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Evidence of SHIP2 S132 phosphorylation, its nuclear localization and stability

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Abbreviations: PI(3,4,5)P₃ phosphatidylinositol 3,4,5-trisphosphate; PI(3,4)P₂ phosphatidylinositol 3,4-bisphosphate; PI(4,5)P₂ phosphatidylinositol 4,5-bisphosphate; bpV(Phen) potassium bisperoxo(1,10-phenanthroline)oxovanadate.

Synopsis

PI(3,4,5)P₃ and PI(3,4)P₂ are major signalling molecules in mammalian cell biology. PI(3,4)P₂ can be produced by PI 3-kinases but also by PI 5-phosphatases including SHIP2. Proteomic studies in human cells revealed that SHIP2 can be phosphorylated at more than 20 sites but their individual function is unknown. In a model of PTEN-null astrocytoma cells, lowering SHIP2 expression leads to increased PI(3,4,5)P₃ levels and Akt phosphorylation. Mass spec analysis identified SHIP2 phosphosites on S132, T1254 and S1258; phosphotyrosine containing sites were undetectable. By immunostaining, total SHIP2 concentrated in the perinuclear area and in the nucleus, while phospho SHIP2 S132 was in the cytoplasm, the nucleus and nuclear speckles depending on the cell cycle. SHIP2 phosphorylated on S132 demonstrated PI(4,5)P₂ phosphatase activity. Endogenous phospho SHIP2 S132 showed an overlap with PI(4,5)P₂ staining in nuclear speckles. SHIP2 S132A was less sensitive to C-terminal degradation and more resistant to calpain as compared to wild type enzyme. We have identified nuclear lamin A/C as a novel SHIP2 interactor. We suggest that the function of SHIP2 is different at the plasma membrane where it recognizes PI(3,4,5)P₃ and in the nucleus where it may interact with PI(4,5)P₂, particularly in speckles.

Introduction

Phosphoinositide (PI) phosphatases belong to a complex network of interacting PIs widely implicated in human physiopathology [1,2]. In particular, PI 3-kinase and PTEN are two of the most frequently mutated proteins in human cancers [3]. The metabolism of PI(3,4,5)P₃ occurs by two pathways: dephosphorylation at the 3-position of the inositol ring catalyzed by PTEN and at the 5-position via the inositol polyphosphate 5-phosphatases [2,4]. Amongst mammalian phosphoinositide 5-phosphatases, SHIP1 and SHIP2 are important enzymes that produce PI(3,4)P₂, a second messenger on its own and possible source of PI3P in the cells [5-7]. SHIP2 is a multi-domain protein comprising a central catalytic domain, an SH2 domain at its N-terminal end, proline rich sequences and SAM domain at its C-terminus [8].

A role of SHIP2 in the control of insulin sensitivity and diet-induced obesity has been characterized in cellular models and in SHIP2 knockout mice *in vivo* [9,10]. Additional functions of SHIP2, unrelated to insulin signalling, have been reported: SHIP2-null mice showed a distinctive truncated facial profile resulting from an abnormality in skeletal growth in that region [10]. Loss of *SHIP2* in zebrafish leads to an increased and expanded expression of outputs of FGF-mediated signalling, suggesting an inhibitory function of SHIP2 in FGF signalling in mammals [11]. A series of proteomic studies provided evidence of a role of SHIP2 in the signalling pathways of growth factors such as EGF [12]. SHIP2 also appears to be one of the core interactors of Abl as shown in human myelogenous leukemia K562 cells [13]. The role of SHIP2 in proliferation or apoptosis is not always clear and could be cell type specific: silencing of SHIP2 gene expression in MDA-231 cells is anti-proliferative [14], whereas inhibition of SHIP2 induces proliferation in insulin producing INS1E cells [15]. Recent data suggested a role of SHIP2 as a tumour suppressor in squamous cell carcinoma. This was achieved by a control of SHIP2 levels by micro-RNAs including mir-205 [16]. SHIP2 is also a docking protein for multiple cytoskeletal proteins such as Cbl, ARAP3, filamin and vinexin [17-20]. Those interactions could be very important in the control of cell adhesion, endocytosis and/or cytoskeletal organization [21].

SHIP2 is phosphorylated on tyrosine residues in response to growth factors EGF, SCF, insulin [8,22,23], an effect that has been positively linked to change in activity, although this remains controversial [24,25]. Tyrosine phosphorylation has also been found responsible for the recruitment of adaptors such as Shc to SHIP2 in p210(bcr/abl)-expressing hematopoietic cells [26]. Global proteomic studies in human cells revealed more than 20 SHIP2 phosphosites (see PhosphositePlus) but their individual function is not known.

SHIP2 phosphorylation and interaction in a network of specific interactors are two mechanisms that could account for its translocation to the membrane and cellular specificity. Here, we used a model of human PTEN-null astrocytoma cells. We identified three phosphosites of SHIP2 S132, T1254 and S1258. pSHIP2 S132 immunoreactivity concentrates in the cytoplasm, the nucleus and in nuclear speckles. Immunoprecipitated phospho-SHIP2, by the use of a pSHIP2 S132 antibody, demonstrated both PtdIns(3,4,5)P₃ and PI(4,5)P₂ phosphatase activity. Evidence is provided that wild type SHIP2, phosphorylated on S132, is more sensitive to proteolytic C-terminal truncation as compared SHIP2 S132A. These observations

show the importance of SHIP2 Ser 132 phosphorylation on intracellular localization, SHIP2 activity and stability.

Materials and Methods

Materials

cDNAs of human SHIP2 wild type, the RNA targeting constructs for SHIP2 and PTEN in the pSUPER.retro.puro vector (OligoEngine) have been described [7,27]. Commercially available antibodies used were as follows: PTEN, GAPDH and Akt, phospho-(Ser473) Akt, phospho-(Thr308) Akt, phospho-(Thr185/Tyr187) Erk1/2 were from Cell Signaling Technology. Phosphotyrosine (4G10) was from Upstate. ERK 2 and lamin A/C were from Santa Cruz Biotechnology. SC35 was from BD Biosciences. Phalloidin-Alexa Fluor594 was from Invitrogen. SHIP2 antibody used in immunostaining was from Abcam (rabbit). Rabbit SHIP2 antibody used in Western and immunoprecipitation was described before [28]. Antibody to pSHIP2 S132 of human SHIP2 was produced in rabbits immunized with keyhole-limpet haemocyanin-conjugated phosphopeptide and affinity purified on the antigenic phosphopeptide (Eurogentec, Belgium). SHIP2 S132A mutant in pcDNA3His was made using the QuickChange site-directed mutagenesis kit (Stratagene). PI(4,5)P₂ (2C11) mouse monoclonal antibody was from Santa Cruz. Calpain was from BioLabs. An antibody (rabbit) to PR130/B"α1 (PR130) regulatory B-type subunit of protein phosphatase 2A was reported before [29]. Bacterial SHIP2 was prepared as before [30]. PI(3,4,5)P₃ di C8 and PI (4,5)P₂ di C8 were from CellSignals.

The fluorescent secondary antibody IRDye 800 anti-goat is from Licor, Lincoln, NE, USA, and the DyLight680 and DyLight800 anti-rabbit or anti-mouse from Thermo Fisher Scientific, Rockford, USA. The fluorescent secondary antibodies DyLight549 anti-rabbit or anti-mouse and DyLight488 anti-mouse or anti-rabbit were from Jackson ImmunoResearch. TLC plates were from Merck. Coomassie® Brilliant Blue R-250 was from Bio-Rad. LY-294002 and puromycin were from Sigma. [³²P] orthophosphate was from Amersham. Potassium bisperoxo(1,10-phenanthroline)oxovanadate bpV(Phen) was from Calbiochem. HeLa cells and human thyroid cells in primary culture were obtained as described before [31]. MEFs +/+ and SHIP2 -/- have been reported [32]. SHIP2 PI(3,4,5)P₃ (or PI (4,5)P₂) phosphatase activity was determined on SHIP2 immunoprecipitates as reported before [30]. X-press tagged SHIP2 was immunoprecipitated by the use of X-press antibody (Invitrogen). pSHIP2 S132 was immunoprecipitated from a nuclear extract of N1 cells. This extract was made thanks to a Kit (Active Motif).

Cell culture and transfection

1321N1 astrocytoma cells kindly provided by Dr Peter Downes (University of Dundee, Scotland, U.K.) were cultured in DMEM (GIBCO, California USA) supplemented with 5% fetal bovine serum (FBS) and 2% penicillin/ streptomycin. COS-7 cells were cultured in DMEM supplemented with 10% FBS, 2% Penicillin/Streptomycin and 1% sodium pyruvate. Transfections in COS-7 cells were performed with FuGENE® (RocheDiagnostics).

Generation of stable shRNA expressing cells

N1 cells were plated at 1×10^5 cells per well in 12 wells plate one day before the transfection. The cells were transiently transfected with the desired pSUPER constructs by using Lipofectamine 2000 (Invitrogen). After recovery, the cells were selected by 3 days incubation with 3 $\mu\text{g/ml}$ puromycin. Cells resistant to puromycin were counted, diluted, and distributed in 96-well plates to have a cell per well. Clonal populations were then expanded and analyzed for SHIP2 expression by Western blotting.

