

SHIP2 phosphoinositol phosphatase positively regulates EGFR-Akt pathway, CXCR4 expression, and cell migration in MDA-MB-231 breast cancer cells

NAGENDRA K. PRASAD

Department of Basic Medical Sciences and Purdue Cancer Center, Purdue University, West Lafayette, IN 47907, USA

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Abstract. The phosphoinositol phosphatase SHIP2 is an important regulator of energy metabolism. SHIP2 dephosphorylates phosphatidylinositol 3,4,5 trisphosphates which are critical second messengers in signaling pathways induced by various extracellular stimuli including insulin. SHIP2 also regulates cytoskeleton remodeling, cell adhesion and spreading. In addition, endogenous SHIP2 in HeLa cells regulates receptor endocytosis and ligand-induced EGFR degradation. Further, SHIP2 in MDA-MB-231 breast cancer cells regulates EGFR levels and supports *in vitro* cell proliferation and *in vivo* tumor growth and spontaneous metastasis. Here we examine the role of SHIP2 in EGF signaling in breast cancer cells using RNA interference. Our results show that suppression of SHIP2 in MDA-MB-231 breast cancer cells alters EGF and EGFR internalization. Upon SHIP2 silencing, EGF-induced Akt activation was reduced causing decreased nuclear levels of activated Akt. Cytokine receptor CXCR4, a downstream element of EGFR-Akt pathway that plays an important role in metastasis, is down-regulated upon SHIP2 knockdown. Finally, cell adhesion and EGF-induced cell migration were suppressed in SHIP2 silenced cells. These results demonstrate a positive role of SHIP2 in EGF-induced Akt activation, CXCR4 expression, and cell migration in breast cancer cells.

Introduction

Phosphatidylinositol lipids are membrane-bound second messengers that transmit intracellular signaling triggered by a variety of external stimuli such as growth factors [e.g., epidermal growth factor (EGF)], hormones (e.g., insulin) as well as extracellular matrix (ECM) proteins (1). Among various lipid molecules, phosphatidylinositol-3,4,5-trisphosphate (PIP3) generated by the phosphatidylinositol 3'-kinase (PI3K) is critically important for cell survival, proliferation, adhesion/migration, glucose transport/metabolism and energy homeostasis (2-4). SH2-containing 5'-inositol phosphatase-2, (SHIP2), is a 5'-phosphoinositol phosphatase that dephosphorylates PIP3 to produce phosphatidylinositol-3,4-bisphosphate (PI-3,4-P2); accordingly, SHIP2 is thought to be a negative regulator of PI3K signaling (5,6).

One of the initial indications that physiological function of SHIP2 may be important for insulin signaling came from the observation that insulin responsive tissues express higher levels of SHIP2 mRNA (5). SHIP2 null mice in fact display mild insulin hypersensitivity, but a pronounced resistance to high-fat diet-induced obesity (7). SHIP2 protein levels were increased by a high-fat diet and in obesity (8). However, *in vitro* studies of SHIP2 in insulin function are far less convincing. Adenoviral-mediated exogenous overexpression of SHIP2 suppresses certain aspects of insulin signaling in adipocytes and skeletal myocytes (9-11), while SHIP2 gene silencing by RNA interference had no effect on insulin signaling in adipocytes (12).

While undoubtedly an important conduit of insulin signaling, PI3K-activated pathway - including Akt, its primary downstream effector, is critical for cancer relevant cellular processes, e.g., survival, proliferation, adhesion/spreading, and migration (13). One of the biologically important downstream components of this pathway is the chemokine receptor CXCR4 (14,15). CXCR4 is a seven-pass trans-membrane G-protein-coupled receptor activated by the chemokine, stromal-derived factor-1 α (SDF-1 α) (16). CXCR4 expressing cancer cells have a propensity for increased metastasis (16,17). Not surprisingly, phosphoinositol phosphatases are expected to play an important role in various aspects of cancer development and progression. Accordingly, studies attribute a classical tumor suppressor function to PTEN (18), however, studies on SHIP2 in cancer cells remain ambiguous

Correspondence to: Dr Nagendra K. Prasad, Department of Basic Medical Sciences and Purdue Cancer Center, Purdue University, 625 Harrison Street, West Lafayette, IN 47907, USA
E-mail: nprasad@purdue.edu

Abbreviations: PI3K, phosphatidylinositol 3'-kinase; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PI-3,4-P2, phosphatidylinositol-3,4-bisphosphate; PIP2, phosphatidylinositol-4,5-bisphosphate; shRNA, small hairpin RNA; siRNA, small interfering RNA; RNAi, RNA interference; AF-EGF, alexa fluor-conjugated EGF

Key words: PI-3' kinase, cell migration, endocytosis, cell adhesion

with regards to its effects on Akt activation and cell cycle progression (19,20).

Recently, we demonstrated that endogenously over-expressed SHIP2 in MDA-MB-231 breast cancer cells paradoxically supports cell proliferation *in vitro* as well as cancer growth and spontaneous lung metastases in nude mice (21). These biological outcomes are linked to SHIP2-mediated changes in EGFR levels. Here, we show that SHIP2 silencing in MDA-MB-231 breast cancer cells decreases EGF-induced Akt activation and suppresses expression of CXCR4, an EGF-Akt downstream effector of major importance to cell migration and metastasis. Furthermore, suppression of SHIP2 levels significantly inhibits breast cancer cell migration.

Materials and methods

Materials. A human SHIP2 antibody (against amino acids 1105-1213 of the C-terminus) used for Western blots has been previously described (22). MDA-MB-231 breast cancer cells were from American Type Culture Collection (ATCC, Manassas, Virginia). Double-stranded siRNA molecules specific for GL3 luciferase and SHIP2 [sequences described in (23)] were purchased from Dharmacon (Lafayette, CO). ShRNA, containing retroviral vectors for human SHIP2 gene (genebank: INPPL1-NM001567) and for EGFP were from Open Biosystems (Huntsville, AL). A recombinant human wild-type SHIP2 [with C-terminus FLAG tag (24)] was rendered RNAi resistant by introducing 6 silent mutations in the 22-ntd target region using PCR was described earlier [Bio-Means, Sugar Land, TX; (21)]. CXCR4 antibody (clone 2090) was from Abcam (Cambridge, MA), vinculin antibody was from Upstate Biotechnology (Charlottesville, VA), and β -actin and M2 anti-FLAG antibodies were from Sigma-Aldrich (St. Louis, MO). Antibodies specific for the activated- and total forms of Akt, were from Cell Signaling Technologies (Danvers, MA). Monoclonal EGFR antibody used for immunofluorescence was from BD Biosciences-Transduction labs (San Jose, CA). Alexa Fluor⁵⁵⁵-EGF was purchased from Molecular probes/Invitrogen (Carlsbad, CA).

Cell culture and RNA interference. Mammary carcinoma cell lines were cultured in MEM supplemented with L-glutamine, 1 nM insulin (only for MDA-MB-231 cells) and 10% fetal bovine serum (Hyclone, Logan, UT). Serum starvation (where indicated) was carried out by incubating cells in serum-free MEM containing 0.5% bovine serum albumin (BSA). Transient RNAi experiments with validated SHIP2 siRNA molecules were performed as previously described (23). Development of single-cell clones from pools of puromycin (1.0 μ g/ml) resistant MDA-MB-231 cells expressing SHIP2shRNA or EGFPshRNA was previously described as well (21). These selected clones were subjected to periodic Western blot analysis to ensure SHIP2 silencing, which was stable for at least 10-12 (8-10 weeks) passages from the time of their isolation at the single colony stage.

