Cellular Signalling 26 (2014) 1258–1268

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Insulin activation of vacuolar protein sorting 34 mediates localized phosphatidylinositol 3-phosphate production at lamellipodia and activation of mTOR/S6K1

Dianne S. Hirsch [a], Yi Shen [a], Milos Dokmanovic [a], Joyce Yu [a,1], Nishant Mohan [a], Mohammed Khair Elzarrad [a,b], Wen Jin Wu [a,*]

[a] Division of Monoclonal Antibodies, Office of Biotechnology Products, Office of Pharmaceutical Science, Center for Drug Evaluation and Research, U.S. Food and Drug Administration (FDA), Bethesda, MD, USA

[b] Interagency Oncology Task Force (IOTF) Fellowship: Program 4 — Cancer Prevention Fellow, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

ARTICLE INFO

Article history:
Received 31 October 2013
Received in revised form 10 February 2014
Accepted 22 February 2014
Available online 27 February 2014

Keywords:
Class III phosphatidylinositol 3-kinase/vacuolar protein sorting 34 (VPS34)
Insulin
Src
ptdins(3)p
S6 kinase 1 (S6K1)
mTOR

ABSTRACT

The class III phosphatidylinositol 3-kinase, VPS34, phosphorylates the D3 hydroxyl of inositol generating phosphatidylinositol 3-phosphate (ptdins(3)p) . Initial studies suggested that ptdins(3)p solely functioned as a component of vesicular and endosomal membranes and that VPS34 did not function in signal transduction. However, VPS34 has recently been shown to be required for insulin-mediated activation of S6 kinase 1 (S6K1). Whether VPS34 activity is directly regulated by insulin is unclear. It is also not known whether VPS34 activity can be spatially restricted in response to extracellular stimuli. Data presented here demonstrate that in response to insulin, VPS34 is activated and translocated to lamellipodia where it produces ptdins(3)p. The localized production of ptdins(3)p is dependent on Src phosphorylation of VPS34. In cells expressing VPS34 with mutations at Y231 or Y310, which are Src-phosphorylation sites, insulin-stimulated VPS34 translocation to the plasma membrane and lamellipodia formation are blocked. mTOR also colocalizes with VPS34 and ptdins(3)p at lamellipodia following insulin-stimulation. In cells expressing the VPS34-Y231F mutant, which blocks lamellipodia formation, mTOR localization at the plasma membrane and insulin-mediated S6K1 activation are reduced. This suggests that mTOR localization at lamellipodia is important for full activation of S6K1 induced by insulin. These data demonstrate that insulin can spatially regulate VPS34 activity through Src-mediated tyrosine phosphorylation and that this membrane localized activity contributes to lamellipodia formation and activation of mTOR/S6K1signaling.

Published by Elsevier Inc.

1. Introduction

The phosphatidylinositol (ptdins) 3-kinases (ptdins(3)K) are a family of lipid kinases that function in numerous cellular processes including proliferation, migration, vesicular trafficking, and signal transduction. The function and regulation of PI3Kα are well characterized. In response to growth factors, PI(3)Kα activation requires translocation of the inactive complex, consisting of the PI3K-p110 catalytic subunit and the p85 regulatory subunit, to the cell membrane and conformational changes that result in activation of the catalytic subunit [1–3]. PI3Kα also plays a critical role in insulin-mediated activation of the Akt/mTOR/S6K1 pathway [2].

Vacuolar protein sorting 34 (VPS34) phosphorylates phosphatidylinositol to yield ptdins(3)p, which is predominantly produced at vesicular membranes [4–7]. VPS34 functions at numerous steps of vesicular trafficking, including early endosomal fusion and autophagosome formation [6,8–11]. Original studies of mammalian VPS34 suggested that VPS34 was not regulated by extracellular stimuli, but rather maintained ptdins(3)p at steady state levels as a component of endosomal membranes [12]. However, recently, regulated activation of VPS34 has been reported. Amino acid stimulation activates VPS34, [13,14] which is required for insulin-mediated activation [13]. VPS34 is also regulated by intracellular signaling molecules. For instance, cyclin dependent kinase (cdk) 1 and cdk5 phosphorylate VPS34 during mitosis to inhibit autophagy [15]. During cytokinesis, ptdins(3)p produced by VPS34 is required for the recruitment of cytoskeletal proteins needed for abscission [16].

c-Src is a non-receptor tyrosine kinase that is activated by numerous cell surface receptors. It is through substrate phosphorylation that Src transduces signals received at the cell surface to regulate critical cellular processes such as proliferation, actin cytoskeleton remodeling, and migration [17]. For instance, the Src family kinase, Lck, phosphorylates the p85 regulatory subunit of PI(3)Kα leading to its recruitment to the cell membrane and subsequent activation [18]. Src also functions in insulin

* Corresponding author at: Division of Monoclonal Antibodies, Office of Biotechnology Products, Office of Pharmaceutical Science, Center for, Drug Evaluation and Research, U.S. Food and Drug Administration, HFD-123, 29B Lincoln Drive, Room 3NN20, Bethesda, MD 20892-4555, USA. Tel.: +1 301 827 0253; fax: +1 301 827 0852.
E-mail address: wen.wu@fda.hhs.gov (W.J. Wu).

[1] FDA Summer Intern 2010.

http://dx.doi.org/10.1016/j.cellsig.2014.02.009
0898-6568/Published by Elsevier Inc.

signaling. Insulin stimulates the formation of a Src, AKT, and β-arrestin complex at the cell membrane [19]. Additionally, following insulin stimulation, Src binds to and phosphorylates Akt contributing to its full activation [20,21]. It was recently demonstrated that Src phosphorylates VPS34 leading to increased lipid kinase activity and that VPS34 lipid kinase activity and VPS34 phosphorylation by Src are required for Src-mediated cellular transformation [22].

Given the model proposed by Cuevas et al. [18], that upon activation of growth factor receptors, Lck regulates the activation and membrane recruitment of PI3Kα, we determined if VPS34 localization and lipid kinase activity are regulated by insulin and Src. The present study demonstrates that in response to insulin, VPS34 was activated and translocated to lamellipodia where it produced ptdins(3)p. VPS34 knockdown reduced ptdins(3)p production at the plasma membrane and inhibited insulin-induced lamellipodia formation. Insulin-induced VPS34 translocation to the plasma membrane and lamellipodia formation was blocked in cells expressing VPS34 with mutations at either Y231 or Y310, which are sites of Src phosphorylation. The localized activity of VPS34 contributed to the recruitment of mTOR to lamellipodia and S6K1 activation induced by insulin. In response to insulin, in cells expressing the VPS34-Y231F mutant, mTOR localization at the plasma membrane was disrupted. Insulin-mediated activation of S6K1 was also reduced when VPS34 function was disrupted. These data demonstrate that VPS34 activity can be spatially regulated by insulin and that this membrane localized activity contributes to lamellipodia formation and activation of mTOR/S6K1signaling.

2. Materials and methods

2.1. Plasmids and siRNA

pcDNA3-Myc-tagged-hVPS34 was a kind gift from Jonathan Backer of Albert Einstein College of Medicine (Bronx, NY, USA). pcDNA3-Myc-tagged-hVPS34 point mutants were generated as previously described [22]. VPS34 targeting siRNAs were purchased from Dharmacon (Lafayette, CO, USA), and Origene (Rockville, MD, USA).

2.2. Antibodies and reagents

The following antibodies were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA): rabbit anti-mTOR, rabbit anti-mTOR-pS2481, rabbit anti-p70 S6K1, and rabbit anti-pT389-S6K1. The mouse anti-IRS-1 monoclonal antibody (L3D12) used for immunofluorescent studies was from Cell Signaling Technology, Inc. Mouse anti-PIP5K3 (PIKFYVE) and mouse anti-PIK3R4 (VPS15) were purchased from Abnova (Walnut, CA). Goat anti-PIP5KIII (C-14) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-ptdins(3)p was purchased from Echelon Biosciences, Inc. (Salt Lake City, UT, USA). Mouse anti-phosphotyrosine antibody (clone 4G10), mouse anti-v-Src (Ab-1) antibody, mouse anti-Src (clone GD11), mouse anti-pY416-Src-488, and mouse anti-mTOR were purchased from EMD Millipore (Billerca, MA, USA). Mouse anti-c-Myc antibody (9E10) ascites and rabbit anti-c-Myc antibody were purchased from Covance, Inc. (Richmond, CA, USA). Insulin was obtained from Gemini Bio Products (West Sacramento, CA, USA). All secondary antibodies, rhodamine-conjugated and coumarin-conjugated phalloidin were purchased from Invitrogen/Molecular Probes® (Carlsbad, CA, USA). Antibiotic/antimycotic solution was obtained from Invitrogen. LabTEKII glass chamber slides were purchased from Fisher Scientific (Pittsburgh, PA, USA). Dulbecco's Modified Eagle Medium (DMEM) was obtained from Lonza (Walkersville, MD, USA). Newborn calf serum (CS) was obtained from Omega Scientific, Inc. (Tarzana, CA, USA).

