elevated levels of SHIP2 is counter-intuitive to the assumption that SHIP2 actions on PI3P down-regulates PI3K/Akt signaling, a well-established onco-genic pathway. HeLa and K562 cancer cells are shown previously to express SHIP2 at high levels (17,35), raising questions about its function as a negative regulator of PI3K signaling in the context of cancer. Exogenous over-expression of SHIP2 in cancer cells has produced contradictory effects on Akt activation and cell cycle progression precluding a clear understanding of SHIP2 role in cancer (8,16).

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It is quite possible that SHIP2 may exert a broad effect on vesicular trafficking in general and may have yet unknown biological consequences. By modulating EGFR turnover, inhibition of SHIP2 increases the effectiveness of EGFR inhibitors, validating EGFR as an important and specific molecule altered by SHIP2 function (Figure 4D). As SHIP2 silencing leads to defects in trafficking of other membrane receptors, perhaps independent of Cbl (e.g. transferrin receptor (19), EphA2 (36)), a potential SHIP2-mediated regulation of additional oncogenic receptors in effect, may broaden the pro-oncogenic mechanisms of SHIP2 over-expression. However, in our study model, we failed to detect significant alterations in the steady state levels of two other receptor tyrosine kinases, EphA2 and IGF-1R (supplementary Figure 6 is available at Carcinogenesis Online). These and perhaps other oncogenic receptors may still be regulated by SHIP2 in a ligand-dependent manner as recently shown for EphA2 (36) and may be of relevance when these ligands are present in sufficient concentrations to drive the cancer development.

The effect of decreased EGFR levels on EGF-induced mammalian target of rapamycin and extracellular signal-regulated kinase activations upon SHIP2 silencing was minimal (data not shown). Activated

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**Fig. 4.** Reduced SHIP2 level inhibits cell proliferation in culture. (A) Time course study of the effect of SHIP2 silencing on cell proliferation. Cells plated in 96-well plate (500 cells per well) were assayed for a period of 5 days for cell numbers using a tetrazolium compound-based assay (Promega CellTiter 96®). Difference in cell numbers is significant (P < 0.001, t-test) both at day 3 (denoted by *) and day 5 (denoted by **) in SHIP2-silenced clones when compared individually to two different control clones (shEGFP). ‘NS’ on day 1 bars indicates that the difference is not significant. (B) SHIP2 RNAi alters cell cycle distribution. DNA histograms of shRNA clones cultured for 5 days in 10% FBS containing medium at a seeding density of 50 000 cells per 100 mm dish. Cell cycle distribution was quantified using ModFit LT v2.0 and average percentage of cells in G1, S and G2/M phases are shown below the histogram (derived from five independent experiments, n = 5). Two-tailed P values per t-test are shown when it is significant. (C) Monolayer clonogenic assay. Stable shRNA clones specific for SHIP2 (S) or control (EGFP; E) MDA-231 cells were plated at a density of 500 cells per 100 mm dish in FBS containing medium and cultured for 14 days. Colonies thus formed were fixed and stained as described in Materials and Methods. The difference in the colony size was quantified by digitally measuring the diameter of the colonies (20 colonies per dish). Average colony sizes shown in the graph are derived from a total of six observations conducted as three separate experiments in duplicates (n = 3, P < 0.0001, t-test). The experiment was reproduced with similar results at least 10 times with varying cell numbers and culture conditions. E27 is a co-cultured population of clones E2 and E7 and S238 is a co-cultured population of clones S2, S3 and S8 (similar to Figure 2C). (D) SHIP2 silencing enhances sensitivity to EGFR inhibitors. Cellular response to anticancer drugs was evaluated by quantifying nuclear condensation and fragmentation of 4′-6-diamidino-2-phenylindole-stained cells after 3 days of treatment with 0.5 μM of indicated inhibitor compounds (PD153035 and AG1478 are inhibitors of EGFR, whereas 17-AAG is an Hsp-90 inhibitor) and expressed as percentage of apoptosis. Cells (50–100) were evaluated per sample and the values shown are mean of three separate experiments and the error bars represent standard error of mean. P values derived by standard t-test are imprinted on top of the bars and ‘NS’ represents statistically ‘non-significant’ differences.
K-Ras present in MDA-231 cells appears to be responsible for the high basal levels of activations of extracellular signal-regulated kinase and mammalian target of rapamycin in an EGF-independent manner (37, 38). Urokinase type plasminogen activator is also shown to be responsible for the activation status of extracellular signal-regulated kinase in MDA-231 cells in an autocrine way (39). Effect of SHIP2 suppression on EGF-induced Akt activation is currently under investigation and is probably to be complex.