Immunofluorescence. Cell treatment with Alexa Fluor⁵⁵⁵-EGF, anti-FLAG immunofluorescence, and 4',6-diamidino-2-

phenylindole (DAPI) staining were carried out as previously described (23,24).

Western blot analyses. Preparation of whole cell lysates in a Triton X-100 lysis buffer as well as protein analyses by SDS-PAGE and Western blot assays were performed as described earlier (25).

Wound migration assays. Confluent monolayers of cells were serum starved (MEM + 0.5% bovine serum albumin) for 24-h, followed by creation of 'wound gaps' in the monolayers using a sterile pipette tip. Cells were further incubated for 6-24 h \pm EGF (20 ng/ml; where indicated) to allow for closure of the wounds. Phase-contrast bright field photomicrographs of the marked areas of the wounds were taken at 0 h and again at the end of the indicated time intervals using a x20 objective. The distance migrated by the cells from the edge of the wound into the gap is quantified by taking 3 random digital measurements of the gaps. The distance migrated is then calculated as arbitrary units by subtracting the gap remaining at the end of an interval from the original size of the gap (26).

Results

Altered EGFR endocytic trafficking in breast cancer cells upon SHIP2 silencing. Previously isolated stable shRNA clones of MDA-MB-231 breast cancer cells consistently showing SHIP2 silencing at both mRNA and protein levels (21), were employed to examine EGF internalization. As previously observed in HeLa cervical carcinoma cells (where SHIP2 was silenced using a transient RNAi approach) (23), both the total amount and the distribution pattern of internalized alexa fluor-conjugated EGF (AF-EGF) were strikingly different in SHIP2 silenced MDA-MB-231 cells expressing SHIP2 shRNA (clone S3) as compared to control cells with shEGFP shRNA (clone E2) (Fig. 1A). Clone E2 cells displayed large vesicles containing AF-EGF at the perinuclear endosomal sorting area at 30-min incubation following a pulse treatment (100 ng/ml for 10 min on ice) (Fig. 1A, panel a). EGF-containing vesicles in control E2 cells were fewer but larger after 45 min of incubation indicating robust endosomal fusion-mediated maturation and/or trafficking (Fig. 1A, panel b). Clone S3 SHIP2-silenced cells had appreciably less fluorescence signal, scattered as fine punctate foci (30-min chase/ Fig. 1A, panel c). After 45 min of follow-up incubation these vesicles remained more widely scattered and substantially smaller than control cells indicating a delayed or disrupted trafficking/maturation (Fig. 1A, panel d).

Exogenous expression of wild-type SHIP2 rendered resistant to RNAi (by introducing mismatches in the form of silence mutations at 6 of the 22 nucleotides in the target sequence) was previously used by us to confirm the direct role of SHIP2 function in regulating the EGFR levels (21). Here, we show that re-expression of this RNAi-resistant version of wild-type SHIP2 containing the FLAG epitope on the C-terminus (detected by anti-FLAG staining; green) (Fig. 1B, panel a) in shSHIP2 cells (clone S3) largely restored the perinuclear accumulation, and to a lesser extent, increased the vesicular fusion of EGF-containing endosomes (as indi-