2.3. Human VPS34 (hVPS34) antibody generation

Rabbit anti-hVPS34 polyclonal antibody (747/748) was generated by Pacific Immunology (Ramona, CA) using the peptide antigen AVVEQIHKFAQYWRK which corresponds to amino acids 873–887 of hVPS34 which was originally cloned by Volinia et al. (GenBank accession #s Z46973) [5]. This antigen was chosen based on the peptide antigen used to generate a previously published rabbit anti-hVPS34 polyclonal antibody that was used for immunofluorescence studies [11]. However, the peptide antigen used to generate the hVPS34 (747/748) antibody used for the current study has a phenylalanine (F) at position 881 which corresponds to the GenBank protein sequences (accession #s Z46973 and NM_002647), whereas the published peptide antigen sequence reports an arginine (R) at this position. Rabbit 747/748 was affinity purified using the peptide antigen and VPS34 antigen specificity was determined by ELISA at Pacific Immunology.

2.4. Cell lines and culture conditions

NIH3T3 and c-Src-Y527F NIH3T3 cells were maintained in DMEM containing 10% calf serum (CS) and antibiotic/antimycotic. MCF10A cells were grown in DMEM containing 10% fetal bovine serum (FBS) and antibiotic/antimycotic. Cells were incubated at 37 °C/5% CO_2.

2.5. Transfections

Cells were transfected using Lipofectamine and PLUS™ reagents (Invitrogen) or by AMAXA™ 4D-Nucleofection (Lonza). siRNA transfections were done using Dharmafect transfection reagent (Dharmacon) or by AMAXA 4D-Nucleofection.

2.6. Insulin response assays

Cells were seeded overnight, or transfected for 24 to 48 h in complete medium, and then switched to either empty DMEM or DMEM containing 1% CS for 18 h. Cells were then treated with 0.8–1.7 μM insulin for 15–30 min. Conditions used for individual experiments are indicated in the figure legends.

2.7. Lamellipodia quantification

The effect of VPS34 knockdown on insulin-induced lamellipodia formation was evaluated for the change in the total percentage of cells that formed lamellipodia and for the percentage of lamellipodia that were ptdins(3)p-positive. VPS34 knockdown was determined by Western blot. Imaging of cells transfected with control non-targeting and VPS34-targeting siRNA constructs were taken using identical settings and image acquisition settings were optimized using cells transfected with the non-targeting siRNA. For lamellipodia quantitation, in cells transfected with VPS34-targeting siRNA, cells with noticeably reduced VPS34 fluorescent intensity were counted. A minimum of ten random fields ($N \geq 25$) were evaluated. Data are presented as the mean from at least two-independent experiments.

2.8. Immunofluorescence staining and microscopy

Cells were fixed in phosphate buffered saline (PBS) containing 3.7% formaldehyde for 15 min, rinsed with PBS, and then permeabilized for 10 min in PBS containing 0.2% Triton-X100. Antibodies were diluted in PBS containing 3% bovine serum albumin (BSA). Immunofluorescent staining was done as previously described [22]. Images were captured on a Zeiss LSM-510 Meta microscope using a 40×/1.4NA plan Apo objective.

3. Results

3.1. VPS34 and ptdins(3)p co-localize at lamellipodia following insulin treatment

Insulin and other growth factors stimulate actin cytoskeletal remodeling leading to the formation of lamellipodia and/or membrane ruffles (Note: following insulin stimulation, VPS34 and ptdins(3)p localized at both membrane ruffles and lamellipodia. For readability, both structures will be referred to as lamellipodia), structures which function in chemotaxis, as well as in concentrating components of activated signaling pathway(s) [23,24]. Insulin-induced lamellipodia formation has been shown to require protein-tyrosine phosphatase (PTP) 1B-mediated c-Src activation, which leads to activation of Rac1 GTPase and reorganization of actin cytoskeleton leading to lamellipodia formation [25]. Previously, we published data that demonstrated VPS34 is

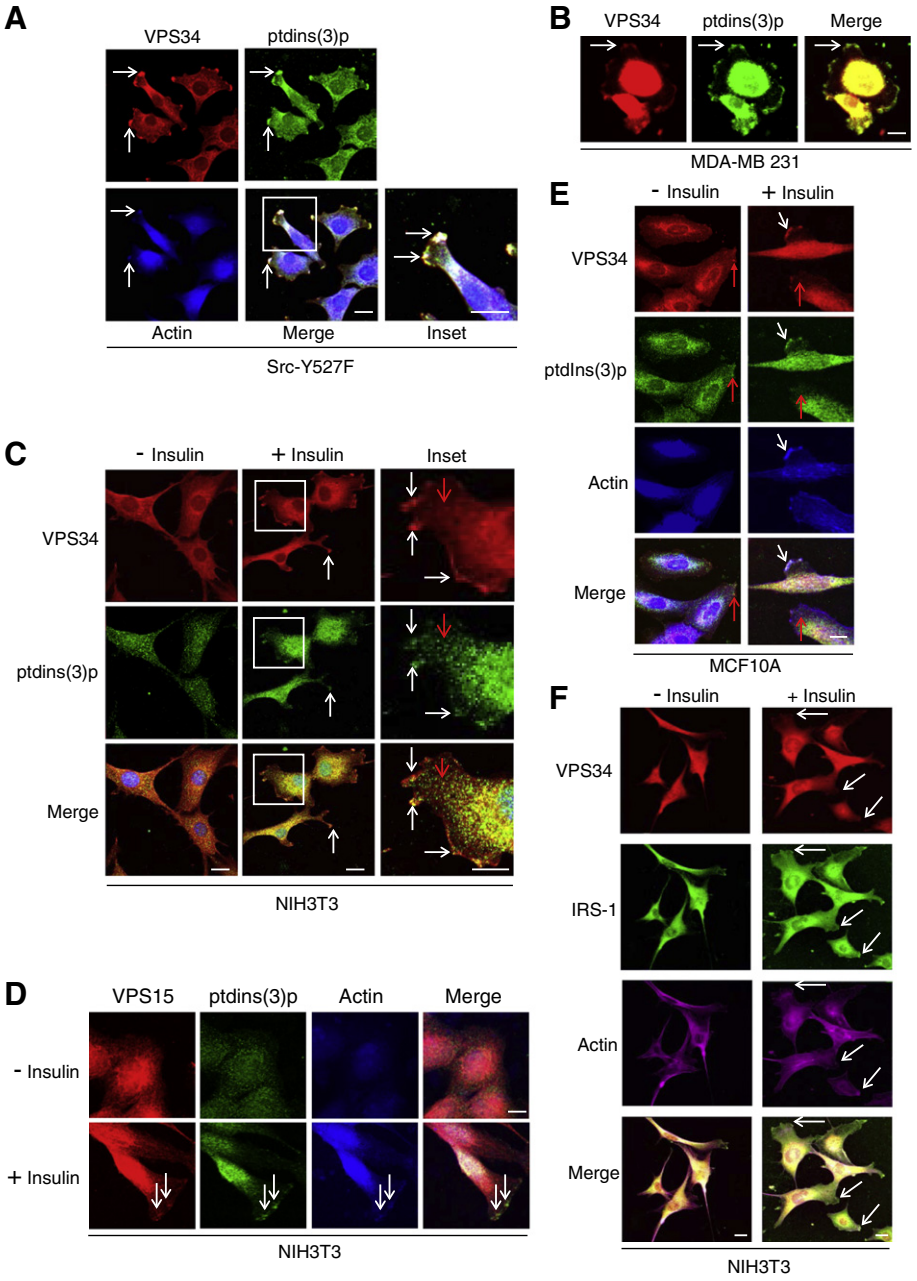


Fig. 1. Insulin treatment stimulates VPS34 kinase activity at lamellipodia. (A) Src-Y527F-transformed NIH3T3 cells were seeded in glass chamber slides coated with fibronectin. Cells were fixed, permeabilized, and stained for VPS34 (red) and ptdins(3)p (green). Actin (blue) was detected with coumarin-conjugated phalloidin (from here on, unless otherwise noted actin was detected with coumarin-conjugated phalloidin). White arrows, VPS34, and ptdins(3)p colocalized at lamellipodia. Bar, 20 μm. Inset, enlargement of boxed region. Bar 10 μm. (B) MDA-MB-231 breast cancer cells were seeded overnight in glass chamber slides coated with fibronectin and then stained for VPS34 (red) and ptdins(3)p (green). (C) NIH3T3 cells were seeded overnight in glass chamber slides coated with fibronectin and then switched to 1% CS for an additional 18–24 h. Cells were then either left untreated or stimulated with 1 μM insulin for 30 min. Cells were then fixed, permeabilized, and stained for VPS34 (red) and ptdins(3)p (green). (D) NIH3T3 cells were seeded overnight in glass chamber slides coated with fibronectin. Cells were then switched to serum-free medium (SFM) for an additional 18–24 h. Cells were then either left untreated or stimulated with 1 μM insulin for 30 min. Cells were fixed, permeabilized, and then stained for VPS15 (red) and ptdins(3)p (green). (E) MCF10A breast epithelial cells were seeded overnight in glass chamber slides coated with fibronectin. Cells were then switched to SFM for 18–24 h and either stimulated with 1 μM insulin or left untreated. The cells were stained for VPS34 (red) and ptdins(3)p (green). (F) NIH3T3 cells were seeded overnight in fibronectin-coated chamber slides and changed to serum-free medium for 18–24 h. Cells were then either treated for 30 min with 1 μM insulin or left untreated. Cells were fixed, permeabilized, and stained for VPS34 (red), IRS-1 (green), and Alexa Fluor 633-conjugated phalloidin. All white arrows in this figure and subsequent figures: colocalization at lamellipodia; all red arrows: colocalization at vesicle-likestructures. All data in this figure are representative of two or more independent experiments. Bar, 20 μm.

required for Src-mediated cellular transformation [22]. However, the subcellular localization of VPS34 in Src-transformed NIH3T3 cells was not determined. In Src-transformed NIH3T3 cells, which have prominent lamellipodia at steady state, VPS34 and ptdins(3)p colocalized with actin at lamellipodia (Fig. 1A). As shown in Fig. 1B, VPS34 and ptdins(3)p also colocalized at lamellipodia in MDA-MB-231 breast cancer cells, which have increased Rac1 activity and prominent lamellipodia/membrane ruffles [22,26]. These data suggested that VPS34 and ptdins(3)p may contribute to signal transduction generated at lamellipodia.