Incubation of N1 cells in Krebs modified medium

Cells were washed three times in Hepes-buffered, modified Krebs-Henseleit buffer (118 mM NaCl, 4.69 mM KCl, 1.18 mM MgSO_4 , 1.29 mM CaCl_2 , 1.18 mM KH_2PO_4 , 11.67 mM glucose, 25 mM Hepes, pH 7.4 at 37 °C) and preincubated for 30 min at 37 °C in the same buffer [34]. They were then incubated in Krebs modified medium in the presence of bpV(Phen), FCS or EGF.

Lipid extraction and separation

2.5×10^6 1321N1 astrocytoma cells were cultured in complete medium overnight. Cells were washed twice in medium without serum and incubated for 24 hours in this medium. Cells were washed twice in medium without serum and without phosphate. Cells were labeled for 4 hours in medium containing [^{32}P] but not phosphate and stimulated with 10 % FCS for 10 minutes. The phosphoinositides were measured as described before [32].

Protein extracts preparation and immunoprecipitation

N1 cells were lysed in buffer A [50 mM Tris (pH 7.5 with HCl), 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium β -glycerophosphate, 1 mM sodium vanadate, 0.1% (v/v) β -mercaptoethanol, 0.5% (w/v) Triton X-100] and protease inhibitors (Roche, Mannheim, Germany). The cells were scraped, and the lysates were cleared by centrifuged at 14000 x g. The lysates were normalized based on total protein content measured by Bradford Assay (BioRad). This was used to compare SHIP2 expression between ShSHIP2 N1 cells. COS-7 transfected and N1 cells were lysed with ice-cold lysis buffer B [10 mM Tris(pH 7.5 with HCl), 150 mM KCl, 12 mM 2-mercaptoethanol, 100 mM NaF, 0.5% (w/v) Nonidet P-40 (NP-40), 0.01 μM sodium vanadate, 2 mM EDTA] and protease inhibitors. Equal amounts of proteins were subjected to incubation with protein A/G Sepharose (Sigma) in the presence of antibodies or pre-immune sera for 2 hours at 4°C.

SHIP2 phosphosites determination

Proteins of interest were separated from contaminating proteins by SDS/PAGE. The band of interest was excised and subjected to an overnight digestion at 37°C with 250 ng trypsin in 200 mM ammonium bicarbonate. The resulting peptide mixture was analysed by a nano LC-MS/MS approach performed on a Dionex Ultimate capillary liquid chromatography system coupled to an Applied Biosystems 4000 QTRAP mass spectrometer. Peptides were separated on a PepMap C18 column developed with a 30 min linear gradient (0.1 % formic acid - 6 % acetonitrile - water to 0.1 % formic acid - 40 % acetonitrile - water). In a first LC-MS run, putative phosphorylated peptides were detected by precursor 79 (-) ion scanning. In a second LC-MS run, MRM (multiple reaction monitoring) - induced product (+) ion scanning was used to

determine peptide sequences and to localize phosphorylated residues. Tandem MS spectra were manually interpreted.

Mass Spec identification of SHIP2 interactors

Immunoprecipitation was performed as described above, and the precipitates were boiled for 5 min in SDS sample buffer and subjected to 8% SDS/PAGE. The gel was stained with Coomassie Brilliant Blue and destained in 30% methanol in HPLC grade water. The candidate interacting protein bands were excised and digested *in situ* with trypsin as described above. The peptide extract was analysed using a ABI 4800 MALDI TOF/TOF analyzer with α -Cyano-4-hydroxycinnamic acid (5 mg/ml in 50% acetonitrile) as matrix. The MS/MS results were submitted to Mascot 2.2 search engine with SwissProt as target database.

Cell fractionation

The incubation medium of N1 cells was replaced with 1 ml of ice-cold hypotonic lysis buffer [10 mM HEPES pH 7.5, 1.5 mM $MgCl_2 \cdot 6H_2O$, 10 mM KCl, 300 mM sucrose, 5% Ficoll, 1 mM EDTA, 0.25 mM EGTA and protease inhibitors] and the cells were allowed to swell on ice for 10 min. The cells were then scraped and homogenized before centrifugation at 600 g for 10 min at 4°C. The supernatant was collected and centrifuged at 13000 rpm for 10 min at 4°C (referred to the cytosolic fraction). The pellet was washed once with 1 ml of PBS then extracted on ice with 0.5 ml of ice-cold lysis buffer B for 1 hour at 4°C. The supernatant was collected and centrifuged at 13000 rpm for 15 min at 4°C (referred to the nuclear fraction).

Immunofluorescence

Cells were grown on coverslips and washed twice with TBS before fixation in paraformaldehyde 4% for 30 min at room temperature. Fixed cells were permeabilized with 0.1% Triton X-100 in TBS for 30 min at room temperature and thereafter blocked with 10% NHS in TBS containing 0.01% Triton X-100 for 1 hour at room temperature. Coverslips were incubated overnight with primary antibody diluted in blocking solution containing 1% NHS. The coverslips were incubated in the dark with the secondary antibodies for 1 hour at room temperature. Nuclei were stained for 3 min with Hoechst. After mounting with FluorSave™ Reagent anti-fade mounting medium (Calbiochem, Nottingham, UK), slides were examined under an AxioImager Z1 equipped with a plan-Neofluar 40x/0.75 N.A. dry objective and-pass filter sets nr 38,15 and 49 for green, red and blue fluorochromes, respectively. Antibody to $PI(4,5)P_2$ (2C11) was applied as described before [33].

Results

Upregulation of $PI(3,4,5)P_3$ in serum starved ShSHIP2 N1 cells

We have used a model of PTEN null human astrocytoma cells to study the properties of SHIP2. A series of ShSHIP2 N1 independent clones, showing a reduction in SHIP2 protein levels, were generated by transfection with a ShRNA for SHIP2 and selected with puromycin. They were compared with two negative control cells: cells transfected with a ShRNA for PTEN or with the pSUPER vector. Fig 1A shows that SHIP2 expression was selectively decreased in ShSHIP2 cells but not in ShPTEN nor in vector transfected cells. We estimated the reduction in SHIP2 to be 70-80 % after normalization with GAPDH. In both serum starved cells (for 24 hours) and serum (FCS) stimulated cells for 10 min, $PI(3,4,5)P_3$ was upregulated in ShSHIP2

cells as compared to the other two control cell types (Fig 1B). Addition of the PI 3-kinase inhibitor LY-294002 for 30 min, completely abolished the production of PI(3,4,5)P₃ (data not shown).

The increased levels of PI(3,4,5)P₃ in SHIP2-depleted cells in starved and serum-stimulated conditions was also reflected in PI(3,4,5)P₃ downstream signalling. Addition of serum for 10 min after overnight starvation stimulated Akt phosphorylation at Ser 473 and Thr 308 in the three types of cells (vector, ShSHIP2 and ShPTEN cells). The upregulation of phospho Akt was the highest in SHIP2-depleted cells (Fig 1C). Basal Akt phosphorylation (after starvation for 24 hours) was always higher in ShSHIP2 cells as compared to the two control cells. Akt phosphorylation in serum stimulated cells was reduced in the presence of the PI 3-kinase inhibitor LY-294002 (data not shown).

Lowering SHIP2 expression in N1 cells has an impact on cell growth

In N1 cells, SHIP2 immunoreactivity (–ir) detected with a total SHIP2 antibody was shown both in the perinuclear area and in the nucleus (Fig 2A). The morphology of the ShSHIP2 N1 cells was also different as compared to control cells. As shown by F-actin staining, cells were less rounded and more elongated as compared to N1 cells (Fig 2A). Cell growth was markedly increased in ShSHIP2 transfected cells as compared to vector. This effect was confirmed in two independent ShSHIP2 N1 clones obtained from different transfections and never seen in vector or ShPTEN N1 clones used as negative controls (Fig 2B). This effect on cell growth upon lowering SHIP2 expression was reversed in cells infected with a lentiviral construct of SHIP2 (data not shown). We concluded that in N1 cells, SHIP2 controls Akt phosphorylation, cell morphology and growth.

SHIP2 phosphorylation in N1 cells on S132, T1254 and S1258

We have shown that SHIP2 controls PI(3,4,5)P₃ levels in N1 cells by comparing SHIP2 depleted cells to normal cells. SHIP2 phosphorylation on tyrosine residues has been often presented as a mechanism to switch on SHIP2 activity and to lower PI(3,4,5)P₃ levels at the plasma membrane [32]. We therefore aimed to identify SHIP2 phosphosites in that specific model. To optimize the identification at endogenous level in N1 cells, we first used a model of SHIP2 transfected COS-7 cells where we would not be limited by the amount of material. We identified SHIP2 phosphosites in SHIP2 transfected COS-7 cells in response to EGF, PDGF, insulin and the vanadate stable analog bpV(Phen) by mass spectrometry. The identity of the phosphosites was established by a combination of precursor ion scanning and multiple reactions monitoring (MRM) induced MS/MS. 8 phosphosites on Tyr, Ser and Thr residues were identified: 6 sites could be found in data bases (PhosphoSitePlus) but 2 (i.e. S1180 and T1254) were new phosphosites (Fig 3A).