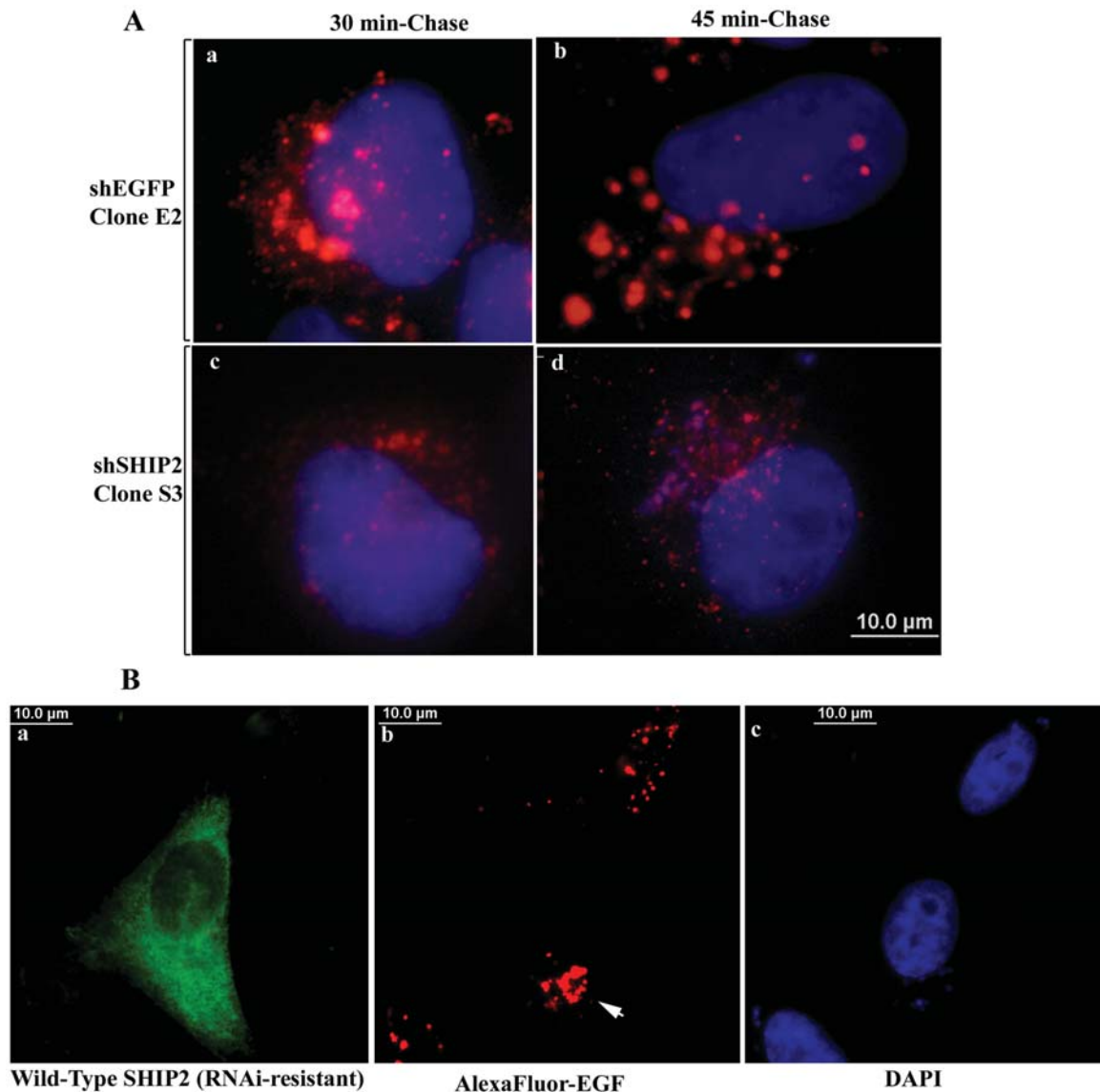


Figure 1. Altered distribution of EGF containing endocytic vesicles upon SHIP2 silencing. (A) ShEGFP (clone E2; panels a and b) or shSHIP2 (clone S3; panels c and d) clones of MDA-MB-231 cells were treated with alexafluor-EGF (100 ng/ml) for 10 min on ice followed by 3 washes with ice-cold serum-free medium to remove unbound EGF and incubated at 37°C for 30 min (panels a and c) or 45 min (panels b and d) in serum-free medium in the absence of EGF (denoted as 'Chase'). Cells were then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and counterstained with DAPI to visualize both internalized EGF and the nuclei using fluorescence microscopy [as detailed in (23)]. The results are representative of three separate experiments. (B) MDA-MB-231 shSHIP2 cells (clone S3) transfected with a RNAi-resistant version of FLAG-tagged wild-type SHIP2 [as described in (21)], were treated with alexafluor-EGF as in 'A' and chased for 45 min in the absence of EGF. Cells were then fixed in 4% paraformaldehyde (in PBS) to visualize internalized EGF (red; panel b) using fluorescence microscopy. Re-expression of wild-type SHIP2 (green; panel a) is detected by anti-FLAG (M2) immunofluorescence and counterstained with DAPI (blue; panel c) (23). White arrow in panel b points to the perinuclear accumulation of endosomes in a SHIP2 re-expressing cell, as compared to a cell situated at the top with no anti-FLAG staining showing a more dispersed distribution of smaller EGF containing vesicles. The experiment was reproduced three times.

cated by larger size) at the endosomal sorting area (Fig. 1B, panel b denoted by white arrow; compare with FLAG-negative cell situated near the top right corner of panel b). These results confirm that SHIP2 regulates receptor-mediated EGF internalization process in MDA-MB-231 breast cancer cells where EGFR plays an important oncogenic and metastatic role.

SHIP2 suppression decreases EGF induced Akt activation. We have previously reported that SHIP2 silencing by transient RNAi in HeLa and by stable RNAi in MDA-MB-231 cells,

decreases EGFR levels (21,23). Although immediate early signaling events downstream of EGFR activation could be reduced due to reduced receptor levels upon SHIP2 suppression, we reason decreased PIP3 dephosphorylation in the absence of SHIP2 could increase certain aspects of EGF signaling such as Akt activation. Determining the overall effect of SHIP2 suppression on EGF-induced signaling was further complicated by the genetic make-up of MDA-MB-231 cells. Activated K-Ras (27,28) and an autocrine pathway, induced by urokinase type plasminogen activator (uPA) (29) are known to cause constitutively high levels

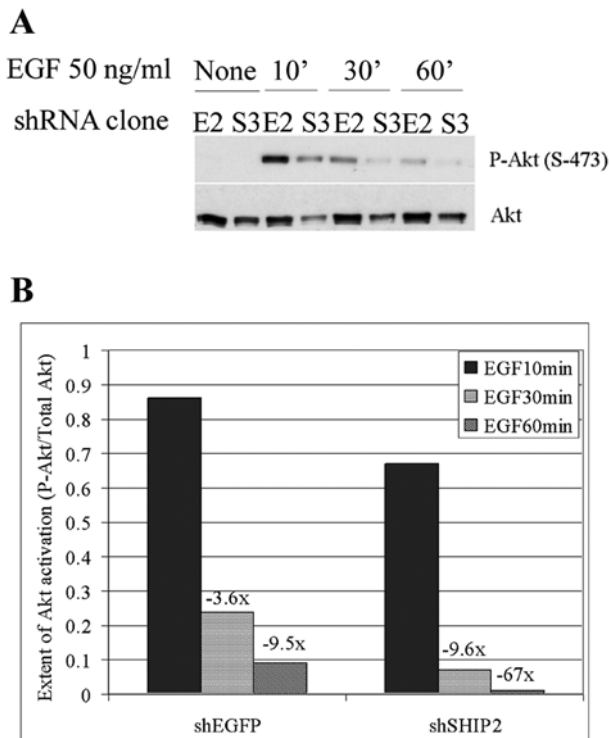


Figure 2. SHIP2 silencing decreases EGF-dependent Akt activation. (A) Protein extracts from MDA-MB-231 shEGFP clone E2 and shSHIP2 clone S3 cells treated with EGF (50 ng/ml) for indicated durations were analyzed in Western blots using phosphor-specific (Ser-473) Akt antibody. The membrane was re-probed with an antibody against total Akt. (B) The extent of Akt activation was determined by calculating the ratio of phosphor-Akt to total Akt of the blot shown in 'A'. The fold (x) decrease in Akt-activation overtime (shown on top of 30 and 60 min columns) is calculated in relation to the maximum activation that occurred at 10-min interval for the respective clone. The experiment was reproduced twice with two shSHIP2 clones (S3 and S8).

of ERK- and mTOR activations in MDA-MB-231 cells. Consistent with these reports, EGF-induced augmentation of mTOR- and ERK activation was negligible in our study, thus making SHIP2 silencing-related changes practically undetectable (21). Therefore, we focused on EGF-induced PI3K-Akt pathway to understand the effect of SHIP2 suppression on EGF signaling. Western blots with antibodies against activated Akt (phosphorylated on serine 473) and total Akt protein show that both control and SHIP2 silenced MDA-MB-231 cells contain no detectable phosphor-Akt under basal condition (i.e., serum-starved for 24 h). SHIP2 silenced cells (clone S3) show reduced Akt activation in response to EGF (50 ng/ml) (Fig. 2A). Specific activation (phosphor-/total) of Akt was lower in SHIP2 suppressed cells than control cells at each of the three time-points tested (Fig. 2B). The signal detected at 10-min EGF treatment in control cells was attenuated by 3.6-fold and 9.5-fold during 30 and 60 min of continuous EGF treatment respectively. In shSHIP2 cells, the signal attenuation was 9.6-fold at 30-min interval (~3 times faster than control) and nearly complete at 60-min interval (~7 times more rapid than control). Interestingly, SHIP2 silenced cells also contain less total Akt protein, indicating possible alterations in Akt protein synthesis and/or stability (Fig. 2A). Immunofluorescence

studies corroborated the outcome of Western analysis where nuclear translocation of phosphor-Akt was notably reduced in clone S3 shSHIP2 cells as compared to control cells (Fig. 3A). In a similar immunofluorescence approach, internalized EGFR containing vesicles in EGF-treated clone S3 cells were markedly less numerous and sparsely accumulated in the perinuclear endosomal sorting area compared to similarly treated control E2 cells (Fig. 3B). Taken together, the overall effect of SHIP2 suppression is a perceptible decrease in Akt activation in response to EGF, an effect that may be linked to decreased EGFR levels. Similar results of decreased EGF-induced Akt activation were obtained for another shSHIP2 clone (data not shown).