VPS34 activity has previously been shown to be required for insulin-mediated signal transduction [13]. However, based on in vitro VPS34 lipid kinase assays, insulin does not activate VPS34 ptdins 3-kinase activity [13]. In agreement with these data, in NIH3T3 cells, as measured using a VPS34 in vitro kinase assay, VPS34 lipid kinase activity either was not or was minimally (≤2-fold) increased in response to insulin stimulation (data not shown). While increases in VPS34 lipid kinase activity were not detected biochemically, it is possible that insulin treatment leads to localized VPS34 enzymatic activity that is below the level of detection biochemically. To test this hypothesis, we assessed the localization of VPS34 and ptdins(3)p following insulin treatment. In NIH3T3 cells treated with insulin, VPS34 and ptdins(3)p colocalized at lamellipodia and at vesicle-like structures that were at or near the lamellipodia (Fig. 1C). VPS15, which is required for VPS34 lipid kinase activity, and ptdins(3)p also colocalized at lamellipodia in response to insulin stimulation (Fig. 1D). VPS34 and ptdins(3)p also colocalized at lamellipodia in MCF10A cells treated with insulin (Fig. 1E).

IRS-1 is a scaffold protein that is recruited to the membrane in response to insulin [27]. Data in Fig. 1F demonstrated that VPS34 colocalized with IRS-1 at lamellipodia in NIH3T3 cells treated with insulin. These data further support the hypothesis that insulin regulates VPS34 intracellular localization and that VPS34 potentially contributes to insulin-mediated signal transduction. It should be noted that binding between VPS34 and IRS-1 was not detected in co-immunoprecipitation assays (data not shown).

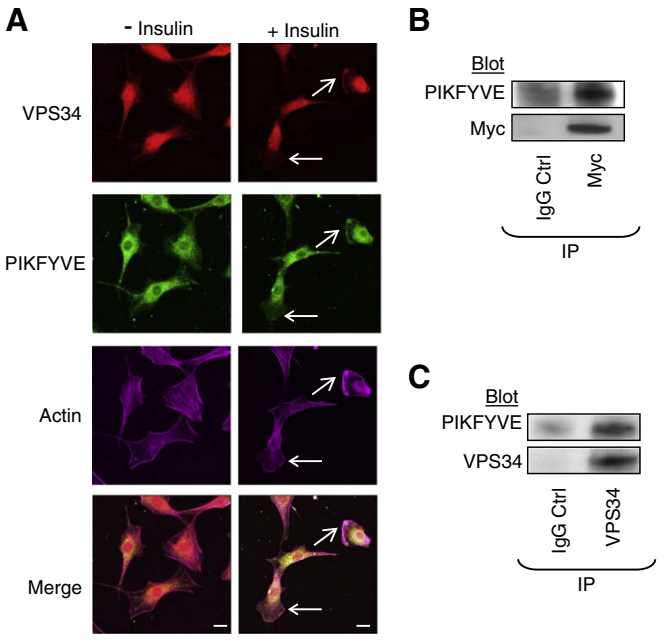


Fig. 2. VPS34 colocalizes with PIKFYVE in response to insulin. (A) The experimental procedures were the same as described in Fig. 1F except that cells were stained for VPS34 (red), PIKFYVE (green), and Alexa Fluor 633-conjugated phalloidin. Bar, 20 μm. (B) NIH3T3 cells were transfected for 48 h with Myc-tagged VPS34-WT. Myc-tagged VPS34 was immunoprecipitated from WCL using an anti-Myc antibody, resolved by SDS-PAGE, and transferred to PVDF. PIKFYVE and Myc-VPS34 were detected by Western blot analysis using the indicated antibodies. (C) NIH3T3 cells were grown overnight, harvested, and then WCL was used for immunoprecipitations using anti-VPS34 antibody or IgG control antibody. Immunoprecipitated proteins were resolved by SDS-PAGE, and transferred to PVDF. PIKFYVE and VPS34 were detected by Western blot analysis using the indicated antibodies. All data in this figure are representative of two independent experiments.

3.2. VPS34 and PIKFYVE co-localize at lamellipodia following insulin treatment

The function of VPS34 in membrane trafficking is in part regulated by the binding of the FYVE (Fab1p, YOTB, Vac1p, and EEA1) domain containing proteins to ptdins(3)p located on endosomal membranes [28]. Three FYVE-domain containing proteins were evaluated for colocalization with VPS34 at lamellipodia following insulin stimulation. The three proteins were EEA1 which functions in early endosomal fusion, Frabin which indirectly contributes to Rac1 activation [29], and the phosphatidylinositol 3-phosphate 5-kinase, PIKFYVE, which following insulin stimulation, converts ptdins(3)p to ptdins(3,5)p [30]. It is also reported that PIKFYVE is necessary for the activation of mTORC1 and its translocation to the plasma membrane in response to insulin stimulation [30]. Of the three proteins screened, only PIKFYVE colocalized with VPS34 at lamellipodia following insulin treatment (Fig. 2A and data not shown). Additionally, PIKFYVE co-immunoprecipitated with ectopically expressed Myc-tagged VPS34 and endogenous VPS34 (Fig. 2B & C). Together, the data presented in Figs. 1 through 2 demonstrated that insulin regulated the localization of VPS34 at lamellipodia.

3.3. VPS34 and Rac1 co-localize at lamellipodia following insulin treatment

Rac1 is a membrane-localized small GTPase that regulates actin cytoskeletal remodeling required for lamellipodia and membrane ruffle formation [31]. Rac1 membrane localization at lamellipodia was confirmed by staining Src-Y527F-transformed NIH3T3 cells, which have prominent lamellipodia at steady state (Fig. 3A). Rac1 also localized at lamellipodia in MDA-MB-231 breast cancer cells (Fig. 3A bottom panels) which also have prominent lamellipodia that are likely due to upregulated Rac1 activity [32]. Fig. 3B showed that VPS34 colocalized with Rac1 in Src-Y527-transformed NIH3T3 cells. Moreover, ptdins(3)p colocalized with Rac1 at lamellipodia in both Src-Y527-transformed NIH3T3 and MDA-MB-231 cells (Figs. 3C). In addition, both VPS34 and ptdins(3)p colocalized with Rac1 at vesicle-like structures (Fig. 3B & C, red arrows). To determine if Rac1 was regulated by insulin, Rac1 localization following insulin treatment was tested. Following insulin stimulation, Rac1 colocalized with actin at lamellipodia (Fig. 3D). VPS34 and Rac1 also colocalized at lamellipodia and occasionally at vesicle-like structures (red arrows) in response to insulin in MCF10 cells (Fig. 3E).

3.4. Insulin stimulates Src activation of VPS34 at lamellipodia

Src activation by protein tyrosine phosphatase 1B (PTP1B) has been shown to contribute to insulin-induced lamellipodia formation [25]. Here, insulin stimulation of NIH3T3 cells led to Src activation as evaluated by the levels of pY416-Src (Fig. 4A). Given that VPS34 is activated by Src-mediated tyrosine phosphorylation, we next determined if VPS34 was tyrosine-phosphorylated following insulin treatment. As shown in Fig. 4B, VPS34 was tyrosine phosphorylated in response to insulin. Additionally, following insulin stimulation, VPS34 and activated Src (pY416-Src) colocalized at lamellipodia and at vesicle-like structures (Fig. 4C). Both Src and ptdins(3)p also localized at lamellipodia; however, the extent of colocalization was less than what was observed between VPS34 and Src (Fig. 4D). VPS34 and Src also colocalized at lamellipodia following insulin stimulation of MCF10A cells (Fig. 4E).