Endogenous SHIP2 phosphosites were determined in N1 cells (Fig 3B). In this model, SHIP2 was strongly phosphorylated on tyrosine in response to the vanadate stable analog bpV(Phen) in agreement with published data [24]: a total cell lysate probed with phosphotyrosine is shown in Fig 3C. However, this increase in tyrosine phosphorylation was only observed in a particular context of cell incubation; it required a preincubation of the cells in Krebs modified medium (medium described by others in [34] and in the Methods section). It was no longer observed in DMEM medium in serum starved cells or cells maintained in 5 % FCS which are the

conditions we have used to study the influence of SHIP2 on PI(3,4,5)P₃ and phospho Akt (Fig 1C). These conditions (in DMEM medium) were also used to identify SHIP2 phosphosites in COS-7 cells (Fig 3A). Starved N1 cells were stimulated by bpV(Phen) for 30 min in DMEM medium: no phosphorylation on tyrosine was detectable by Western blotting (Fig 3C). This contrasted with similar experiments performed in SHIP2 transfected COS-7 cells where SHIP2 was found to be tyrosine phosphorylated, particularly in response to EGF.

SHIP2 phosphosites were identified by mass spec in N1 cells at endogenous level (Fig 3B): phosphorylation sites on S132, T1254 and S1258 were detected but no phosphosites on tyrosine as expected from our Western blot analysis (Fig 3C). MS/MS data confirmed the presence of these phosphopeptides in serum stimulated cells.

The specificity of a novel pSHIP2 S132 antibody

A purified antibody was prepared against the phosphopeptide CPDDRDApS(132)DGEDE in the sequence of human SHIP2. Its specificity was estimated in transfected COS-7 cells with a S132A SHIP2 mutant (Supplementary Fig S1A on line). When probed with pSHIP2 S132 antibody, there was no signal in cells transfected with the SHIP2 S132A mutant (in contrast to the signal obtained with SHIP2 wild type at 150 kDa). The 150 kDa band was also present in MEFs +/+ for SHIP2 but absent in -/- cells (Supplementary Fig S1B). In both N1 and in HeLa cells (at endogenous level), SHIP2 immunoreactivity with pSHIP2 S132 antibody was shown at the expected molecular weight (i.e. 150 kDa) (the data in N1 cells are shown in Fig 4A). The influence of EGF and serum was estimated in N1 cells: SHIP2 phosphorylation was not modified in response to EGF and serum up to 60 min stimulation. To assess the specificity of the signal in N1 cells, we used the antigenic peptide in competition experiments: the pSHIP2 S132 signal on Westerns was no longer present when the purified antibody was mixed with the antigenic phosphorylated but not the nonphospho peptide (Figs 4A and 4B, lower part of the Figures). This was not observed with another phosphospecific antibody p-Akt S473 mixed with the same peptides (i.e the pSHIP2 S132 phospho peptide, Fig 4B and nonphospho peptide, Fig 4A).

pSHIP2 S132 co-localization with the splicing factor SC35

Fixed N1 cells were stained with pSHIP2 S132 antibody: the staining was both cytoplasmic and in nuclear speckles as demonstrated by co-staining with the splicing factor SC35 (Fig 4C). pSHIP2 S132-ir was totally prevented by the addition of phosphorylated antigenic peptide but not with the nonphospho peptide (Supplementary Fig S2). Data reported by others indicated a speckle cell cycle: the interphase speckled pattern disperses as cells enter prophase of mitosis [35]. Nuclear pSHIP2 S132-ir co-localized with SC35 and was also markedly influenced by the cell cycle (Fig 5). It was maximal at interphase and anaphase.

Constitutive tyrosine phosphorylation of SHIP2 has been reported in p210 (bcr/abl)-expressing haematopoietic cellss [26]. In this model, no phosphorylation on S132 was detected on SHIP2 (data not shown). In HeLa cells, pSHIP2 S132-ir was also detected in the cytoplasm, the nucleus and speckles in both starved and serum stimulated cells. This was also the case in normal human thyroid cells in primary culture (Supplementary Fig S3). Again as shown in N1 cells, the fraction of cells for

which we see a staining in speckles was very much influenced by the cell cycle: pSHIP2 S132 -ir in speckles was at best seen in interphase (data not shown).

We tried to overexpress SHIP2 wild type and SHIP2 S132A in N1 cells by lentiviral transduction: cells infected by wild type SHIP2 appeared to cluster together. This was not shown with mutated S132A. Therefore, the morphology of the cells was markedly affected in the model of N1 cells (data not shown). We feel that this is a major effect of SHIP2 overexpression. It will influence the association between SHIP2 and protein partners that occurs in equilibrium at endogenous level. This will complicate any interpretation of overexpressing mutated forms of SHIP2 in N1 cells.

SHIP2 interaction with lamin A/C

The fact that our pSHIP2 S132 antibody was able to stain nuclear entities prompted us to look for the presence of SHIP2 in a nuclear fraction of N1 cells. We prepared cytoplasmic and nuclear extracts of N1 cells and could indeed detect SHIP2 in the nuclear fraction as the nuclear marker lamin A/C which was not detected in the cytoplasmic fraction (Fig 4D). Moreover, pSHIP2 S132 was enriched in the nuclear fraction in contrast to total SHIP2 which was more abundant in the cytoplasmic fraction (Fig 4D). We also looked for possible nuclear proteins interacting with SHIP2 in N1 cells after SHIP2 immunoprecipitation: lamin A/C was identified by mass spec analysis, and the interaction with both SHIP2 and pSHIP2 S132 confirmed by reciprocal co-immunoprecipitation (Figs 4E and F for IP-SHIP2 and IP-Lamin A/C, respectively). Stimulation of the cells with serum did not affect the interaction.

Another SHIP2 interactor is PR130/B α 1 (PR130) regulatory B-type subunit of protein phosphatase 2A. This protein was previously reported to interact with SHIP2 in the model of COS-7 cells and fibroblasts 3T3 cells [29]. In N1 cells, PR130 immunostaining is largely nuclear (Supplementary Fig S4) and we confirmed the interaction between PR130 and SHIP2 at endogenous level by co-immunoprecipitation (data not shown). Together, we show the interaction between SHIP2 and two nuclear proteins: lamin A/C and PR130.

pSHIP2 S132 phosphatase activity

Evidence has been provided that SHIP2 is a PI(3,4,5)P₃ phosphatase but it is also able to dephosphorylate PI(4,5)P₂ [25,36]. The nuclear fraction of N1 cells was used as starting material to immunoprecipitate pSHIP2 S132 (Fig. 6). The immunoprecipitated phospho-enzyme was shown to be active with respect to both PI(3,4,5)P₃ and PI(4,5)P₂ as substrate (Fig. 6B and C). PtdIns(4,5)P₂ -ir in N1 cells (detected by the use of a mouse monoclonal antibody 2C11) was in the nucleus and partly in speckles identified by SC35 co-staining (Supplementary Fig. S5). pSHIP2 S132 -ir showed an overlap with the 2C11 staining (Fig. 7). A comparison of PtdIns(4,5)P₂ staining in the nucleus of N1 and ShSHIP2 N1 cells showed that 2C11 staining could be seen in the two types of cells but that it was always stronger in ShSHIP2 N1 cells as compared to N1 cells.

Influence of S132 phosphorylation on SHIP2 sensitivity to calpain in COS-7 cells

Crude lysates prepared from SHIP2 transfected COS-7 cells show the presence of two immunoreactive bands at 150 and 135 kDa when probed with an N-terminal antibody X-press, Fig 8A. The ratio between the high and low molecular weight forms was always higher in SHIP2 S132A mutant (17.3 % \pm 5.5, n=5) as compared to wild type (3.6 % \pm 0.7).

The sequence of SHIP2 showed the presence of sequence areas with unusually high concentrations of proline, glutamic/aspartic acid, serine and threonine, i.e., PEST sequences that might be target for calpain cleavage [37]. Incubation of wild type SHIP2 in the presence of calpain resulted in the loss of the 150 kDa SHIP2 protein that was time and calpain concentration dependent. This was not observed with SHIP2 S132A mutant that was more resistant to proteolysis by calpain as compared to wild type (Fig 8B). We concluded that wild type SHIP2 at 150 kDa, phosphorylated on S132, appeared more sensitive to C-terminal truncation as compared to the S132A mutant.

Influence of S132 phosphorylation on SHIP2 activity in COS-7 cells

We next questioned whether SHIP2 phosphorylation on S132 had an impact on phosphatase activity in the model of COS-7 cells. In this model, EGF stimulates SHIP2 tyrosine phosphorylation [8]. PI(3,4,5)P₃ phosphatase activity was determined in immunoprecipitates of SHIP2 transfected cells (Fig 8C). Phosphatase activity was normalized with respect to SHIP2 protein expression. SHIP2 phosphatase activity of wild type SHIP2 was increased in EGF-stimulated cells. This was not observed in cells transfected with SHIP2 S132A nor SHIP2 Δ SH2 where SHIP2 was not phosphorylated on S132 (Fig 8C). SHIP2 phosphorylation on tyrosine in response to EGF stimulation was present on SHIP2 wild type and S132A mutant. We concluded that S132 phosphorylation has an impact on SHIP2 conformation and sensitivity to EGF stimulation of phosphatase activity in the model of COS-7 cells.

Discussion

SHIP2 is involved in many human diseases such as diabetes and cancer [1,2,4,38]. Therefore, the mechanisms that control SHIP2 activity and localization are fundamental questions to address. In this study, we identified three phosphosites of SHIP2 in astrocytoma cells: S132, T1254 and S1258. These findings were unexpected as SHIP2 was essentially reported to be tyrosine phosphorylated [24,32]. A phosphospecific antibody for pSHIP2 S132 showed a very specific staining pattern in the nucleus and in speckles. Interestingly, a co-staining between pSHIP2 S132-ir and PI(4,5)P₂ was observed in the nucleus. Finally, we show that SHIP2 S132 phosphorylation influences proteolytic C-terminal truncation of the protein.