CXCR4 levels are decreased in SHIP2 silenced cells. SHIP2 silenced MDA-MB-231 cells not only formed tumors slowly in mammary fat pad implantation assays in nude mice but also led to dramatically reduced spontaneous lung metastases (21). Given that chemokine receptor CXCR4 is an important determinant of metastasis (30) whose expression is regulated by EGFR or Her2-induced PI3K-Akt pathway (15,31), we reasoned that changes in EGFR levels (21) and EGF-Akt signaling (Fig. 2) due to SHIP2 silencing, may ultimately down-regulate CXCR4 expression and suppressed metastasis. As less than 10% of *in vitro* cultured MDA-MB-231 cells express CXCR4 on the surface at low levels (which is shown to increase only upon *in vivo* passage) (17), we employed Western blot assays using an antibody shown to be specific for total cellular CXCR4 (15) to detect changes in CXCR4 expression. In transient and stable RNAi assays, our results show that CXCR4 expression was substantially reduced upon suppression of SHIP2 levels in MDA-MB-231 cells (Fig. 4A and B). The extent of CXCR4 suppression was directly correlated with the extent of SHIP2 suppression. Prolonged treatment with SDF-1 α (for 24 h) elevated CXCR4 expression (80% above untreated cells) perhaps due to persistent activation of PI3K-Akt pathway (32); this induction was abolished by SHIP2 silencing (Fig. 4A). In addition, puromycin-resistant cells derived from xenografts developed from control or SHIP2-silenced cells [from a previously reported study (21)] also displayed suppressed CXCR4 levels (Fig. 4C). This result correlated well with significant suppression of lung metastases in mice harboring these tumors (21). Taken together, our results showing SHIP2-induced changes in EGFR levels (21,23) and EGF-signaling (Figs. 2 and 3) are associated with changes in expression of the downstream gene CXCR4, reveal a potential molecular mechanism by which SHIP2 could regulate metastatic behavior of breast cancer cells.

SHIP2 is important for breast cancer cell adhesion and migration. As EGFR-PI3K-Akt and CXCR4 signaling are critical for cancer cell adhesion and migratory behaviors influencing the metastasis (33,34), we studied the effect of SHIP2 silencing on adhesion and migratory properties of breast cancer cells (Fig. 5A). SHIP2-silenced cells (Fig. 5A; panels b and d) were morphologically different, revealing frequent spindle-shaped or rounded cells compared to the control shEGFP cells (white arrowheads, Fig. 5A; panels a and c). In addition, small island-like clusters of MDA-MB-

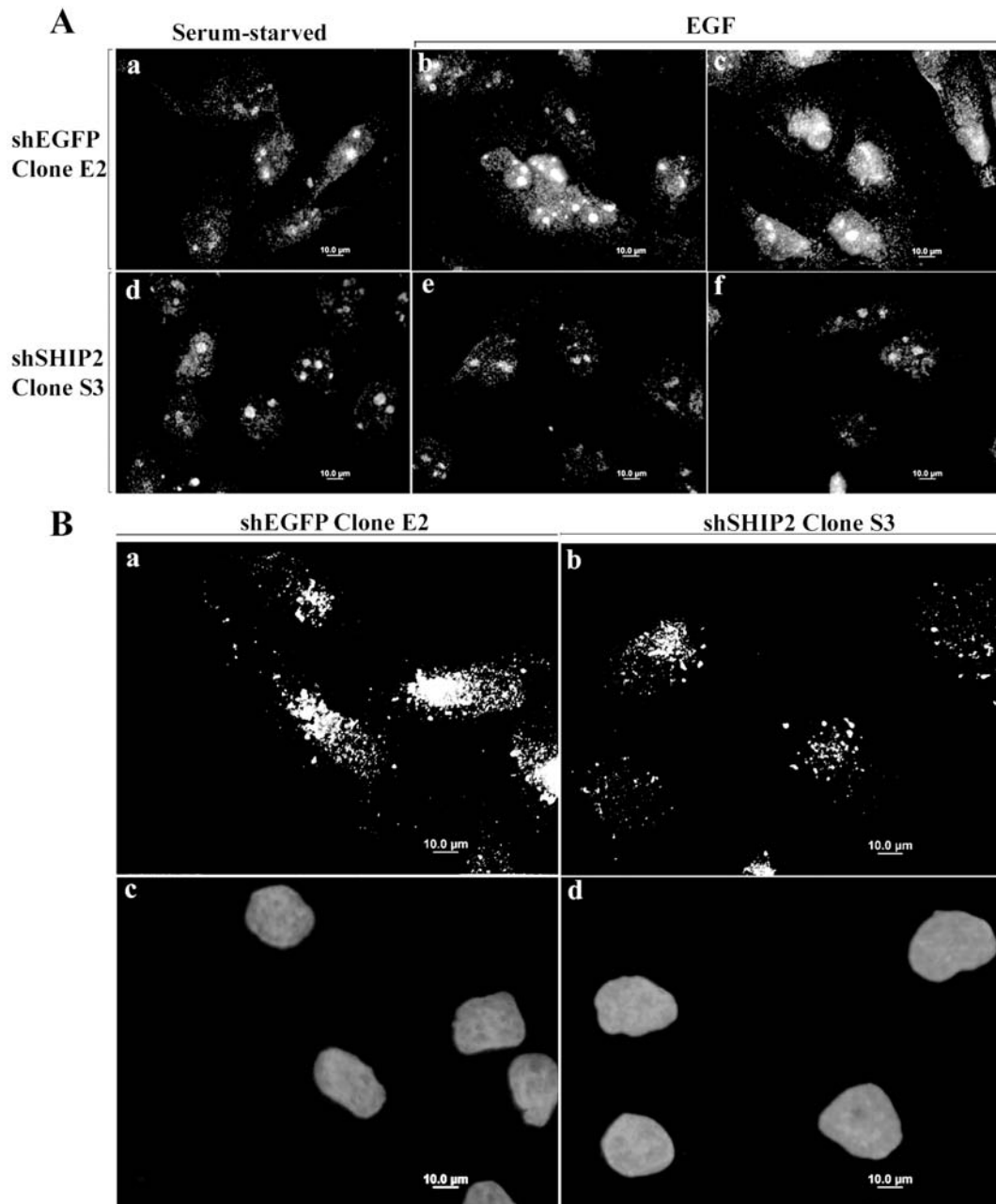


Figure 3. SHIP2 suppression causes reduced activated Akt in the nucleus associated with decreased EGFR in endosomal sorting area. (A) Immunofluorescence analyses of serum-starved (for 24 h in MEM + 0.5% BSA, panels a and d) or EGF treated (100 ng/ml for 15 min, subsequent to 24-h serum starving; panels b, c, e and f) control shEGFP (clone E2, panels a-c) and shSHIP2 (clone S3, panels d-f) MDA-MB-231 cells using an antibody specific for activated Akt (phosphor-Ser-473). Two different fields of EGF-treated culture are shown for each clone. (B) Immunofluorescence analyses of control shEGFP (clone E2, panels a and c) and shSHIP2 (clone S3, panels b and d) MDA-MB-231 cells that were treated with EGF as above (100 ng/ml for 15 min, subsequent to 24-h serum starving) using an anti-EGFR antibody (green; panels a and b). Counterstaining with DAPI is also shown (blue; panels c and d) to illustrate the distribution of EGFR-positive vesicles in relation to the nucleus.

231 cells (black arrowheads in panels a and c) were largely missing upon SHIP2 silencing (panels b and d). Instead, SHIP2 silenced cells remain as single cell entities. Similar morphological changes were observed upon SHIP2 silencing in 4T-1 mouse mammary carcinoma cells as well. Upon replating of MDA-MB-231 shRNA clonal cells in the presence of 10% FBS on regular plastic, significantly fewer SHIP2-silenced cells attached to the surface as compared to control cells after 60 min of plating (Fig. 5B). Importantly, in scratch

wound closure assays (26), SHIP2 RNAi cells migrated significantly slower than control cells (Fig. 6A). The effect was seen in serum-free medium (34-50% of control) as well as in the presence of EGF (25-50% of control) (Fig. 6B). Thus, our experiments clearly show that endogenously expressed SHIP2 in breast cancer cells play an important role in determining cell adhesion and migratory behavior. These results in addition highlight the biological relevance of SHIP2-mediated changes in the EGF-Akt pathway.