Although SHIP2 knock down potentially elevates PIP3 levels consequently increasing the Akt activation, decreased EGFR levels seen in these cells could oppose this effect owing to decreased PI3K activation and lower PIP3 levels. In addition, decreased formation of phosphatidylinositol-3,4-bisphosphate due to lack of SHIP2 may also contribute to decreased Akt activation as phosphatidylinositol-3,4-bisphosphate is essential for full activation of Akt both in vitro and in vivo (6, 7).

**Fig. 5.** SHIP2 is important for in vivo tumor growth and metastasis. (A) SHIP2 silencing suppresses tumorigenesis in vivo. One million MDA-231 cells of control shEGFP clone or shSHIP2 clone were implanted in the mammary fat pads of 7- to 8-week-old nude mice. Average estimated tumor mass values are shown for 14 mice in each group. P value (two-tailed t-test) < 0.0001 for all data pairs from 7 to 11 week. *Denotes that there was only one animal with a tumor size of 75 mg at 6 week in SHIP2 shRNA group. (B) Reduced lung metastases upon SHIP2 silencing: representative images of lung tissues from two animals of each group of mice with mammary tumors developed from shRNA clones. Black arrows point to the metastatic lesions. (C) Scoring for the presence and extent of spontaneous lung metastases: 0, negative; 1, micro with few isolated cells; 2, macro masses covering < 25% of lung area and 3, macro masses covering > 25% of lung area. *Denotes that two mice in each of these groups allowed to grow for five additional weeks than controls. (One mouse died during the extended observation period due to non-tumor related cause leaving only 13 lung specimens in SHIP2 RNAi group.) (D) Suppression of SHIP2 in cells derived from tumor xenografts—correlation with lower EGFR receptor levels. Cells derived from tumors of MDA-231 breast cancer cells developed by shSHIP2 (149-1, 149-2, 152-1, 152-2) or control shEGFP (155-1) clones were cultured in the presence of puromycin for 7-10 days following the excision of tumors. Protein extracts from these freshly derived cells from the tumors as well as the pre-implantation in vitro cultured cells ("parental cells") were analyzed for SHIP2 levels using a specific SHIP2 antibody in a western blot. Same membrane was reprobed with an antibody against EGFR receptor (EGFR) and β-actin (loading control). The normalized expression values for SHIP2 and EGFR are shown in the graph.
Functional interaction between PTEN and SHIP2, two inositol phosphatases acting on PIP3 is another interesting question of high relevance to oncogenesis. In prostate cancer cells, SHIP2 function is required for basal Akt phosphorylation in PTEN-positive PNT2 and P4E6 cell lines (40). PTEN levels remained largely unaffected upon SHIP2 silencing in MDA-231 cells, indicating the lack of a compensatory response at least at the protein levels (supplementary Figure 1A is available at Carcinogenesis Online). In another study (N. Prasad, M. Tandon, G. Moore, C. Babbs, and S. Bose, submitted for publication), we show that the expression of SHIP2 is unrelated to the PTEN status of the breast cancer cells and primary cancer tissues and that SHIP2 silencing in a PTEN-negative but EGFR-amplified MDA-468 breast cancer cells suppresses cell proliferation. Interestingly, SHIP2 silencing decreases EGFR levels and enhances EGF-induced receptor degradation in MDA-468 cells (supplementary Figure 7 is available at Carcinogenesis Online). Besides, allelic loss of PTEN, although frequent in many types of cancer, is less frequent in breast cancers leading to alternate hypotheses for overcoming its tumor suppressor function (10,41). Given the shared substrate preference between two enzymes, we suggest, dominance exerted by high levels of SHIP2 (induced by yet unknown mechanisms) over co-existing PTEN to dephosphorylate PIP3 could divert the signaling from PTEN-mediated down-regulation of PI3K/Akt pathway to SHIP2-mediated pro-oncogenic pathway (e.g. potentiation of EGFR function). Thus, elevated expression of SHIP2 can, we propose, act as a novel molecular mechanism where PTEN tumor suppressive function can be circumvented to facilitate oncogenesis and metastasis.

While SHIP2 RNA expression in most tissues of mouse was detected by in situ hybridization approaches, no such information is currently available for the protein. As detected in western blots, in vitro-cultured non-transformed cell types (pre-adipocyte, myoblast, fibroblast) typically contain very low levels of SHIP2 protein (N. Prasad, unpublished data, (23,31)), whereas crude extracts of liver, fat and skeletal muscle are shown to contain detectable SHIP2 protein (15). Clearly, more studies are needed to examine the distribution of SHIP2 protein in various cell types and tissues of different organs. To the best of our knowledge, this is the first report on relative SHIP2 protein expression in the mammary gland.