Previous evidence has been provided that both SHIP1/2 act on agonist-provoked stimulated levels of PI(3,4,5)P₃, whereas PTEN acts at basal level and is still active after long-term stimulation [32]. This was observed in SHIP2-deficient MEF cells. As shown here, PI(3,4,5)P₃ and Akt phosphorylation were upregulated in ShSHIP2 N1 cells as compared to control cells but this was already shown in serum-starved cells. Since astrocytoma cells are PTEN-null, our data suggest that SHIP2 function is directly or indirectly influenced by PTEN expression levels, a situation which is particularly critical in numerous cancer cells [3]. We have observed that lowering SHIP2 expression in astrocytoma cells increases cell growth. The fact that SHIP2 negatively controls PI(3,4,5)P₃ and Akt in our model is consistent with this observation. In glioblastoma cells (where PTEN is mutated as well), SHIP2 overexpression caused a potent cell cycle arrest in G1 [25].

Global proteomic studies in human cells revealed that SHIP2 can be phosphorylated at more than 20 sites (Tyr, Ser and Thr): the significance of this is however not understood. SHIP2 phosphorylation on tyrosine has been reported in response to EGF in HeLa or COS-7 cells, FGF or insulin [8,22]. In N1 cells, this was not observed in any condition we tested (in starved cells or in response to EGF or serum). Instead, we have identified three phosphosites on Ser and Thr residues. SHIP2 phosphosite at S132 had been reported in HeLa cells stimulated by EGF in a global proteomic approach but it was not characterized [12]; the two other sites identified here (T1254 and S1258) were unknown in data bases (e.g. in PhosphoSitePlus) thus far.

The specific activity of SHIP2 in N1 cells was reported to be increased by the vanadate stable analog bpV(Phen) [24]. This was proposed to occur through an increase in SHIP2 tyrosine phosphorylation. Our data clearly indicate that in N1 cells, SHIP2 tyrosine phosphorylation is probably supramaximal due to a preincubation step of the cells in Krebs modified medium used in the studies of Batty *et al.* We did not observe any SHIP2 tyrosine phosphorylation at all when the cells were kept in DMEM. This was shown both by immunodetection with an antiphosphotyrosine antibody and by phosphosite identification. Interestingly, when the same mass spec approach was applied in COS-7 cells in DMEM, a series of phosphosites were identified on Ser/Thr and Tyr residues in serum starved and EGF (or insulin) stimulated cells. This suggests that the identity of SHIP2 phosphosite(s) is very much cell type specific and differs in relation with the expression of SHIP2 kinases/phosphatases across tissues. Our data in astrocytoma cells indicate that SHIP2 is active to modulate PI(3,4,5)P₃ levels in cells in the absence of any tyrosine phosphorylation.

Could SHIP2 phosphorylation on Ser be linked to its location to the cell membrane close to PI(3,4,5)P₃ ? We tested this hypothesis in starved and serum stimulated astrocytoma cells using a novel phosphospecific antibody pSHIP2 S132. We never detected any signal at the cell periphery at endogenous level. Instead, pSHIP2 S132-ir concentrates in the cytoplasm, in the nucleus and in nuclear speckles where it co-stained with SC35 in the three cell models we tested. The fact that we could confirm the data in normal human thyroid cells in culture suggests that a nuclear staining also occurs in normal cells and is not limited to cancer cell lines. The staining of pSHIP2 S132 in speckles is however depending on the cell cycle being at best observed in interphase. The data are consistent with another report where SHIP2 was expressed in vascular smooth muscle cell nuclei and associated with speckles [39] except that we only see co-staining of SC35 with pSHIP2 S132 but not with total SHIP2. The use of different cells or antibodies may explain the discrepancy. However, in our hands it was crucial to use the purified antibody against the phosphorylated peptide on S132 to show a staining in speckles. We could also detect total SHIP2 in the nucleus in N1 cells using total SHIP2 antibody. In this case however, we used a commercial antibody (from Abcam catalogue number ab70267) made against a non phosphorylated peptide sequence as antigen (a synthetic peptide, corresponding to amino acids 100-150 of human SHIP2 and thus encompassing a non-phospho S132). This could be the reason why SHIP2 -ir could hardly be detected in speckles using a total SHIP2 antibody as compared to our pSHIP2 S132 antibody.

SHIP2 does not possess a classical NLS. The presence of phosphorylated SHIP2 in speckles was therefore surprising given the fact that PtdIns(3,4,5)P₃ itself is not

present in speckles. $\text{PtdIns}(3,4,5)\text{P}_3$ has been reported in the nuclear matrix but the expected 5-phosphatase product $\text{PtdIns}(3,4)\text{P}_2$ was not present at that location [40]. We show here that $\text{PtdIns}(4,5)\text{P}_2$ is also a substrate of SHIP2 as determined in immunoprecipitates of pSHIP2 S132. The importance of nuclear $\text{PtdIns}(4,5)\text{P}_2$ is now well established from the studies of the Anderson's lab and others [41,42]. Its presence in nuclear speckles has been reported in many different cells [43] and confirmed in N1 cells. A comparison of 2C11 staining in the nucleus of N1 and ShSHIP2 N1 cells shows that $\text{PtdIns}(4,5)\text{P}_2$ staining was always stronger in ShSHIP2 N1 cells as compared to N1 cells. This would suggest a negative control of nuclear SHIP2 on $\text{PtdIns}(4,5)\text{P}_2$ in the nucleus.

SHIP2 interaction with nuclear proteins is also characteristic of its nuclear localization. Two of such proteins were identified in this study: lamin A/C and PR130 regulatory subunit of protein phosphatase 2A. In N1 cells, PR130 is largely nuclear. Interestingly, PR130 shows the presence of three NLS [44,45]. So the interaction between SHIP2 and at least two nuclear proteins lamin and PR130 could explain the fact that part of SHIP2 staining is nuclear. We suggest that a fraction of total SHIP2 is phosphorylated in the cytoplasm and then concentrates in the nucleus and in speckles in association with other proteins. It was proposed that signalling effectors related to lamin A/C may be implicated in the pathogenesis of laminopathies [46]. Our data indicate that SHIP2 and phosphorylated SHIP2 could be part of these effectors. They also provide the molecular basis for a nuclear localization of SHIP2 in a speckled pattern: it is of interest that a localization of lamin A in speckles has been reported in HeLa cells [47].

The presence of SHIP2 multiple forms has been reported before in human leukemia cells [26]. We always detect two forms of SHIP2 by Western blot with an N-terminal antibody: an estimate of the ratio of SHIP2 high and low molecular weight forms between SHIP2 wild type and S132A transfected cells shows SHIP2 S132A to be less sensitive to C-terminal truncation. This was confirmed by the effect of calpain on SHIP2 expression as determined by Western blotting. Our data suggest that wild type SHIP2 phosphorylated on S132 displays a conformational change that has an impact on protein C-terminal truncation. Interestingly, the activity of SHIP2 can be regulated at the protein level i.e via its degradation in response to interleukin-4 [48] and PTEN phosphorylation on Thr366 was also less stable as compared to a mutated enzyme at that position [49].

In HeLa cells, SHIP2 activity appears to be stimulated by EGF [50]. This was confirmed in our study in SHIP2 wild type COS-7 transfected cells. It was not observed in SHIP2 S132A mutant (that cannot be phosphorylated on S132) despite the fact that this mutant was tyrosine phosphorylated in EGF-stimulated cells. Therefore, SHIP2 phosphorylation on S132 has an impact on its activity at least in the model of COS-7 cells stimulated by EGF.

In conclusion, by the use of an antibody against a pSHIP2 S132 in astrocytoma cells, our data indicate that a fraction of SHIP2 is located in the nucleus and in speckles. Our data suggest that SHIP2 function is very different at the plasma membrane where it recognizes $\text{PtdIns}(3,4,5)\text{P}_3$ and in the nucleus where it may control $\text{PtdIns}(4,5)\text{P}_2$, particularly in speckles. Therefore, SHIP2 activators, Ser/Thr kinase

and phosphatase modulators may be effective in cancers where PTEN is deleted and more generally in SHIP2 signalling functions.

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Legends to the Figures

Figure 1 Status of SHIP2 expression in ShSHIP2, ShPTEN and vector transfected N1 cells. (A) 50 µg of protein was applied to each lane for Western blotting. The blot was probed with SHIP2 and GAPDH antibodies. Cl1, Cl2 and Cl3 represent three isolated clones in the three set of cells, (B) Cells were starved overnight and labeled with [³²P] orthophosphate. PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ levels were determined following addition of FCS 10 % for 10 min and compared to control unstimulated cells, (C) N1 cells were serum starved followed by the addition of 10 % FCS (10 min) as compared to control (CTL) cells. The blots were probed with SHIP2, p-Akt T308, p-Akt S473, total Akt and GAPDH. The data are representative of two independent experiments performed in duplicate.