3.5. VPS34 is required for insulin-mediated ptdins(3)p production at lamellipodia

Data presented in Figs. 1 through 3 raised the question of whether the ptdins(3)p at lamellipodia was produced by VPS34. To address

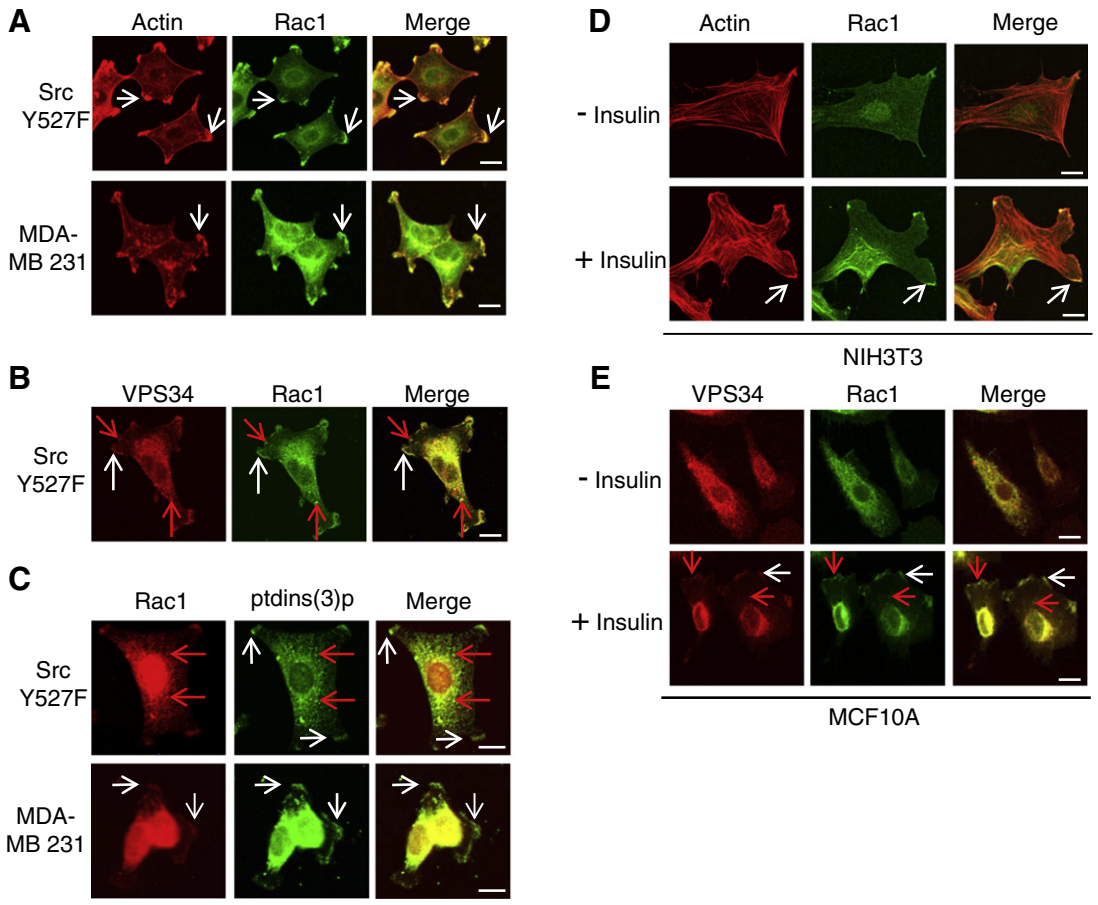


Fig. 3. VPS34 colocalizes with Rac1 at lamellipodia following insulin treatment. (A) Src-Y527F-transformed NIH3T3 cells and MDA-MB-231 cells were seeded overnight in fibronectin-coated chamber slides. Cells were fixed, permeabilized, and then stained for Rac1 (green); actin was detected with rhodamine-conjugated phalloidin. (B) Src-Y527F-transformed NIH3T3 cells were seeded as described in "A" and then stained for VPS34 (red) and Rac1 (green). (C) Src-Y527F-transformed NIH3T3 cells and MDA-MB-231 cells were seeded overnight in fibronectin-coated chamber slides. Cells were stained for Rac1 (red) and ptdins(3)p (green). (D) NIH3T3 cells were seeded overnight in fibronectin-coated chamber slides. Cells were then switched to empty DMEM for an additional 18–24 h. Cells were either treated with 1 μM insulin or left untreated for 30 min. Cells were stained for Rac1 (green). Actin was detected with rhodamine-conjugated phalloidin. (E) The methods were as described in Fig. 1D except that the experiment was run using MCF10A cells. Cells were stained for VPS34 (red) and Rac1 (green).

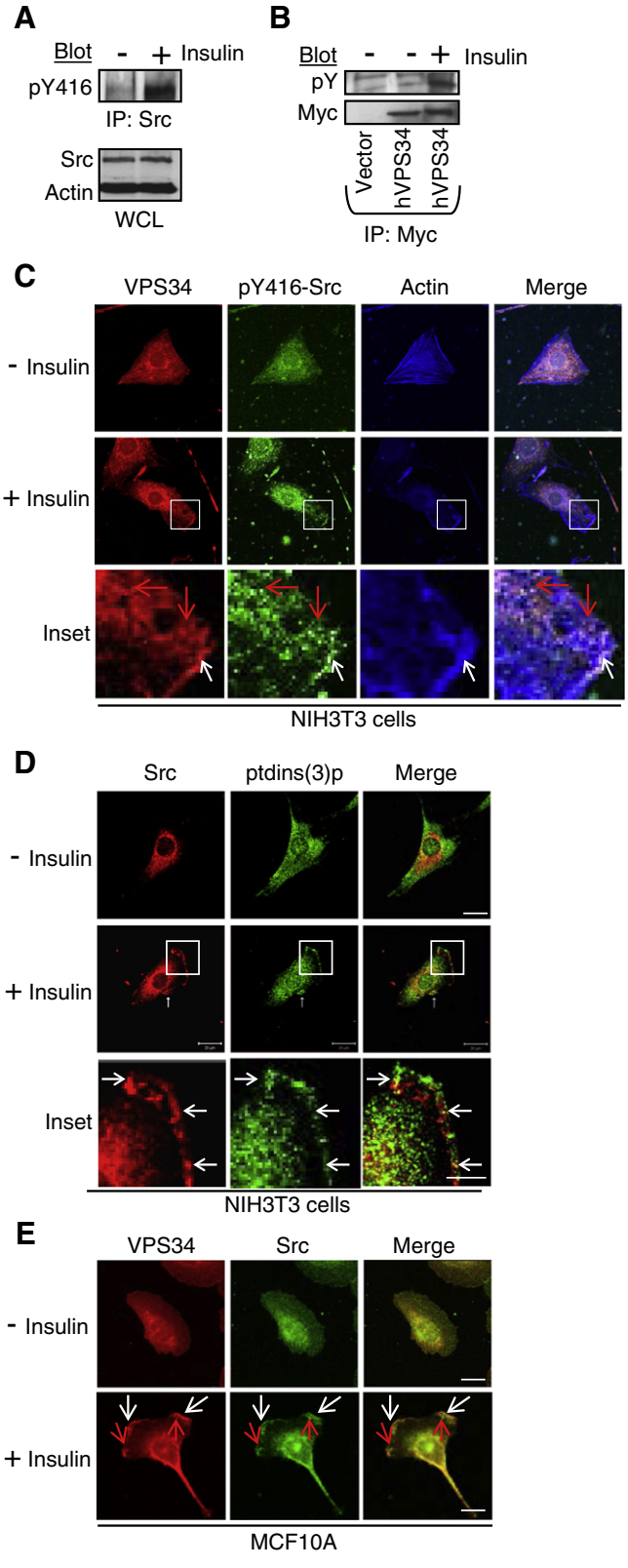
this question, VPS34 protein levels were reduced by transfecting cells with VPS34-targeting siRNA. Based on Western blot analysis of WCL, VPS34 protein levels were reduced ≥50% in cells transfected with VPS34-targeting siRNA as compared to cells transfected with control siRNA (Fig. 5A). Following insulin treatment, two effects were evident. First, in cells transfected with VPS34-targeting siRNA with decreased VPS34 fluorescent intensity that was clearly evident by fluorescent microscopy, the percentage of cells with ptdins(3)p-positive lamellipodia following insulin treatment was reduced to the levels observed in untreated control cells (Fig. 5A; *, representative cells with reduced VPS34 protein). Reduced ptdins(3)p fluorescent intensity was also observed. Second, we observed that in response to insulin, fewer cells formed ruffles when VPS34 protein levels were reduced as compared to control cells.

To confirm our observation of the effect of VPS34 knockdown on lamellipodia formation and to control for the potential that off-target effects were occurring, the experiment was repeated using different control and VPS34-targeting siRNAs. In these experiments, the effect of VPS34 knockdown on the total percentage of NIH3T3 cells that formed lamellipodia in response to insulin was determined. In response to insulin treatment, the percentage of control cells with lamellipodia increased from 25% in untreated cells to 60% of cells treated with insulin (Fig. 5B). In cells transfected with VPS34-targeting siRNA, with decreased VPS34 fluorescent intensity that was clearly evident by fluorescent microscopy, the percentage of cells with insulin-induced lamellipodia was reduced to that of control untreated cells. We noted that higher percentages of untreated control and VPS34 knockdown cells had lamellipodia as compared to the previous study (Fig. 5A). Because only cells with lamellipodia containing ptdins(3)p were scored as positive in the first study and all cells with lamellipodia were scored positive in this experiment, we determined if the increased background in the second study was due inclusion of cells with lamellipodia that did not contain VPS34. When the percentage of control cells with lamellipodia was divided into VPS34-positive lamellipodia (black bars) and VPS34-negative lamellipodia (gray bars), the background levels in the untreated control cells were comparable between the two studies (compare column 1 of Fig. 5A to the black bar in column 1 of 5B). Together, these data demonstrated that VPS34 was not only required for insulin-stimulated ptdins(3)p production at the plasma membrane, but was also required for insulin-induced lamellipodia formation.