Mutations in the SHIP2 gene are known to occur in the context of diabetes (42). These mutations are suggested to cause SHIP2

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**Fig. 6.** Immunohistochemical analysis of SHIP2 expression in primary breast cancers. (A) Representative images of SHIP2 staining: formalin-fixed sections of normal or mammary carcinoma tissue were stained with control rabbit serum or polyclonal anti-SHIP2 serum. Images of breast tissues stained with a control rabbit serum or polyclonal anti-SHIP2 antibody are shown at ×60 magnification. (a) Normal mammary gland stained with control rabbit serum, (b) normal mammary gland stained with anti-SHIP2. (c-e) breast cancer tissues stained with anti-SHIP2 and (f) breast cancer tissue stained with control rabbit serum. (b and c) Show a low level staining (1+), (d) displays a moderate level staining (2+) and (e) is an example of high level staining (4+) for SHIP2. (B) Comparison of SHIP2 expression levels between normal and cancer tissues. A normal and a mammary carcinoma tissue were stained with control rabbit serum or polyclonal anti-SHIP2 serum. Images are shown at ×40 magnification. (C) Antigenic competition of SHIP2 staining: breast cancer specimens stained with polyclonal SHIP2 antibody in the absence (left panel) or presence (right panel) of excess soluble antigen in the form of purified recombinant full-length human SHIP2 protein. Images shown are at ×60 magnification. (D) Summary of immunohistochemical analyses of SHIP2 expression in normal and cancerous breast tissues. SHIP2 expression was scored on a scale of 0 (absence) to 4+ (intense and extensive). The pilot study (n = 20) included five normal and 15 cancerous tissues. The tissue microarray (TMA; Imgenex) contained 10 normal and 50 cancerous tissues. *P = 0.0001 as per Fisher’s exact test indicating that association between high SHIP2 expression and cancer is statistically significant.
up-regulation through protein stabilization, although such mutations are yet to be found in cancers. SHIP2 expression is known to be altered by diet. SHIP2 protein level in skeletal muscle and adipose tissues increases when normal mice are fed on a high-fat diet and SHIP2 protein level in the aforementioned tissues of obese db/db strain of mice is higher than those found in heterozygous littermates (15). Resistance to obesity observed in SHIP2 knockout mice (12), considering the insulin actions of energy conservation in the peripheral tissues, SHIP2 appears to be necessary for fat storage during energy abundance. Although the molecular mechanisms are yet to be elucidated, resistance to obesity in SHIP2+/− mice is suggested to be due to unchecked insulin functions at the central nervous system promoting greater energy expenditure (43). In any case, SHIP2 presents an intriguing link between diet/energy metabolism and cancer. Obesity is a recognized risk factor for the development of cancers of many organs including endometrium, breast, gastrointestinal tract, kidney, pancreas and prostate (44). This is an important issue given the epidemic proportion of obesity in the developed world (45). Molecular mechanisms underlying this risk are not yet clear; however, studies suggest adipocytes-secreted estrogen may play a major role as the aromatase-mediated conversion of adrenal androstenedione into estrone by adipose tissue is an important source of estrogen for the postmenopausal woman (46). Cross-talk between estrogen and EGFR is an important mechanism of therapeutic importance (47) and SHIP2 up-regulation in response to obesity/high-fat diet perhaps provides additional mechanism to prolong/sustain this estrogen-activated EGFR function (19). Independent of EGFR, SHIP2 may directly promote invasiveness through cytoskeleton remodeling and the regulation of cell adhesion and lamellipodia formation during spreading on collagen (18,19). Alternatively, as disease progresses, SHIP2 regulation of oncogenic receptors may become a dominant underlying molecular event.

Targeted suppression of SHIP2 is being pursued as a therapeutic approach for diabetes and more recently for obesity (4). Our data describes SHIP2 as a cancer target with high therapeutic index given its over-expression in cancer and its dispensability for normal cell survival (12). While this manuscript was in preparation, a positive role for the tyrosine phosphatase PTP-1b in Her2-mediated oncogenesis was reported (48,49). PTP1-b negatively regulates insulin signaling whose genetic knockout in mice prevents high-fat diet-induced obesity, a phenotype similar to that of SHIP2 knockout (50). Thus, SHIP2 and PTP-1b may constitute a new class of drug targets with applications for both obesity/diabetes and cancer.

Supplementary material
Supplementary Figures 1–7 and Table 1 can be found at http://carcin.oxfordjournals.org/

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References


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