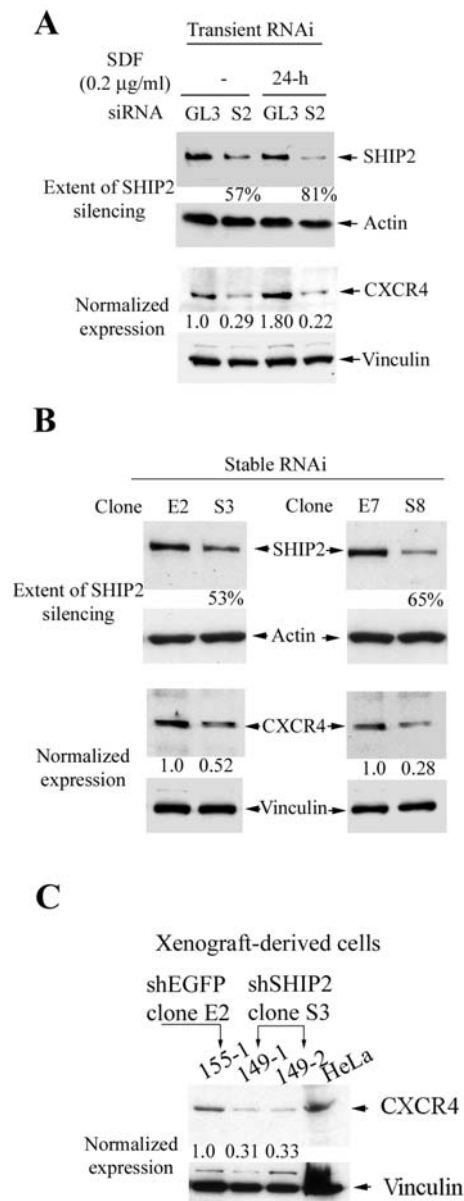


Figure 4. Changes in CXCR4 expression upon SHIP2 silencing. MDA-MB-231 cells were subjected to SHIP2 specific gene silencing using RNA interference approach. SHIP2 gene silencing at the protein level using a SHIP2-specific antibody (membrane re-probed with β -actin as loading control) is shown along with the analysis for CXCR4 levels carried out on a separate gel where re-probing was done with antibodies specific to vinculin as loading control. CXCR4 detection required greater amounts of protein loading where β -actin signal became saturating; hence, vinculin was used as control. (A) Transient RNA interference. Two-days post-transfection, MDA-MB-231 cells transfected with indicated siRNA molecules were serum starved for 24 h followed by treatment with \pm SDF-1 α (0.2 µg/ml) for 24 h. GL3 refers to control luciferase-specific siRNA and S2 is SHIP2-specific siRNA (23). SHIP2 expression was normalized to that of β -actin and the extent of SHIP2 silencing in SHIP2-specific siRNA transfected cells was estimated in relation to control GL3 siRNA. CXCR4 expression is normalized to vinculin levels. (B) Stable RNA interference. Stable puromycin-resistant clones of MDA-MB-231 cells expressing shEGFP (clone E2 and E7) or shSHIP2 (clones S3 and S8) [developed in a previous study (21)] cultured in serum containing culture medium were examined for SHIP2 and CXCR4 levels as described for 'A'. (C) Cells derived from orthotopic mammary tumors induced by shSHIP2 clone S3 (animal ID 149-1 and 149-2) or control shEGFP clone E2 (155-1) were cultured in the presence of puromycin for 7-10 days (21). Protein extracts from these cells as well as from HeLa cells (as positive control) were analyzed for CXCR4 and vinculin (loading control) in Western blots [presence of SHIP2 silencing correlating with EGFR suppression in these cells has been previously reported (21)].

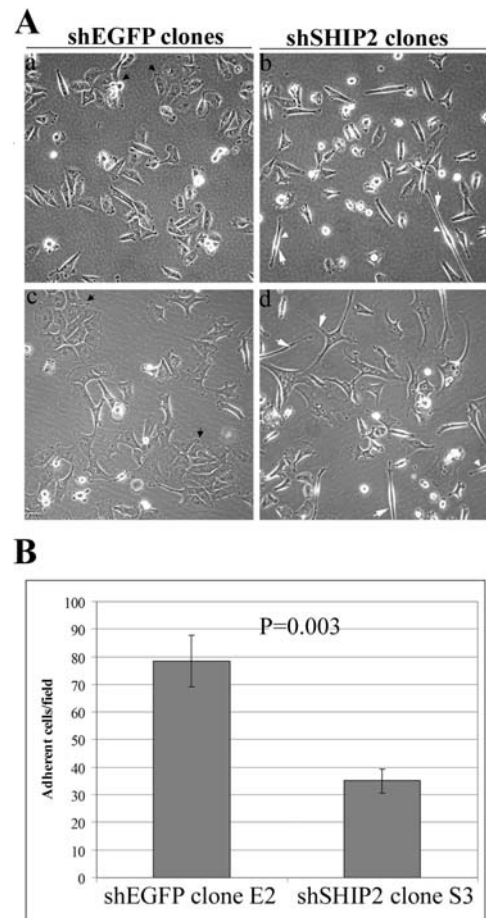


Figure 5. Altered cell morphology and reduced cell adhesion in SHIP2-silenced cells. (A) Photomicrographs (x20) of shEGFP (panels a and c) or shSHIP2 clones (panels b and d) of MDA-MB-231 cells cultured on tissue culture plastic in 10% FBS containing culture medium. Black arrows denote cell clusters of small island-like clusters in MDA-MB-231 cells (panels a and c). White arrows point to spindle-shaped morphology, rounded cells or axon-like membrane protrusions frequently seen upon SHIP2 silencing (panels b and d). (B) ShRNA clones of MDA-MB-231 cells plated (1000-cells/cm²) on tissue culture-treated plastic for 60 min. Cells were then photographed and number of cells attached per x20 field was enumerated (n=6). P-value was estimated using 2-tailed t-test.

Discussion

SHIP2-mediated regulation of EGF signaling. We have earlier reported that SHIP2 in HeLa cells negatively regulates EGF-induced EGFR endocytic internalization and degradation (23). Similar function of SHIP2 in ligand-induced EphA2 receptor endocytosis is reported in MDA-MB-231 cells (35), although a role for SHIP2 in cancer-relevant functions of EphA2 is yet to be demonstrated. Here, we describe a novel positive regulation by SHIP2, a proposed negative regulator of insulin signaling, of the EGFR-Akt-CXCR4 pathway. MDA-MB-231 cells reveal no basal levels of Akt activation following serum starvation for 24 h, thereby permitting easy detection of EGF-induced activation. SHIP2 suppression attenuated both the magnitude and persistence of EGF-induced Akt activation. There are at least two possible explanations for SHIP2 silencing-mediated suppression of the EGF-induced Akt pathway.