3.6. Insulin-mediated ptdins(3)p produced at lamellipodia requires VPS34 tyrosine phosphorylation

The data shown in Figs. 1 and 5 suggested that insulin modulated the localization and activation of VPS34 and that decreased VPS34 protein levels reduced both lamellipodia formation and ptdins(3)p production at lamellipodia. The amino acid Y231 of VPS34 is a Src phosphorylation site that is essential for both Src-catalyzed activation of VPS34 lipid kinase activity and for Src-mediated cellular transformation [22]. In VPS34-Y231F expressing NIH3T3 cells, following insulin treatment,

lamellipodia formation was reduced (Fig. 6A). VPS34-Y310 was identified as a second potential Src phosphorylation site using the web-based tool Scansite [33]. VPS34 with a Y310A mutant had reduced tyrosine phosphorylation when expressed in Src-Y527F transformed NIH3T3 cells (Fig. 6B). Similar to the effect of VPS34-Y231F, cells expressing VPS34-Y310A did not form lamellipodia in response to insulin and did not produce ptdins(3)p at the plasma membrane (Fig. 6A). Furthermore, insulin-mediated VPS34 translocation to plasma membrane was blocked when either of the Src phosphorylation defective mutants were expressed in NIH3T3 cells. These data were consistent with results shown in Fig. 5B where reduced VPS34 protein levels reduced lamellipodia formation in response to insulin. In addition, we observed that overexpression of VPS34-Y231F or VPS34-Y310A caused increased numbers of binucleate and multinucleate cells, suggesting that tyrosine phosphorylation of VPS34 is required for cytokinesis, which is consistent with our previous report [22]. These data provided evidence that tyrosine phosphorylation of VPS34 is necessary for its translocation to the plasma membrane, ptdins(3)p production, and lamellipodia formation following insulin stimulation. Loss of VPS15 binding to the VPS34 tyrosine mutants could be one mechanism behind the loss of insulin-stimulated VPS34 activation. However, neither mutant disrupted VPS34 binding to VPS15 (Fig. 6C). We next determined if coexpressing HA-tagged constitutively activated Rac1-Q61L with the VPS34 tyrosine mutants affected Rac1-mediated lamellipodia formation (Fig. 6D). In NIH3T3 cells coexpressing constitutively active Rac1-Q61L and wildtype VPS34, no effect on lamellipodia was observed. In contrast, coexpression of either VPS34 tyrosine mutant with constitutively active Rac1-Q61L disrupted lamellipodia formation. These data demonstrated that VPS34 activity was required for lamellipodia formation.



3.7. VPS34 recruits mTOR to lamellipodia and contributes to insulin-mediated S6K1 activation

Insulin stimulates activation of the mTOR/S6K1 signaling pathway, which is critical to insulin-mediated signal transduction [34]. It has been recently reported that mTOR and Rac1 can colocalize at plasma membrane in response to serum stimulation [35]. We addressed whether the locally produced ptdins(3)p by VPS34 induced the translocation of mTORC1 to lamellipodia. While VPS34 and mTOR did not co-immunoprecipitate (data not shown), we did find that mTOR colocalized with VPS34 at lamellipodia following insulin stimulation in NIH3T3 cells (Fig. 7A). Both mTOR and activated mTOR (pS2481-mTOR) colocalized with ptdins(3)p at lamellipodia following insulin stimulation (Fig. 7B and C). However, the insulin-induced translocation of mTOR was blocked in cells expressing Myc-tagged VPS34–Y231F, which indicates that tyrosine phosphorylation and lipid kinase activity of VPS34 were necessary for insulin-induced mTOR translocation to the plasma membrane (Fig. 7D). mTOR1, as well as S6K1, also colocalized with ptdins(3)p in MCF10A cells following insulin stimulation (Fig. 7 E & F).

c-Src inhibition has previously been shown to inhibit S6K1 activation [36]. Consistent with the published literature, pre-treatment of serum-starved cells with the Src inhibitor SU6656 prior to insulin stimulation reduced S6K1 activation as evaluated by the levels of

Fig. 4. VPS34 is phosphorylated by Src and colocalizes with Src in response to insulin treatment. (A) NIH3T3 cells were seeded overnight in six well plates and then switched to DMEM with 1% calf serum (CS) for 18–24 h. Cells were then either treated for 30 min with 1 μM insulin or left untreated. Src was immunoprecipitated from whole cell lysates (WCL), resolved by SDS-PAGE, and transferred to PVDF. Activated Src was detected by Western blot analysis using an anti-Src-pY416 antibody (top blot). Levels of Src and actin in WCL were detected by Western blot analysis using anti-Src and anti-actin antibodies (bottom blot). (B) NIH3T3 cells were transfected for 24 h with vector control or Myc-tagged-pcDNA3-hVPS34 plasmid and then switched to DMEM containing 1% CS for an additional 18–24 h. Cells were then either treated for 30 min with 1 μM insulin or left untreated. Myc-hVPS34 was immunoprecipitated using anti-Myc antibody. Following SDS-PAGE and transfer to PVDF, blots were probed for tyrosine-phosphorylated Myc-hVPS34 (pY) using anti-phosphotyrosine (4G10) antibody. Blots were then stripped and reprobed with anti-Myc antibody. (C) The experiment was as described in Fig. 1C with the exception that cells were stained for VPS34 (red) and 488-conjugated-pY416-Src (green). Blue is actin. Arrows, colocalized VPS34 and pY416-Src. D. The experiment was as described in Fig. 1C with the exception that cells were stained with Src (red) and ptdins(3)p (green). Arrows, colocalized ptdins(3)p and total Src. E. MCF10A were seeded in fibronectin-coated chamber slides overnight and then switched to SFM for an additional 18–24 h. Cells were then either treated for 30 min with 1 μM insulin or left untreated and then fixed, permeabilized, and stained with VPS34 (red) and Src (green).