Figure 2 SHIP2 immunolocalisation in N1 and N1/ShSHIP2 cells. (A) SHIP2 (green) was stained with the Abcam SHIP2 antibody and a secondary DyLight488 antibody. Actin cytoskeleton was stained with phalloidin-Alexa Fluor594 (red). Nuclei were stained with Hoechst in blue. Bar 10 µm, (B) The growth of N1 cell was increased in ShSHIP2 cells as compared to control cells (vector and N1/ShPTEN cells). 10,000 N1 cells of each series were seeded in triplicates in the absence of serum for 24 h. The medium was then made in 10 % serum and the cells were counted daily (n=3) after 24 hours. Results are means ± S.E.M.

Figure 3 (A) SHIP2 phosphosites in SHIP2 transfected COS-7 cells and (B) at endogenous level in N1 cells. The phosphosites identified by mass spec are indicated above SHIP2. Three phosphosites were identified in N1 cells at endogenous level, (C) Absence of tyrosine phosphorylation in N1 cells when cultured in DMEM medium in response to bpV(Phen) 100 µM, 10 % serum or 50 ng/ml EGF (10 min each except Phen which was added for 30 min). Tyrosine phosphorylation (p-Tyr) was observed in response to Phen in Krebs modified medium only. The same blot was probed with p-Akt S473, total Akt, p-Erk 1/2, total Erk 2 and GAPDH.

Figure 4 Specificity of pSHIP2 S132 staining and lamin A/C interaction with SHIP2. Western blot analysis of SHIP2 (A) and pSHIP2 S132 (B); FCS was added for 30 min. P-Akt S473 was used to probe the blots for Akt phosphorylation. The lower part of the Figure shows an immunoblot of N1 cells probed with a mix of pSHIP2 S132 antibody and either antigenic phospho peptide or nonphospho peptide (both at 0.5 µg/ml). The same blots were also probed with p-Akt S473 antibody mixed with the same two peptides. GAPDH was used a loading control, (C) Fixed N1 cells were used for immunostaining. pSHIP2 S132 (red) was stained with the rabbit antibody and a secondary DyLight549 antibody. SC35 (green) was stained with the mouse monoclonal antibody to SC35 and a secondary DyLight488. Bar 5 µm, (D) Cytoplasmic and nuclear fractions were prepared from 1 mg of N1 cells (control and FCS stimulated, 10 min) and analyzed by Western blotting with SHIP2, pSHIP2 S132, lamin A/C and GAPDH antibodies. 50 µg of protein was applied to each lane, (E) A total lysate of N1 cells (1 mg) made in 0.5 % NP-40 was immunoprecipitated with SHIP2 antibody (IP-SHIP2) and probed for pSHIP2 S132, SHIP2 and lamin A/C, (F) Immunoprecipitation was performed as in (B) but with a lamin A/C antibody (mouse monoclonal, IP-Lamin A/C). The negative control was made in the presence of lysate but no antibody. The data are representative of three independent experiments.

Figure 5 pSHIP2 S132 staining through the cell cycle. N1 cells were fixed and subjected to immunostaining. The various phases of the cell cycle are indicated. pSHIP2 S132 and SC35-ir were probed as in Fig 4. Nuclei were stained with Hoechst in blue. Bar 5 μ m.

Figure 6 pSHIP2 S132 PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ phosphatase activity. (A) 1 mg of a nuclear extract of N1 cells was used in immunoprecipitation by pSHIP2 S132 antibody. The immunoprecipitate (IP pSHIP2 S132) was subjected to SDS/PAGE and probed with pSHIP2 S132. The supernatant after the IP and first centrifugation, and the pre-immune serum were used as negative controls, (B and C), The immunoprecipitate from 1 mg of protein was divided in 5 equal fractions; those were used for determination of activity (either PI(3,4,5)P₃ or PtdIns(4,5)P₂ phosphatase activity) and blank values. Activity was expressed as mean values \pm S.E.M. Bacterial SHIP2 was used as positive control of activity.

Figure 7 PtdIns(4,5)P₂ (2C11) immunolocalisation in N1 and N1/ShSHIP2 cells. N1 cells were fixed and stained for nuclear PtdIns(4,5)P₂ (2C11) in green and pSHIP2 S132 in red. Nuclei were stained with Hoechst in blue. Bar 5 μ m

Figure 8 Influence of SHIP2 S132 phosphorylation on SHIP2 C-terminal truncation and phosphatase activity. (A) Evidence of SHIP2 multiple forms in COS-7 transfected cells by SHIP2 wild type (WT), SHIP2 Δ SH2 and SHIP2 S132A. Total lysates were probed for X-press. The ratio between the upper and lower band of SHIP2 was expressed as mean values \pm S.E.M., (B) Sensitivity of SHIP2 WT and SHIP2 S132A transfected COS-7 cells to calpain. Calpain was added for the indicated time at 30°C. Blots were probed for X-press, (C) SHIP2 immunoprecipitation of 1 mg of COS-7 transfected cell lysates stimulated by EGF at 50 ng/ml (5 min). Half of the immunoprecipitate was used for Western, 1/10 for PI(3,4,5)P₃ phosphatase activity. Activity was expressed as mean values \pm S.E.M. Statistical analysis was performed using ANOVA. ** $p < 0.01$; and *** $p < 0.001$; n.s. not significant. The data are representative of three independent experiments.

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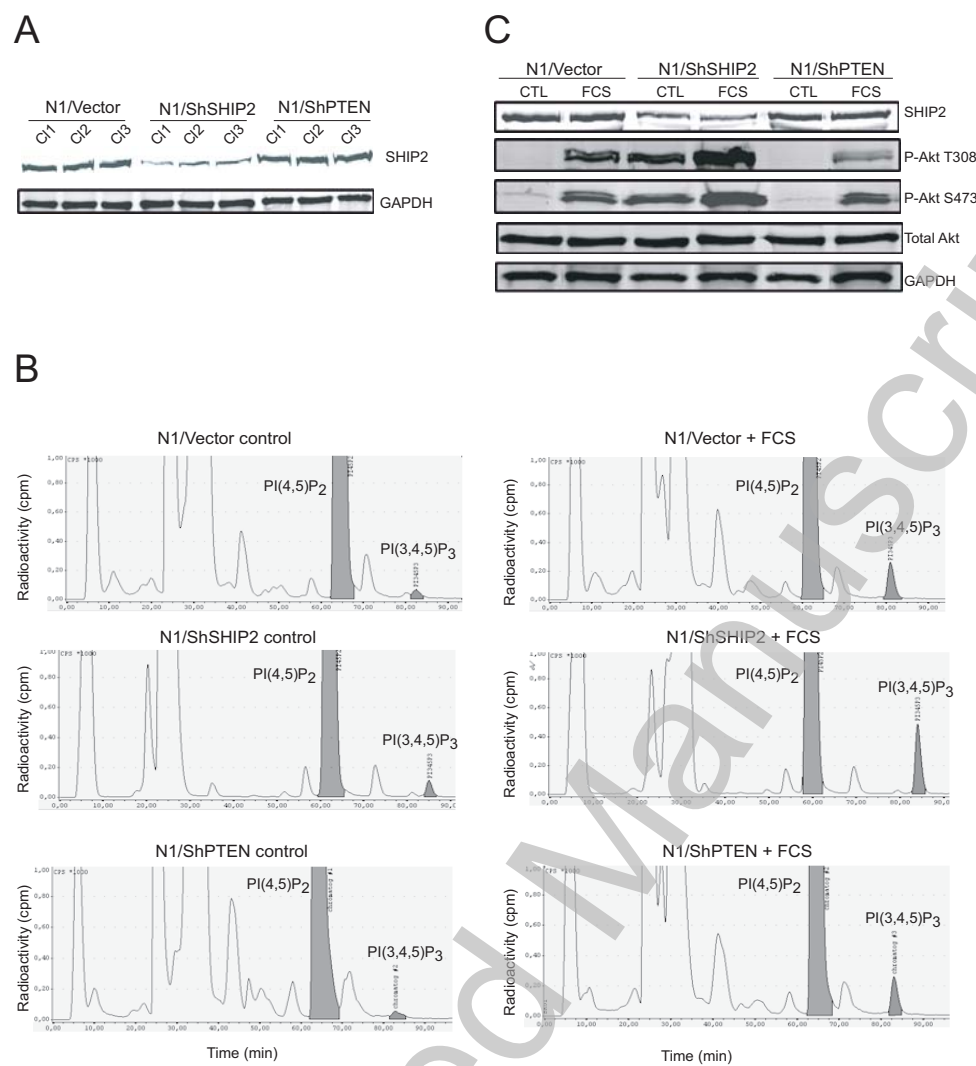