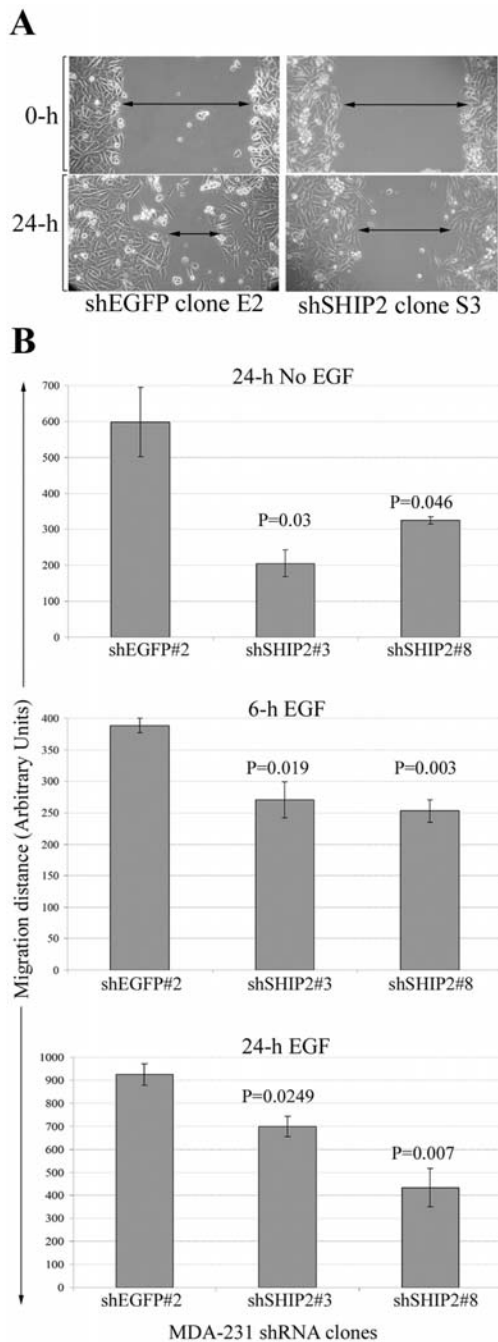


Figure 6. Reduced cell migration of MDA-21 cells with decreased SHIP2. Scratch wounds were created in the serum starved (for 24 h) monolayer of shRNA clones of MDA-MB-231 cells plated to confluency, followed by incubation \pm EGF (20 ng/ml) for indicated durations. Marked areas of the gap were photographed at 0 h and at the end of indicated intervals. The average width of the gap in the photomicrographs was estimated by taking three random digital measurements. (A) Representative images from a wound closure assay used to monitor cell migratory behavior of MDA-MB-231 cells. Experiment was repeated three times with similar results. The arrows illustrate the extent of the wound closure during the course of 24-h incubation in serum-free medium. (B) Average distance migrated in the absence of EGF after 24 h or in the presence of EGF after 6 and 24 h is shown for control shEGFP clone (E2) and two shSHIP2 clones (S3 and S8) (n=3, each with triplicate measurements). P-value on top of the columns represents the significance of comparison for each of the shSHIP2 clone with the control shEGFP clone as estimated from 2-tailed t-test.

First, it has been shown that internalized EGF-bound EGFRs present in the endosomal compartment do not activate

PI3K or PLC- γ (36,37). PI-4,5-P₂, the substrate for PI3K and PLC- γ , is absent in the endosomal membranes thereby restricting the signaling activity from these molecules to the plasma membrane. Therefore, we reason the SHIP2 silencing-induced decrease in EGF-induced Akt activation in MDA-MB-231 cells is potentially due to the combined effects of previously reported enhanced EGFR degradation (21) (Fig. 3B), leading to decreased surface EGFR levels over time, as well as altered endocytic trafficking (23) (Fig. 1), rapidly sequestering the surface EGFRs in the endosomal compartments. Interestingly, enhanced EGFR degradation observed upon knockout of a E3 ubiquitin ligase *Parkin* similarly decreases Akt activation, but not ERK phosphorylation in mouse brain (38).

Second, decreased Akt activation upon SHIP2-silencing may be due to decreased formation of lipid PI3,4P₂, known to be essential for full activation of Akt, both *in vitro* and *in vivo* (39,40). In PTEN-positive PNT2 and P4E6 prostate cancer cells, SHIP2 function is required for serum-induced Akt phosphorylation (41). This study, however, does not examine the changes in upstream components of this pathway and changes in receptor tyrosine kinases upon SHIP2 silencing were not considered in the interpretation. MDA-MB-231 cells are PTEN-positive and our results indicate a positive role for SHIP2 in Akt activation that can be clearly linked to changes in EGFR levels and in receptor endocytosis (21).

Decreased levels of total Akt protein upon SHIP2-silencing is another intriguing observation, indicating a possible additional mechanism by which the Akt pathway may be regulated by SHIP2. As levels of many other cellular proteins including PTEN, ERK1/2, mTOR, vinculin, and β -actin remain unaltered (21), the changes in Akt levels are likely a specific effect. As Akt stability could be determined by phosphorylation by an mTOR-containing protein complex (mTORC2) and Hsp-90 mediated mechanisms (42,43), it is intriguing to postulate a role for SHIP2 in maintaining Akt stability, possibly through novel protein interactions. More studies are clearly needed to better dissect the underlying molecular mechanisms of this interesting observation SHIP2 function.

SHIP2 role in of cell adhesion and migration - implications for cancer biology. Another important finding of our study is that endogenously over-expressed SHIP2 in breast cancer cells augments adhesion, spreading and migration. This substantially expands our previous finding in HeLa cells, showing that exogenously expressed wild-type SHIP2 promotes cell adhesion and a truncated SHIP2 gene, deleted in phosphatase domain, prevents cell spreading on type I collagen (24). In HeLa cells SHIP2 function in cell spreading is regulated by a type I collagen-specific signaling pathway involving Src kinases, leading to interactions with Shc adapter protein (23,25). Interestingly, cell morphology upon stable SHIP2-silencing reveals features associated with enhanced migration such as spindle shaped and/or rounded cells. However, these cells display dramatically deficient cell adhesion and migration behavior. Although this seems counterintuitive, our results are in agreement with reports demonstrating the critical importance of dynamic changes in the local phosphoinositide pools in determining the final outcomes of cell

adhesion and migration (44,45). Therefore, reduced SHIP2 function may also enhance specific early events in cell migratory behavior (for e.g., adhesion disassembly) while preventing successful completion of migration by interfering with later events (for e.g., lamellipodia formation, nascent focal contact formation). Indeed, SHIP2 suppression or inhibition has shown to interfere with focal contact formation, actin polymerization, formation of lamellipodia and cell scattering (23-25,46).

Alternatively, SHIP2 could regulate cell adhesion and migration via interactions with cytoskeleton regulators [such as p130Cas; (24)] independent of its lipid phosphatase activity. In fact, specific aspects of regulation of cell migration by PTEN has been shown to be independent of its lipid phosphatase activity (47). Other studies have reported SHIP2-mediated regulation of actin remodeling in fibroblasts via interactions with filamin (48) or vinexin (49) and induction of cell scattering and formation of lamellipodia via interactions with c-Met in MDCK (Madin-Darby Canine Kidney) cells (46). Specific interactions with Arp3, a GTPase activating protein (GAP) for both Arf and Rho G-proteins, mediated by the C-terminally located SAM (sterile alpha motif) domain suggest yet another mechanism for the cytoskeleton regulatory function of SHIP2 (50). Therefore, there is a strong structural and mechanistic basis for a novel cytoskeleton regulatory function of SHIP2. On the other hand, cell motility is known to be linked closely to dynamics of endocytosis (51,52). There is solid evidence for the role of both PI3K-Akt and PLC- γ pathways in the cytoskeletal remodeling responsible for cell adhesion and migration (53). Decreased cell adhesion and migration in SHIP2-silenced cells could also be attributed to deregulated EGFR endocytosis and consequent impairment of the PI3K pathway.