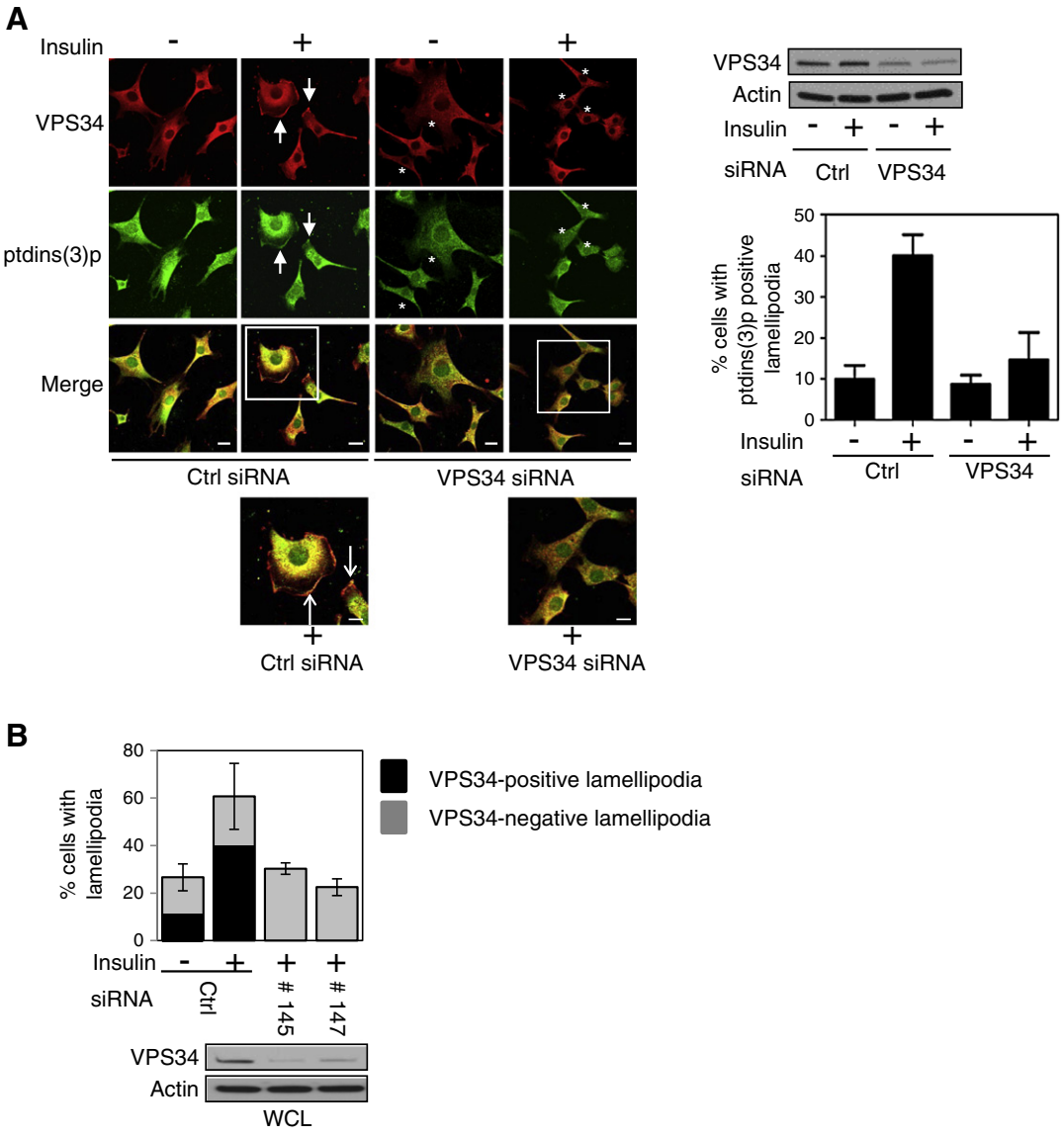


Fig. 5. VPS34 is required for insulin-mediated lamellipodia formation. (A) NIH3T3 cells were transfected for 48 h with either control siRNA or pooled VPS34-targeting siRNA [SMARTpool] VPS34-targeting siRNA (Dharmacon) and then switched to serum-free DMEM medium for an additional 18–24 h. Cells were then either treated for 30 min with 1.7 μM insulin or left untreated. Cells were fixed, permeabilized, and stained for VPS34 (red) and ptdins(3)p (green). The percent cells with lamellipodia that contained ptdins(3)p were determined from a minimum of 10 random fields (N ≥ 25 cells). Histogram, percentage of cells with ptdins(3)p localized at lamellipodia. *, cells with reduced VPS34 protein levels. VPS34 knockdown was confirmed by Western blot of WCL. Data are the mean from two independent experiments ± SEM. (B) NIH3T3 cells were transfected for 24 h with control non-targeting siRNA or VPS34-targeting siRNA [(#145 and #147) (Origene)] and then serum-starved overnight. Cells were then either treated for 30 min with 1.7 μM insulin or left untreated. Cells were fixed, permeabilized, and stained for VPS34 and actin. The percentage of cells with lamellipodia was determined from a minimum of 10 random fields (N ≥ 25cells). Histogram, the percentage of cells with lamellipodia. For cells transfected with control siRNA, the percentage of cells with lamellipodia is divided into the % cells with VPS34-positive lamellipodia (black bars) and % cells with VPS34-negative lamellipodia. VPS34 knockdown was confirmed by Western blot. Data are the mean of two independent experiments ± Stdev.

phosphorylated S6K1 (pT389-S6K1) (Fig. 8A). VPS34 knockdown also decreased insulin-stimulated activation of S6K1 (Fig. 8B, second panel from the top), indicating that VPS34 is required for full activation of S6K1 following insulin stimulation. These data are similar to published results using other cell lines [4,15]. We next determined if activation of S6K1 required Src phosphorylation of VPS34. In insulin-treated cells overexpressing Myc-tagged VPS34 wild type, levels of activated S6K1 were higher than in vector transfected cells (Fig. 8C, second panel from the top). A slight increase in total S6K1 and activated S6K1 also occurred in untreated cells expressing wild type VPS34. In contrast, overexpression of Myc-tagged VPS34-Y231F reduced S6K1 activation following insulin treatment (Fig. 8D). These data indicate that VPS34 phosphorylation by c-Src contributes to the downstream activation of S6K1.

4. Discussion

This study presents the first evidence that VPS34 is regulated by insulin. A working model for insulin regulation of VPS34 is presented in Fig. 8E. In this model, insulin activates Src, leading to VPS34 phosphorylation and activation. Activated VPS34 is then translocated to the plasma membrane where VPS34 produces ptdins(3)p and contributes to lamellipodia formation. mTOR is then recruited to the lamellipodia where it contributes to S6K1 activation. The requirement of VPS34 tyrosine phosphorylation by Src in the regulation of insulin signaling is underscored by the fact that the phosphorylation defective VPS34–Y231F mutant blocks insulin-induced lamellipodia formation and S6K1 activation. These data indicate that VPS34 is an effector of insulin-mediated signal transduction and that Src phosphorylation of VPS34 is required for this

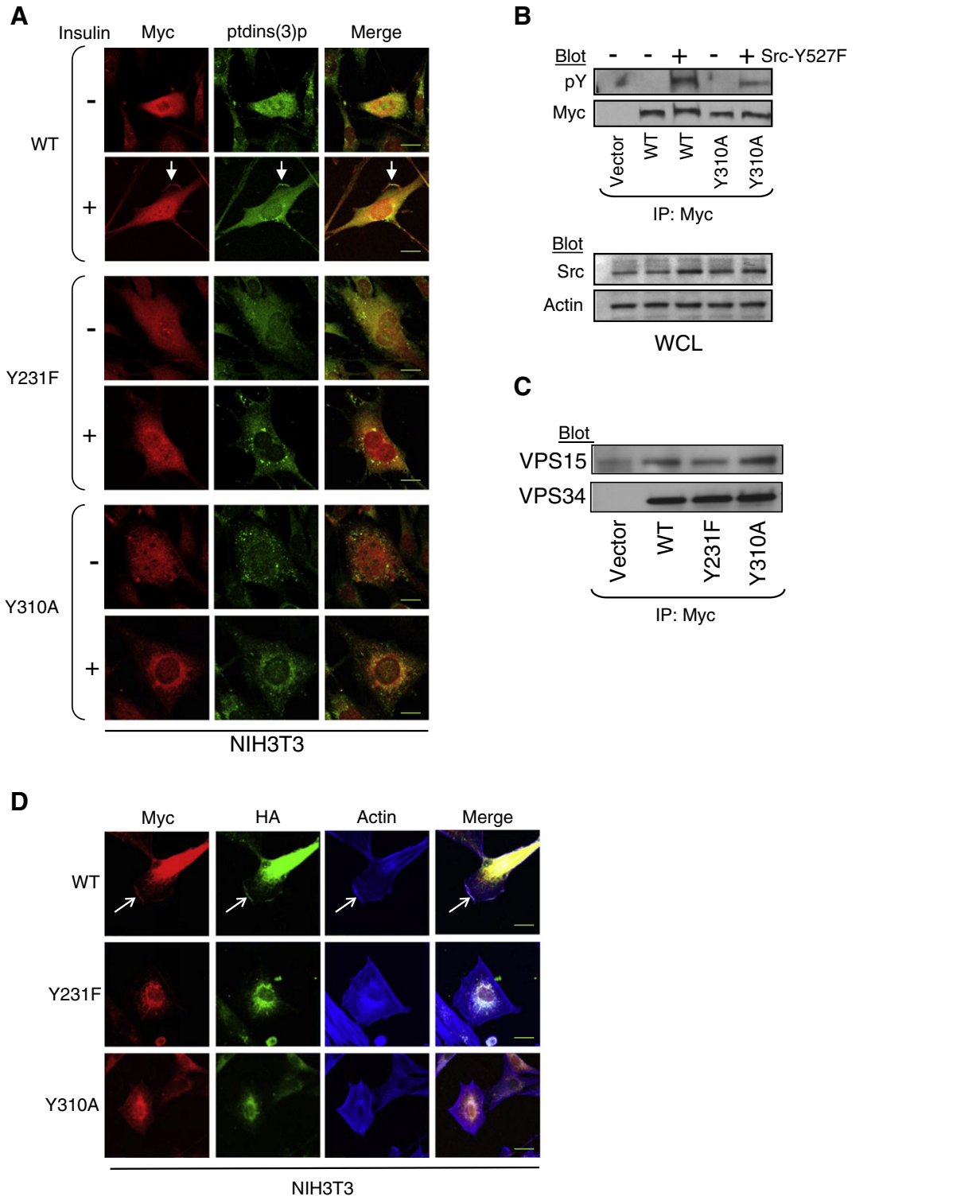


Fig. 6. VPS34 tyrosine phosphorylation by Src is required for ptdins(3)p production and lamellipodia formation following insulin treatment. (A) NIH3T3 cells were transfected for 24 h with pcDNA3-Myc-VPS34 (WT), pcDNA3-Myc-VPS34-Y231F (Y231F), or pcDNA3-Myc-VPS34-Y310A (Y310A) plasmids and then switched to DMEM containing 1% CS for 18–24 h. Cells were then either treated with 1 μM insulin for 30 min or left untreated. Cells were fixed, permeabilized, and then stained with anti-Myc antibody (red) and ptdins(3)p (green). Arrows, Myc-VPS34 and ptdins(3)p at lamellipodia. (B) Y310 of VPS34 is a Src phosphorylation site. NIH3T3 cells were transfected overnight with vector, pcDNA3-Myc-VPS34, or pcDNA3-Myc-VPS34-Y310A ± pcEFL-Src-Y527F. Myc-tagged VPS34 proteins were immunoprecipitated from WCL using an anti-Myc antibody. Tyrosine-phosphorylated Myc-VPS34 was detected using anti-phosphotyrosine antibody (clone 4G10). Blots were then stripped and reprobed for Myc-tagged VPS34. Lower blots, WCL Western blot for Src and Actin. Data are representative of two independent experiments. (C) Cos7 cells were transfected for 24 h with pcDNA3-Myc-VPS34 (wild type), pcDNA3-Myc-VPS34-Y231F (Y231F), or pcDNA3-Myc-VPS34-Y310A plasmids. Myc-tagged VPS34 proteins were immunoprecipitated from WCL using an anti-Myc antibody. VPS15 was detected by Western blot analysis; immunoprecipitated Myc-VPS34 was detected by blotting with VPS34 antibody. (D) NIH3T3 cells were co-transfected overnight with HA-tagged Rac1-Q61L and the indicated VPS34 plasmids. Cells were stained using anti-Myc antibody to detect the Myc-tagged VPS34 proteins (red) and HA to detect HA-Rac1-Q61L (green) overexpression. Actin (blue). All data in this figure are representative of two independent experiments.