Figure 1

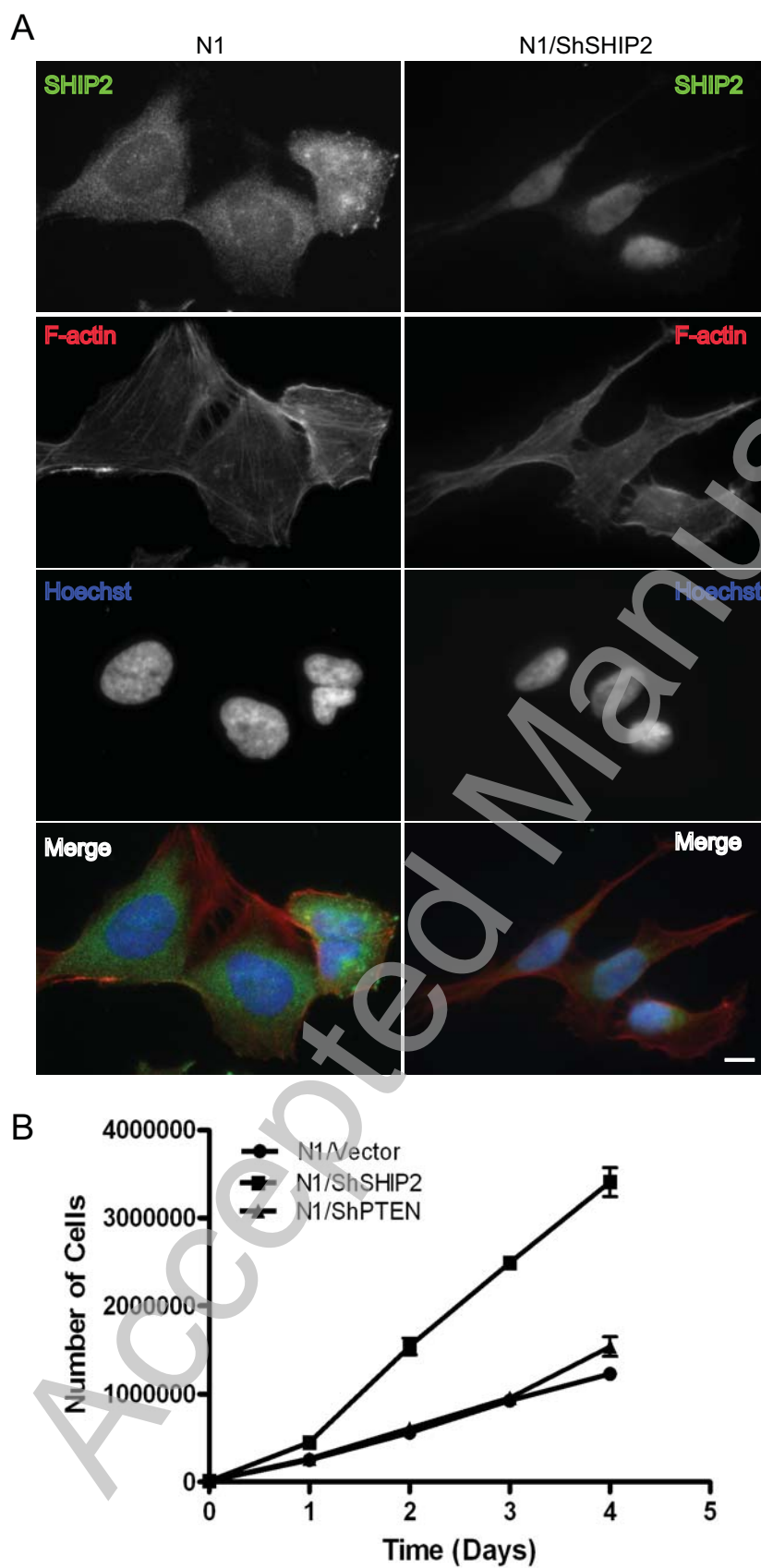


Figure 2

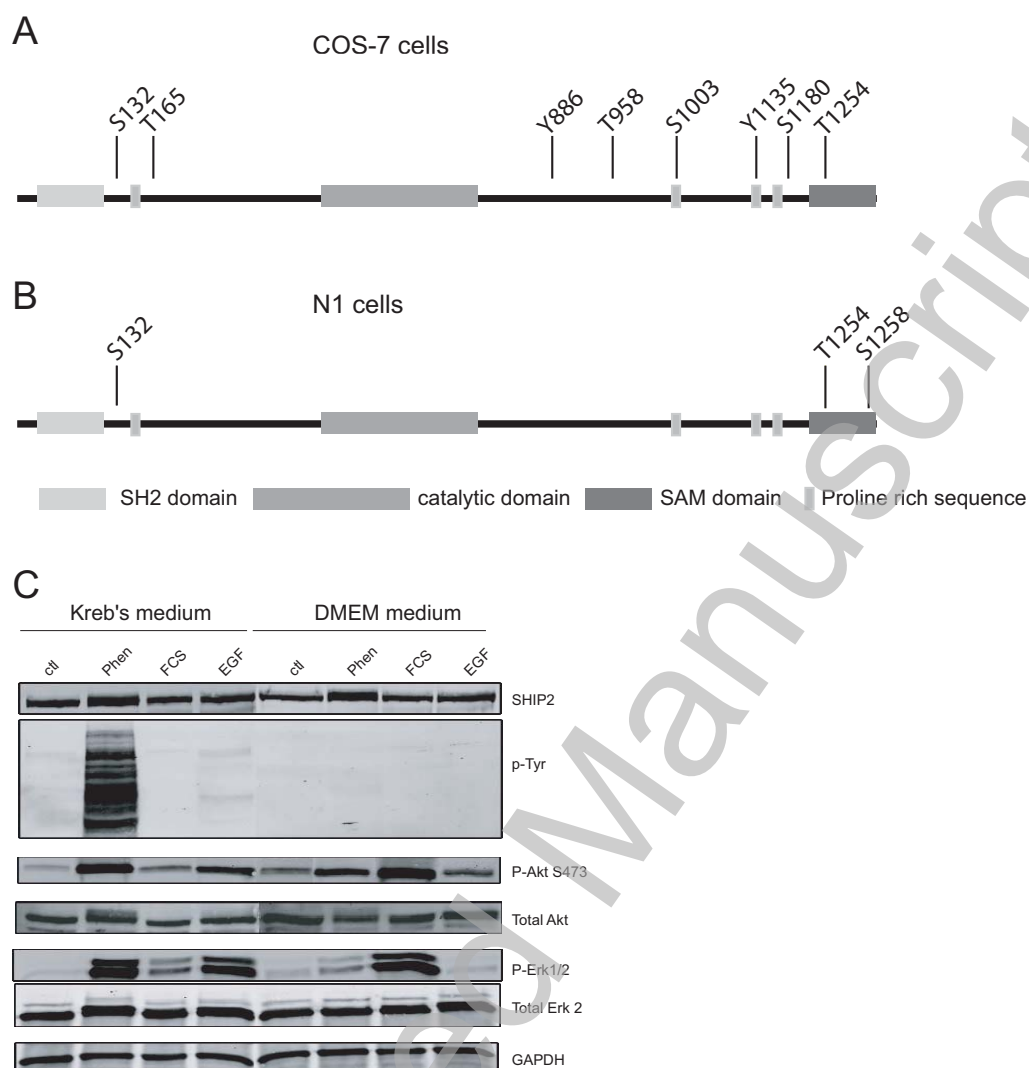


Figure 3

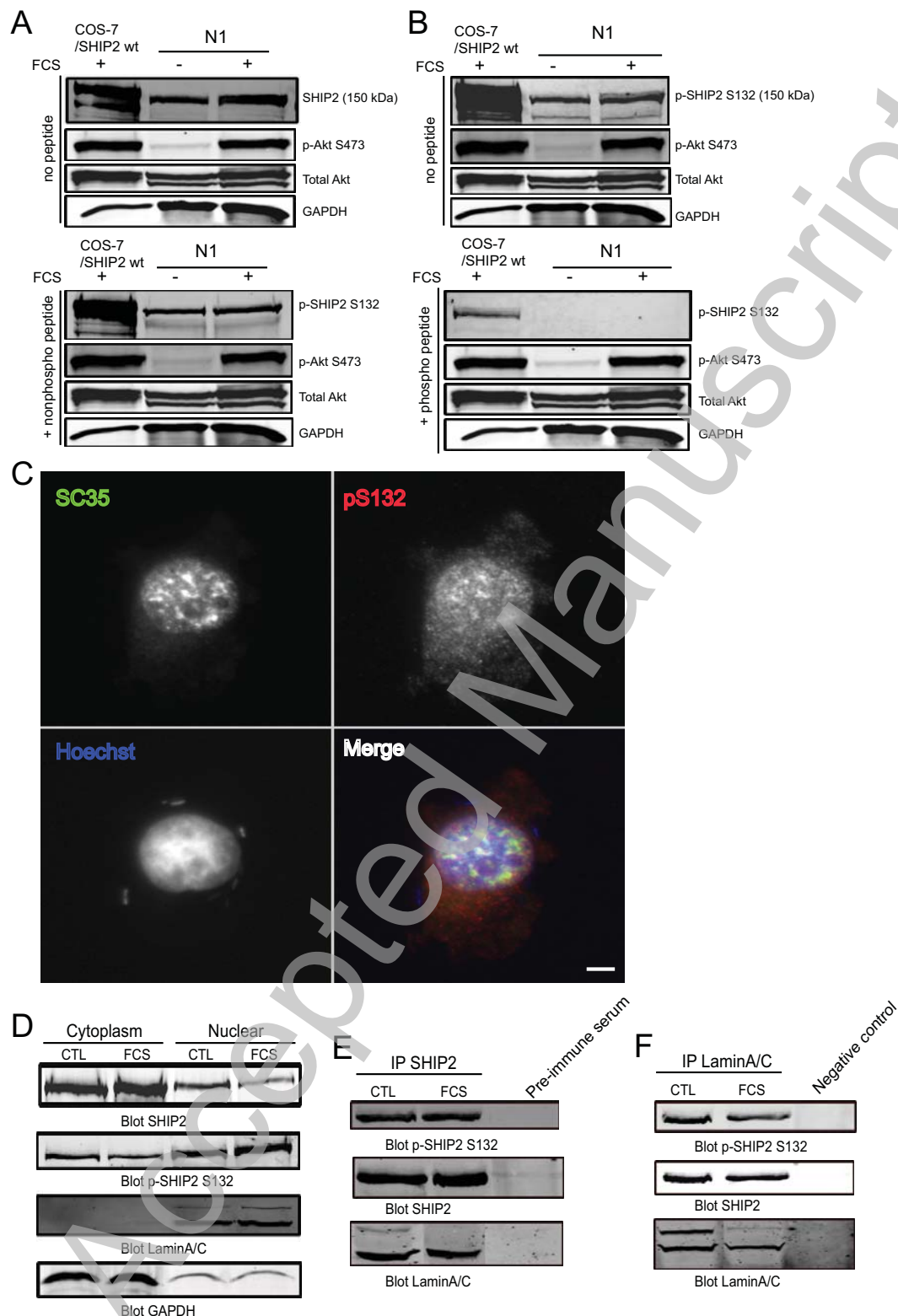