CXCR4 as the possible downstream effector of SHIP2 function in metastasis. Interestingly, our study identifies CXCR4 as a potential relevant downstream target gene of SHIP2 function in breast cancer cells. As SHIP2 silencing caused a pronounced decrease in spontaneous lung metastases in an orthotopic mammary tumor model, changes observed in CXCR4 expression provide a mechanistic basis for this clinically relevant aspect of cancer biology. However, we were unable to determine the role of SHIP2 in SDF-1 α -induced directed cell migration or invasion using our study model as SDF-1 α induced chemotaxis is substantial only in metastatic variants, but not in parental MDA-MB-231 cells (17,32). As the role of CXCR4 in breast cancer metastases is increasingly recognized (14,17,30,32,34), reports show that EGFR or Her2 signaling via PI3K-Akt regulates the expression of CXCR4 (15,31). Thus, reduced CXCR4 levels observed in SHIP2 silenced MDA-MB-231 cells are congruent with changes in the EGFR-Akt pathway, including reduced EGFR levels, impaired EGF internalization, and reduced EGF-induced Akt-signaling. SDF-1 α signaling via CXCR4 involves activation of the PI3K-Akt pathway (54), and given that the CXCR4 receptor undergoes ligand-induced endocytic down-regulation (55), SHIP2 may regulate SDF-1 α signaling in a manner similar to EGF signaling.

In conclusion, our study reveals that EGFR-internalization process, EGF-Akt pathway, and expression of a downstream

gene CXCR4 are positively regulated by the endogenously expressed SHIP2 phosphoinositol phosphatase in breast cancer cells. Further, we show, for the first time that SHIP2 in breast cancer cells is an important regulator of cell migration. Thus, our study provides a mechanistic basis for the recently reported positive role of SHIP2 in breast cancer growth and metastasis (21). Further evidence for the translational significance of this aspect of SHIP2 function is provided in another recent report from our group where immunohistochemical analysis of clinical samples of primary breast cancers (N=285) reveal that SHIP2 expression levels correlate with shortened disease-free survival in invasive breast cancer patients and that SHIP2 levels positively correlate with EGFR expression *in vivo* (56).

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References

1. Sasaki T, Sasaki J, Sakai T, Takasuga S and Suzuki A: The physiology of phosphoinositides. *Biol Pharm Bull* 30: 1599-1604, 2007.
2. Cantley LC: The phosphoinositide 3-kinase pathway. *Science* 296: 1655-1657, 2002.
3. Vanhaesebroeck B, Ali K, Bilancio A, Geering B and Foukas LC: Signalling by PI3K isoforms: insights from gene-targeted mice. *Trends Biochem Sci* 30: 194-204, 2005.
4. Vanhaesebroeck B, Leevers SJ, Ahmadi K, *et al*: Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem* 70: 535-602, 2001.
5. Pesesse X, Deleu S, De Smedt F, Drayer L and Erneux C: Identification of a second SH2-domain-containing protein closely related to the phosphatidylinositol polyphosphate 5-phosphatase SHIP. *Biochem Biophys Res Commun* 239: 697-700, 1997.
6. Rohrschneider LR, Fuller JF, Wolf I, Liu Y and Lucas DM: Structure, function and biology of SHIP proteins. *Genes Dev* 14: 505-520, 2000.
7. Sleeman MW, Wortley KE, Lai KM, *et al*: Absence of the lipid phosphatase SHIP2 confers resistance to dietary obesity. *Nat Med* 11: 199-205, 2005.
8. Hori H, Sasaoka T, Ishihara H, *et al*: Association of SH2-containing inositol phosphatase 2 with the insulin resistance of diabetic db/db mice. *Diabetologia* 44: 1258-1267, 2002.
9. Murakami S, Sasaoka T, Wada T, *et al*: Impact of Src homology 2-containing inositol 5'-phosphatase 2 on the regulation of insulin signaling leading to protein synthesis in 3T3-L1 adipocytes cultured with excess amino acids. *Endocrinology* 145: 3215-3223, 2004.
10. Sasaoka T, Hori H, Wada T, *et al*: SH2-containing inositol phosphatase 2 negatively regulates insulin-induced glycogen synthesis in L6 myotubes. *Diabetologia* 44: 1258-1267, 2001.
11. Wada T, Sasaoka T, Funaki M, *et al*: Overexpression of SH2-containing inositol phosphatase 2 results in negative regulation of insulin-induced metabolic actions in 3T3-L1 adipocytes via its 5'-phosphatase catalytic activity. *Mol Cell Biol* 21: 1633-1646, 2001.