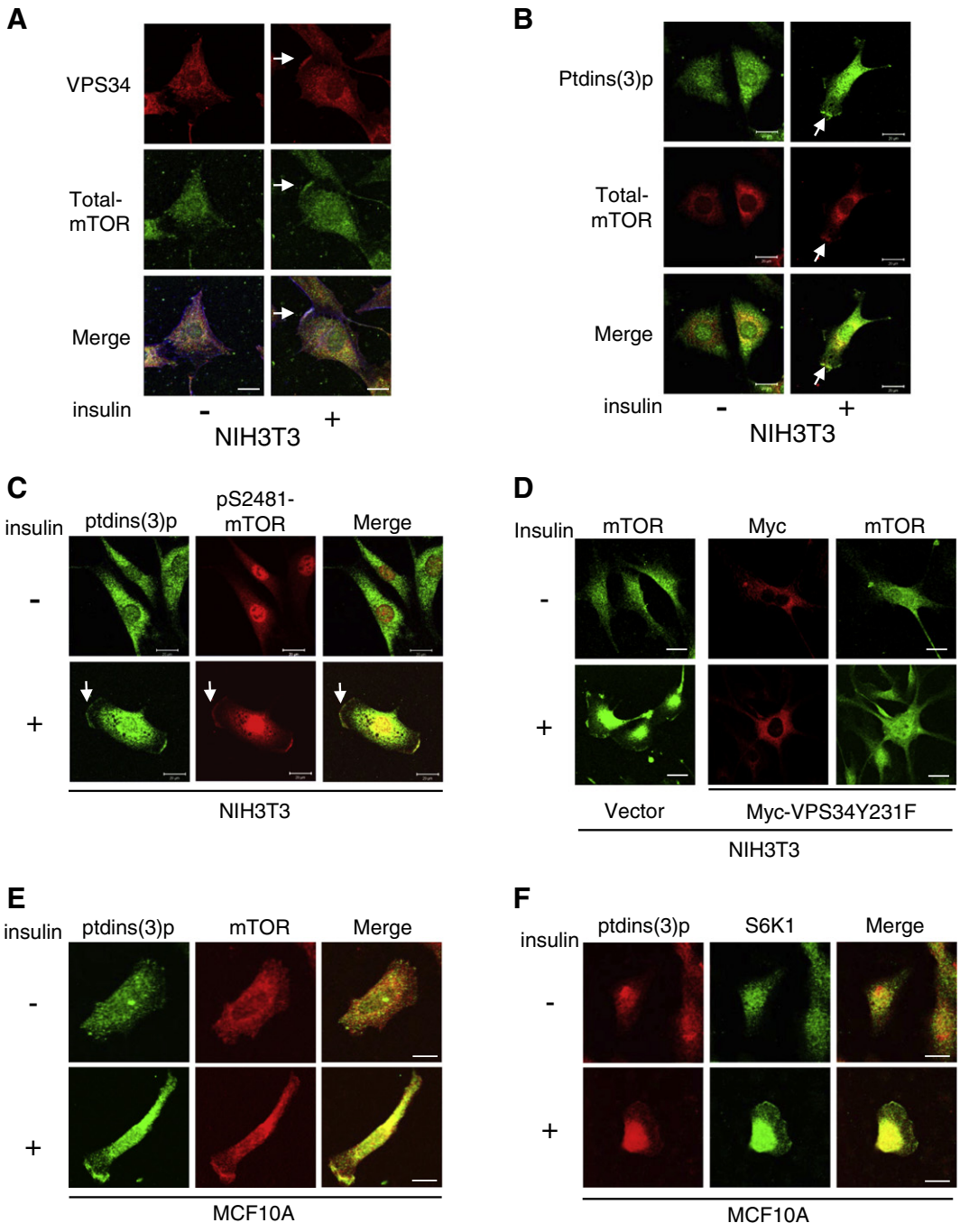


Fig. 7. mTOR colocalizes with VPS34 and ptdins(3)p following insulin stimulation. (A–C) NIH3T3 cells were seeded overnight in fibronectin-coated chamber slides and then switched to serum-free medium for 18–24 h. Cells were then either treated with 1 μM insulin for 30 min or left untreated. Cells were fixed, permeabilized, and stained as indicated. (A) VPS34 (red), total mTOR (green); blue in merge is actin. (B) Total mTOR (red) and pdtins(3)p (green). (C) pS2481-mTOR (red) and ptdins(3)p (green). (D) NIH3T3 cells were seeded overnight in fibronectin-coated chamber slides and then transfected for 24 h with the indicated plasmids. Cells were switched to serum-free medium for 18–24 h and then either treated with 1 μM insulin for 30 min or left untreated. Cells were fixed, permeabilized, and then stained for Myc (red) and total mTOR (green). (E-F) MCF10A cells were seeded overnight in fibronectin-coated chamber slides. Cells were switched to serum-free DMEM for an additional 18–24 h and then either treated for 30 min with 1 μM insulin or left untreated. Cells were fixed, permeabilized, and then stained as indicated. All data in this figure are representative of two independent experiments.

function. VPS34 is well characterized for its role in vesicular trafficking where ptdins(3)p produced by VPS34 binds to FYVE domain-containing proteins to regulate processes such as autophagy and early endosomal fusion [6]. Here, the ptdins(3)p 5-kinase PIKFYVE colocalized with VPS34 at lamellipodia following insulin stimulation which suggests that both ptdins(3)p and PI(3,5)p2 contribute to insulin-mediated signaling. This idea is supported by data generated in adipocytes that demonstrated that PIKFYVE is required for insulin-mediated translocation of mTORC1 to the plasma membrane and for mTORC1 activation [30]; however, the localization of PIKFYVE was not determined in this study. Here, we demonstrate that insulin stimulates PIKFYVE colocalization with VPS34 at lamellipodia which provides further evidence that membrane localized production of ptdins(3)p and PI(3,5)p2 contributes to the localization and activation of mTORC1. Due to the conversion between ptdins(3)p and PI(3,5)p2, additional studies are needed to determine the distinct function of each in insulin-mediated signaling.

Initial studies suggested that VPS34 was not regulated by extracellular stimuli and that it maintained ptdins(3)p at steady state levels [12].