Figure 4

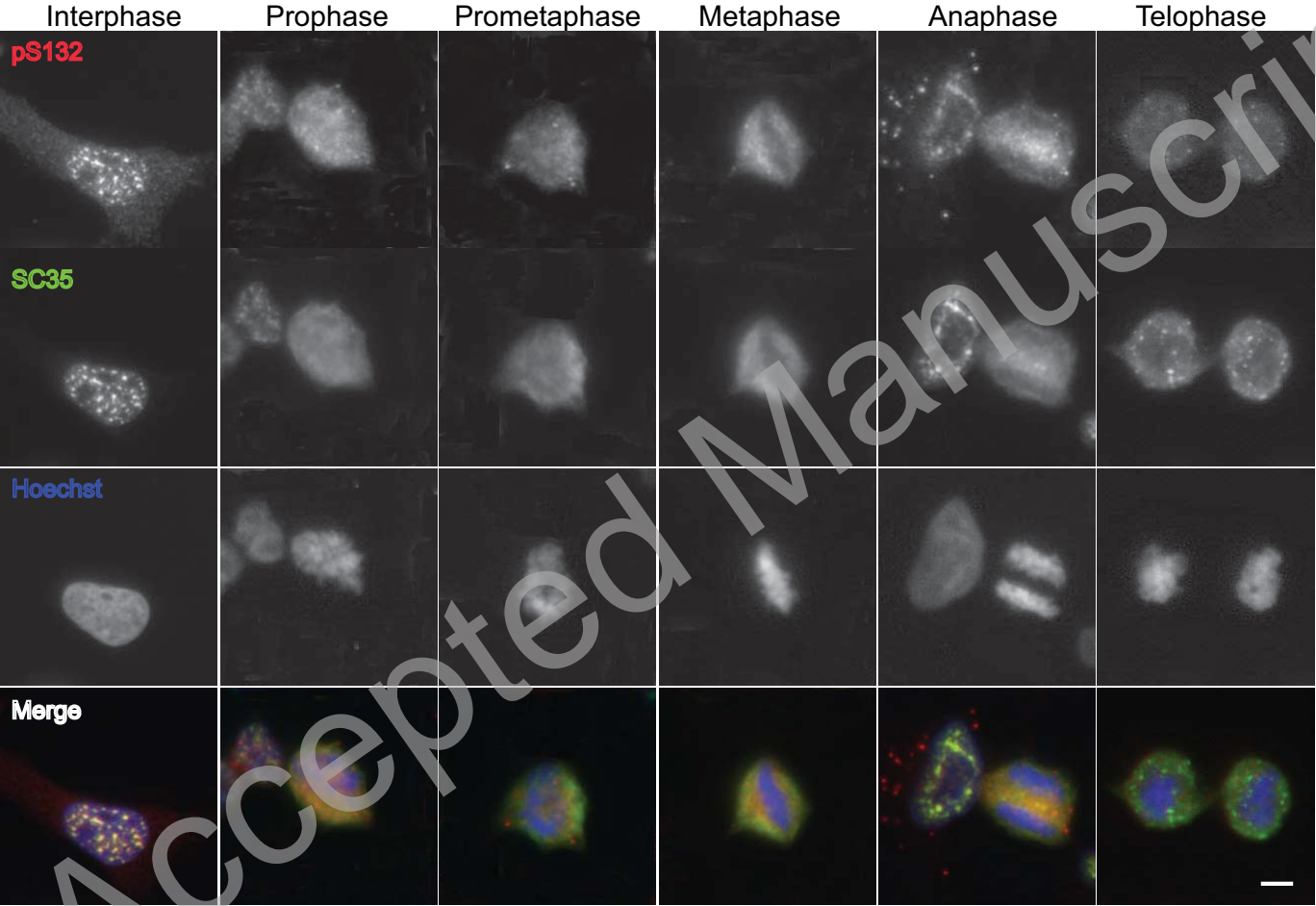


Figure 5

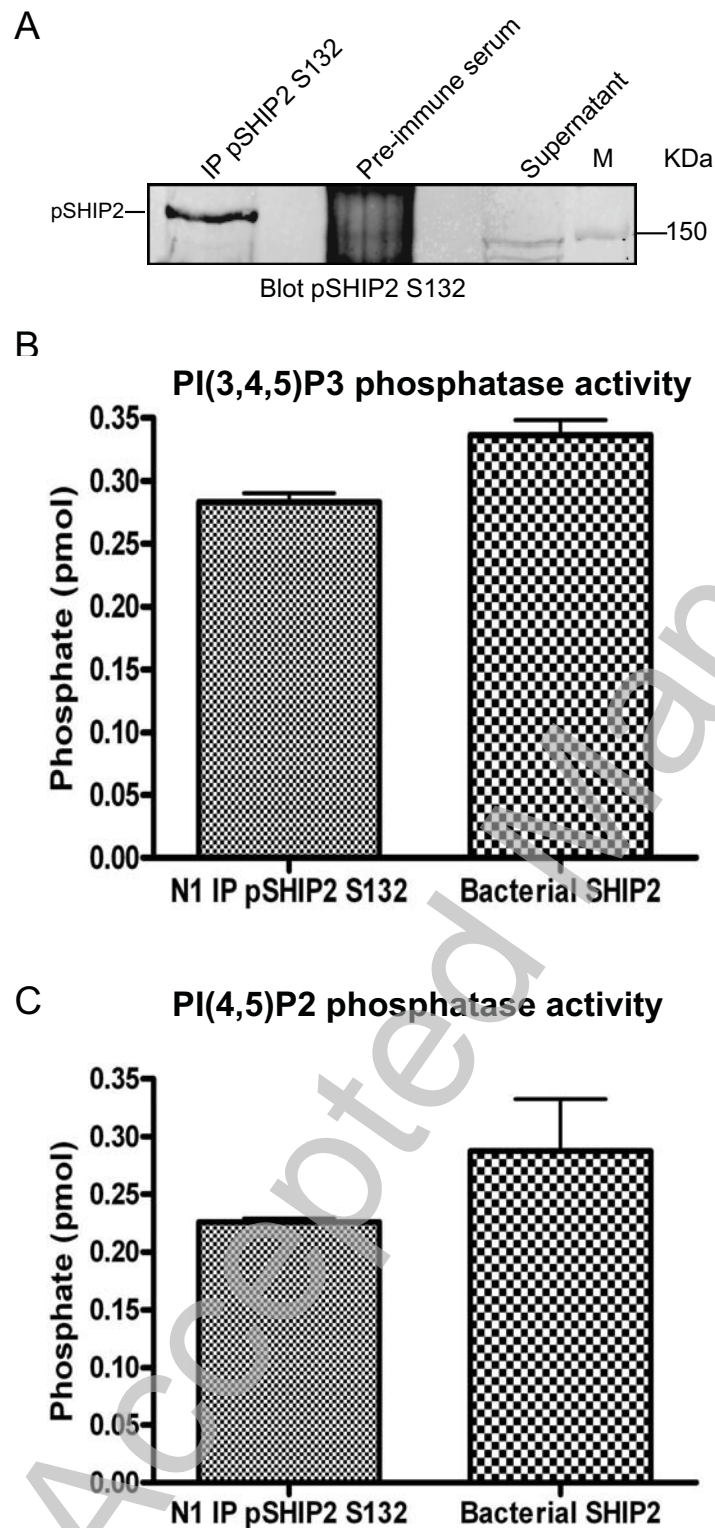


Figure 6

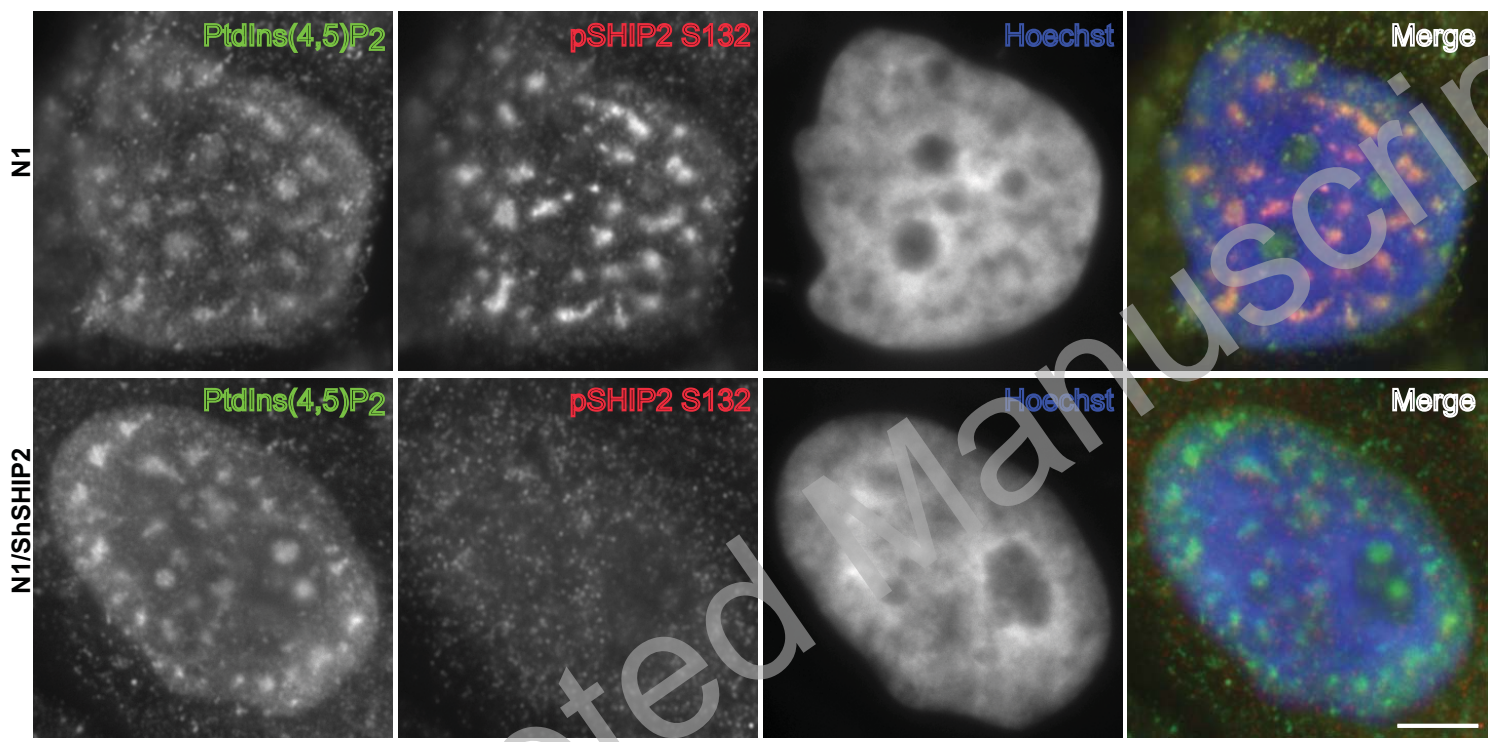