12. Tang X, Powelka AM, Soriano NA, Czech MP and Guilherme A: PTEN, but not SHIP2, suppresses insulin signaling through the phosphatidylinositol 3-kinase/Akt pathway in 3T3-L1 adipocytes. *J Biol Chem* 280: 22523-22529, 2005.
13. Cantrell DA: Phosphoinositide 3-kinase signalling pathways. *J Cell Sci* 114: 1439-1445, 2001.
14. Benovic JL and Marchese A: A new key in breast cancer metastasis. *Cancer Cell* 6: 429-430, 2004.
15. Li YM, Pan Y, Wei Y, *et al*: Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis. *Cancer Cell* 6: 459-469, 2004.
16. Dewan MZ, Ahmed S, Iwasaki Y, Ohba K, Toi M and Yamamoto N: Stromal cell-derived factor-1 and CXCR4 receptor interaction in tumor growth and metastasis of breast cancer. *Biomed Pharmacother* 60: 273-276, 2006.
17. Helbig G, Christopherson KW II, Bhat-Nakshatri P, *et al*: NF-kappaB promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4. *J Biol Chem* 278: 21631-21638, 2003.
18. Leslie NR and Downes CP: PTEN function: how normal cells control it and tumour cells lose it. *Biochem J* 382: 1-11, 2004.
19. Choi Y, Zhang J, Murga C, *et al*: PTEN, but not SHIP and SHIP2, suppresses the PI3K/Akt pathway and induces growth inhibition and apoptosis of myeloma cells. *Oncogene* 21: 5289-5300, 2002.
20. Taylor V, Wong M, Brandts C, *et al*: 5' phospholipid phosphatase SHIP-2 causes protein kinase B inactivation and cell cycle arrest in glioblastoma cells. *Mol Cell Biol* 20: 6860-6871, 2000.
21. Prasad NK, Tandon M, Badve S, Snyder PW and Nakshatri H: Phosphoinositid phosphatase SHIP2 promotes cancer development and metastasis coupled with alterations in EGF receptor turnover. *Carcinogenesis* 29: 25-34, 2008.
22. Habib T, Hejna JA, Moses RE and Decker SJ: Growth factors and insulin stimulate tyrosine phosphorylation of the 51C/SHIP2 protein. *J Biol Chem* 273: 18605-18609, 1998.
23. Prasad NK and Decker SJ: SH2-containing 5'-inositol phosphatase, SHIP2, regulates cytoskeleton organization and ligand-dependent down-regulation of the epidermal growth factor receptor. *J Biol Chem* 280: 13129-13136, 2005.
24. Prasad N, Topping RS and Decker SJ: SH2-containing inositol 5'-phosphatase SHIP2 associates with the p130(Cas) adapter protein and regulates cellular adhesion and spreading. *Mol Cell Biol* 21: 1416-1428, 2001.
25. Prasad N, Topping RS and Decker SJ: Src family tyrosine kinases regulate adhesion-dependent tyrosine phosphorylation of 5'-inositol phosphatase SHIP2 during cell attachment and spreading on collagen I. *J Cell Sci* 115: 3807-3815, 2002.
26. Liang CC, Park AY and Guan JL: *In vitro* scratch assay: a convenient and inexpensive method for analysis of cell migration *in vitro*. *Nat Protoc* 2: 329-333, 2007.
27. Gilhooly EM and Rose DP: The association between a mutated ras gene and cyclooxygenase-2 expression in human breast cancer cell lines. *Int J Oncol* 15: 267-270, 1999.
28. Agbunag C and Bar-Sagi D: Oncogenic K-ras drives cell cycle progression and phenotypic conversion of primary pancreatic duct epithelial cells. *Cancer Res* 64: 5659-5663, 2004.
29. Ma Z, Webb DJ, Jo M and Gonias SL: Endogenously produced urokinase-type plasminogen activator is a major determinant of the basal level of activated ERK/MAP kinase and prevents apoptosis in MDA-MB-231 breast cancer cells. *J Cell Sci* 114: 3387-3396, 2001.
30. Burger JA and Kipps TJ: CXCR4: a key receptor in the cross-talk between tumor cells and their microenvironment. *Blood* 107: 1761-1767, 2006.
31. Phillips RJ, Mestas J, Gharaee-Kermani M, *et al*: Epidermal growth factor and hypoxia-induced expression of CXCR4 chemokine receptor 4 on non-small cell lung cancer cells is regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin signaling pathway and activation of hypoxia inducible factor-1alpha. *J Biol Chem* 280: 22473-22481, 2005.
32. Zhao M, Mueller BM, Discipio RG and Schraufstatter IU: Akt plays an important role in breast cancer cell chemotaxis to CXCL12. *Breast Cancer Res Treat* 110: 211-222, 2008.
33. Yoeli-Lerner M and Tokar A: Akt/PKB signaling in cancer: a function in cell motility and invasion. *Cell Cycle* 5: 603-605, 2006.
34. Zlotnik A: New insights on the role of CXCR4 in cancer metastasis. *J Pathol* 215: 211-213, 2008.
35. Zhuang G, Hunter S, Hwang Y and Chen J: Regulation of EphA2 receptor endocytosis by SHIP2 lipid phosphatase via phosphatidylinositol 3-Kinase-dependent Rac1 activation. *J Biol Chem* 282: 2683-2694, 2007.
36. Haugh JM and Meyer T: Active EGF receptors have limited access to PtdIns(4,5)P(2) in endosomes: implications for phospholipase C and PI 3-kinase signaling. *J Cell Sci* 115: 303-310, 2002.
37. Varnai P, Rother KI and Balla T: Phosphatidylinositol 3-kinase-dependent membrane association of the Bruton's tyrosine kinase pleckstrin homology domain visualized in single living cells. *J Biol Chem* 274: 10983-10989, 1999.
38. Fallon L, Belanger CM, Corera AT, *et al*: A regulated interaction with the UIM protein Eps15 implicates parkin in EGF receptor trafficking and PI(3)K-Akt signalling. *Nat Cell Biol* 8: 834-842, 2006.
39. Klippel A, Kavanaugh WM, Pot D and Williams LT: A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. *Mol Cell Biol* 17: 338-344, 1997.
40. Scheid MP, Huber M, Damen JE, *et al*: Phosphatidylinositol (3,4,5)P3 is essential but not sufficient for protein kinase B (PKB) activation; phosphatidylinositol (3,4)P2 is required for PKB phosphorylation at Ser-473: studies using cells from SH2-containing inositol-5-phosphatase knockout mice. *J Biol Chem* 277: 9027-9035, 2002.
41. Sharrard RM and Maitland NJ: Regulation of protein kinase B activity by PTEN and SHIP2 in human prostate-derived cell lines. *Cell Signal* 19: 129-138, 2007.
42. Basso AD, Solit DB, Chiosis G, Giri B, Tschlis P and Rosen N: Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function. *J Biol Chem* 277: 39858-39866, 2002.
43. Facchinetti V, Ouyang W, Wei H, *et al*: The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. *EMBO J* 27: 1932-1943, 2008.
44. Haugh JM, Codazzi F, Teruel M and Meyer T: Spatial sensing in fibroblasts mediated by 3' phosphoinositides. *J Cell Biol* 151: 1269-1280, 2000.
45. Merlot S and Firtel RA: Leading the way: directional sensing through phosphatidylinositol 3-kinase and other signaling pathways. *J Cell Sci* 116: 3471-3478, 2003.
46. Koch A, Mancini A, El Bounkari O and Tamura T: The SH2-domain-containing inositol 5-phosphatase (SHIP)-2 binds to c-Met directly via tyrosine residue 1356 and involves hepatocyte growth factor (HGF)-induced lamellipodium formation, cell scattering and cell spreading. *Oncogene* 24: 3436-3447, 2005.
47. Leslie NR, Yang X, Downes CP and Weijer CJ: PtdIns(3,4,5)P(3)-dependent and -independent roles for PTEN in the control of cell migration. *Curr Biol* 17: 115-125, 2007.
48. Dyson JM, O'Malley CJ, Becanovic J, *et al*: The SH2-containing inositol polyphosphate 5-phosphatase, SHIP-2, binds filamin and regulates submembraneous actin. *J Cell Biol* 155: 1065-1079, 2001.
49. Paternotte N, Zhang J, Vandenbroere I, *et al*: SHIP2 interaction with the cytoskeletal protein Vinexin. *FEBS J* 272: 6052-6066, 2005.
50. Raaijmakers JH, Deneubourg L, Rehmann H, *et al*: The PI3K effector Arap3 interacts with the PI(3,4,5)P3 phosphatase SHIP2 in a SAM domain-dependent manner. *Cell Signal* 19: 1249-1257, 2007.
51. Jones MC, Caswell PT and Norman JC: Endocytic recycling pathways: emerging regulators of cell migration. *Curr Opin Cell Biol* 18: 549-557, 2006.
52. Le Roy C and Wrana JL: Signaling and endocytosis: a team effort for cell migration. *Dev Cell* 9: 167-168, 2005.
53. Ling K, Schill NJ, Wagoner MP, Sun Y and Anderson RA: Movin' on up: the role of PtdIns(4,5)P(2) in cell migration. *Trends Cell Biol* 16: 276-284, 2006.
54. Busillo JM and Benovic JL: Regulation of CXCR4 signaling. *Biochim Biophys Acta* 1768: 952-963, 2007.
55. Signoret N, Oldridge J, Pelchen-Matthews A, *et al*: Phorbol esters and SDF-1 induce rapid endocytosis and down modulation of the chemokine receptor CXCR4. *J Cell Biol* 139: 651-664, 1997.
56. Prasad NK, Tandon M, Handa A, *et al*: High expression of obesity-linked phosphatase SHIP2 in invasive breast cancers correlates with reduced disease-free survival. *Tumor Biol* (In Press).