Figure 7

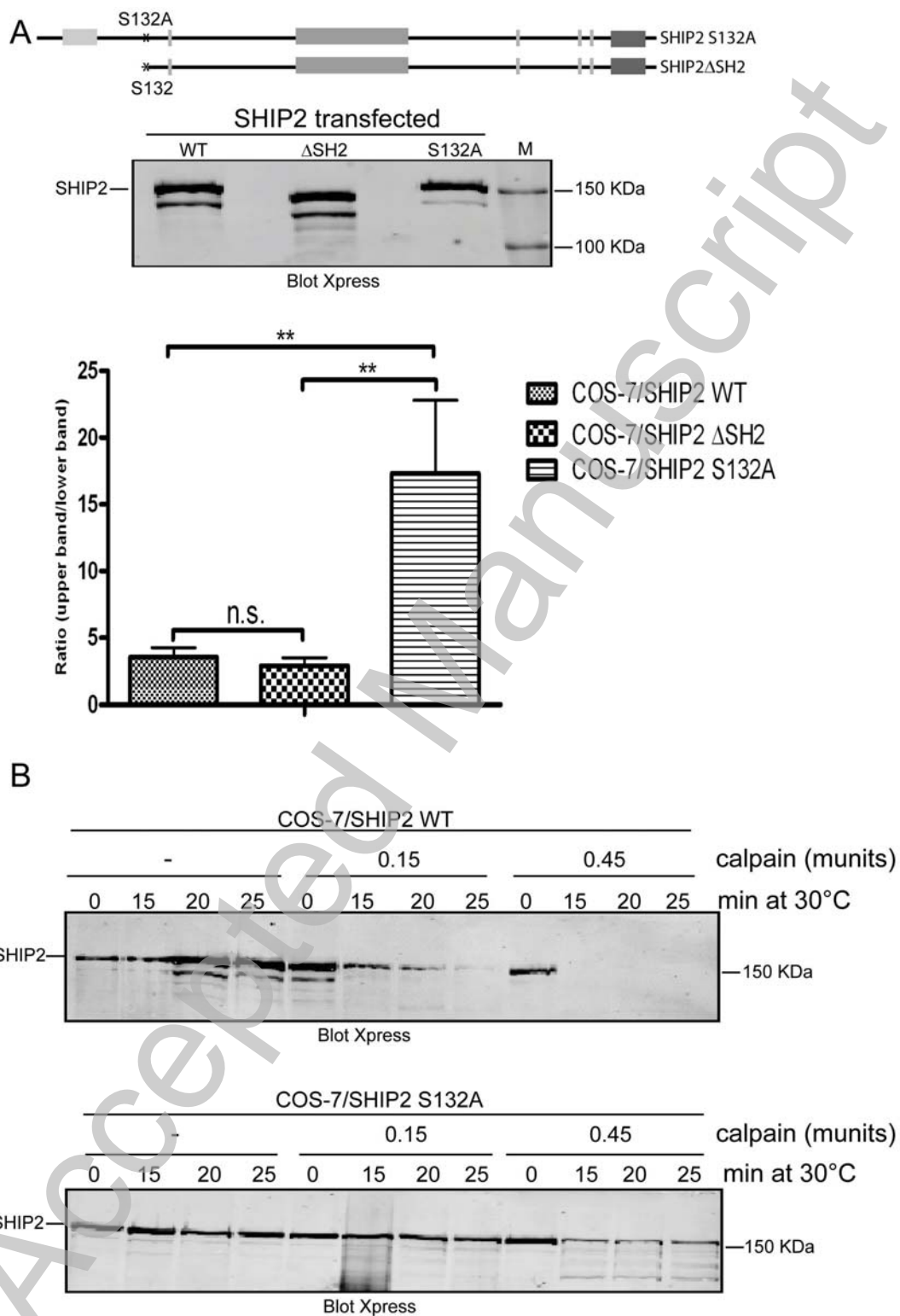


Figure 8

C

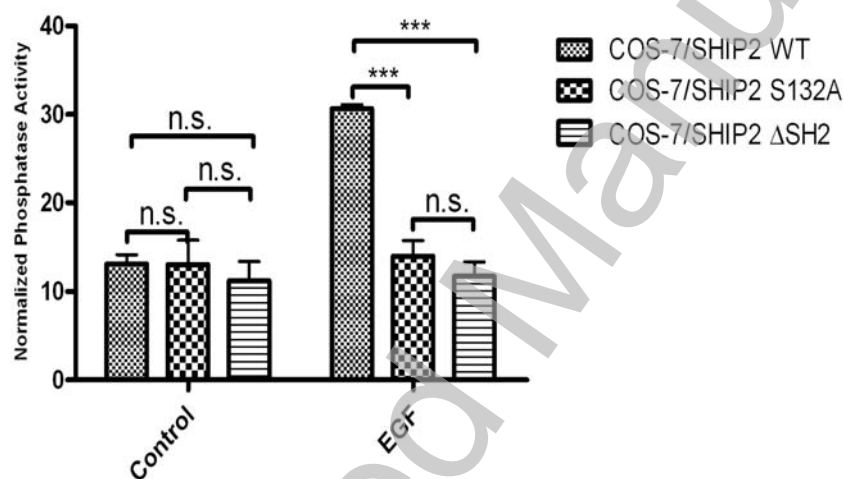
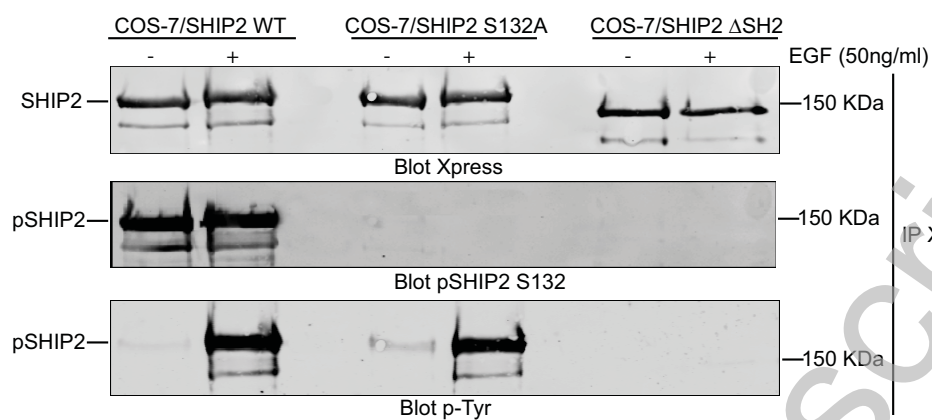


Figure 8