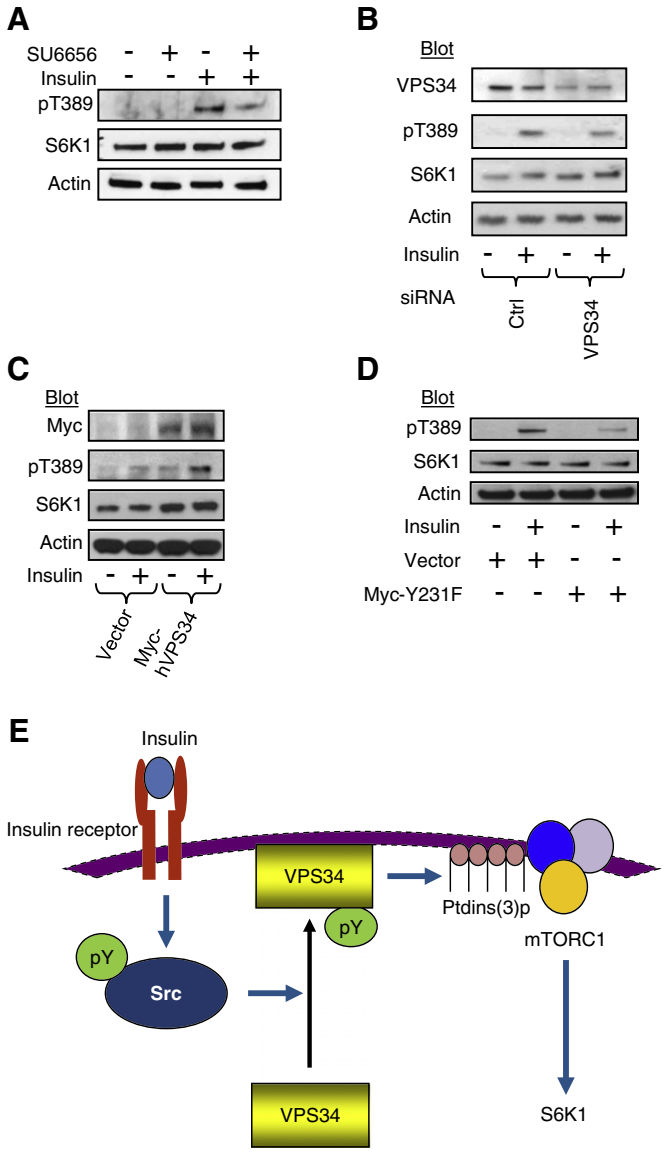


Fig. 8. VPS34 tyrosine phosphorylation is required for activation of S6K1 following insulin stimulation.(A) NIH3T3 cells were seeded overnight in six-well plates and switched to serum-free medium for an additional 18–24 h and then either pretreated with 2.5 μM SU6656 for 4 h or left untreated. The cells were then either treated with 1 μM insulin for 30 min or left untreated. WCL was resolved by SDS-PAGE, transferred to PVDF, and then blotted for pT389-S6K1. The blots were then stripped and reprobed for total S6K1. Actin Western blotting confirmed equal protein loading. (B) NIH3T3 cells were transfected for 48 h with either control siRNA or VPS34-targeting siRNA and then switched to DMEM containing 1% CS for an additional 18–24 h. Cells were then either treated for 30 min with 1 μM insulin or left untreated. WCL was resolved by SDS-PAGE, transferred to PVDF, and then Western blotted for the indicated proteins. (C) NIH3T3 cells were transfected overnight with either vector control or Myc-tagged-hVPS34 wild type (Myc-hVPS34). 48 h post transfection, cells were switched to 1% CS in DMEM for 18–24 h. Cells were then either treated with 1 μM insulin or left untreated. WCL was resolved by SDS-PAGE, transferred to PVDF, and then Western blotted for the indicated proteins. (D) Cells were transfected for 24 h with Myc-tagged pcDNA3-VPS34Y231F (Myc-Y231F), switched to 1% CS in DMEM for an additional 18–24 h, and then either treated for 30 min with 0.8 μM insulin or left untreated. WCL was resolved by SDS-PAGE, transferred to PVDF, and then Western blotted for the indicated proteins. (E) Proposed model: insulin stimulation activates Src, which then phosphorylates VPS34. Activated VPS34 produces ptdins(3)p which is localized at lamellipodia and is required for lamellipodia formation and activation of the mTOR/S6K1 pathway.

However, recent studies have demonstrated that cell stimulation activates VPS34. For instance, insulin-mediated activation of S6K1 requires VPS34 activation by amino acids [13]. While VPS34 is required for insulin-stimulated S6K1 activation, VPS34 activation by insulin has not been detected using in vitro kinase assays [13]. The data presented here demonstrate that insulin stimulates increased ptdins(3)p production by VPS34 which is localized predominantly at lamellipodia. Based on the low level of VPS34 activity detected biochemically in NIH3T3 cells grown in complete media [22], we predict that the level of ptdins(3)p generated by VPS34 in serum-starved NIH3T3 cells treated with insulin is below the level of detection using the currently available in vitro kinase assays.

Following insulin stimulation, Src functions in both lamellipodia formation and activation of the mTOR/S6K1 pathway. For instance, following insulin stimulation, protein-tyrosine phosphatase 1B (PTP1B) activates Src resulting in Rac1 activation and subsequent ruffle formation [25]. Insulin also stimulates the formation of a complex containing Src, Akt, and the scaffold protein β-arrestin at the plasma membrane positioning Src to phosphorylate Akt resulting in full Akt activation [19–21]. Whether VPS34 functions in either of these pathways remains to be determined.

Data from this study demonstrate that insulin-induced lamellipodia formation requires Src phosphorylation of VPS34. This is evidenced by the data showing that both knockdown of VPS34 protein levels and expression of the VPS34 tyrosine mutants block lamellipodia formation in response to insulin. Additionally, co-expression of constitutively active Rac1-Q61L with either of the VPS34 tyrosine mutants reduced membrane ruffle formation. The mechanisms by which VPS34 regulates Rac1-induced lamellipodium formation remain to be determined. However, this study provides an important clue that VPS34 may function as a downstream target of Rac1 that regulates lamellipodium formation.

Data in these studies reveal similarities between the regulation and function of VPS34 and PI(3)Kα in insulin signaling. Following insulin stimulation, both are recruited to discrete regions of the plasma membrane where they are activated. Both PI(3)Kα and VPS34 activate the mTOR/S6K1 signaling pathway. Lastly, both VPS34 and PI(3)Kα [37] are required for lamellipodium formation. In light of these similarities, comparisons between VPS34 and PI(3)Kα may lead to elucidation of other functions of VPS34.

5. Conclusion

In conclusion, these data demonstrate that insulin stimulates localized activation of VPS34 through Src-mediated tyrosine phosphorylation and that ptdins(3)p generated by VPS34 functions in lamellipodia formation and S6K1 activation.

Disclaimer

The views expressed in this article are those of the authors and do not reflect the views or policies of the U.S. Food and Drug Administration.

Statement of conflicts of interest

The authors have no conflicts of interest.

Author contributions

Conceived project: WJW. Designed and performed experiments: DSH and WJW. Performed experiments and analyzed data: DSH and WJW. Performed experiments: YS, MD, NM, JY and MKE. Wrote the manuscript: DSH and WJW.

References

[1] C.H. Huang, D. Madelker, S.B. Gabelli, L.M. Amzel, Cell Cycle 7 (2008) 1151–1156.
[2] O. Vadas, J.E. Burke, X. Zhang, A. Berndt, R.L. Williams, Sci. Signal. 18 (2011) re2.
[3] R. Katso, K. Okkenhaug, K. Ahmadi, S. White, J. Timms, M.D. Waterfield, Annu. Rev. Cell Dev. Biol. 17 (2001) 615–675.
[4] L. Stephens, F.T. Cooke, R. Walters, T. Jackson, S. Volinia, I. Gout, M.D. Waterfield, P.T. Hawkins, Curr. Biol. 4 (1994) 203–214.

[5] S. Volinia, R. Dhand, B. Vanhaesebroeck, L.K. MacDougall, R. Stein, M.J. Zvelebil, J. Domin, J.C. Panaretou, M.D. Waterfield, EMBO J. 14 (1995) 3339–3348.
[6] J.M. Backer, Biochem. J. 410 (2008) 1–17.
[7] D.J. Gillooly, I.C. Morrow, M. Lindsay, M.R. Gould, N.J. Bryant, J.M. Gaullier, R.G. Parton, H. Stenmark, EMBO J. 19 (2000) 4577–4588.
[8] W.J. Brown, D.B. DeWald, S.D. Emr, H. Plutner, W.E. Balch, J. Cell Biol. 130 (1995) 781–796.
[9] S. Christoforidis, M. Miaczyns ka, K. Ashman, M. Wilm, L. Zhao, S.C. Yip, M.D. Waterfield, J.M. Backer, M. Zerial, Nat. Cell Biol. 1 (1999) 249–252.
[10] E. Nielsen, F. Severin, J.M. Backer, A.A. Hyman, M. Zerial, Nat. Cell Biol. 1 (1999) 376–382.
[11] U. Siddhanta, J. McIlroy, A. Shah, Y. Zhang, J.M. Backer, J. Cell Biol. 143 (1998) 1647–1659.
[12] J.-M. Gaullier, A. Simonsen, A. D'Arrigo, B. Bremnes, H. Stenmark, Nature 394 (1998) 422–433.
[13] M.P. Byfield, J.T. Murray, J.M. Backer, J. Biol. Chem. 280 (2005) 33076–33082.
[14] T. Nobukuni, M. Joaquin, M. Roccio, S.G. Dann, S.Y. Kim, P. Gulati, M.P. Byfield, J.M. Backer, F. Natt, J.L. Bos, F.J. Zwartkruis, G. Thomas, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 14238–14243.
[15] T. Furuya, M. Kim, M. Lipinski, J. Li, D. Kim, T. Lu, Y. Shen, L. Rameh, B. Yankner, L.H. Tsai, J. Yuan, Mol. Cell 38 (2010) 500–511.
[16] A.P. Sagona, I.P. Nezis, N.M. Pedersen, K. Liestol, J. Poulton, T.E. Rusten, R.I. Skotheim, C. Raiborg, H. Stenmark, Nat. Cell Biol. 12 (2010) 362–371.
[17] S.J. Parsons, J.T. Parsons, Oncogene 23 (2004) 7906–7909.
[18] B.D. Cuevas, Y. Lu, M. Mao, J. Zhang, R. LaPushin, K. Siminovitch, G.B. Mills, J. Biol. Chem. 276 (2001) 27455–27461.
[19] B. Luan, J. Zhao, H. Wu, B. Duan, G. Shu, X. Wang, D. Li, W. Jia, J. Kang, G. Pei, Nature 457 (2009) 1146–1149.
[20] R. Chen, O. Kim, J. Yang, K. Sato, K.M. Eisenmann, J. McCarthy, H. Chen, Y. Qiu, J. Biol. Chem. 276 (2001) 31858–31862.
[21] T. Jiang, Y. Qiu, J. Biol. Chem. 278 (2003) 15789–15793.
[22] D.S. Hirsch, Y. Shen, M. Dokmanovic, W.J. Wu, Cancer Res. 70 (2010) 5974–5983.
[23] A.J. Ridley, H.F. Paterson, C.L. Johnston, D. Dickmann, A. Hall, Cell 70 (1992) 401–410.
[24] X. Gao, P.R. Lowry, X. Zhou, C. Depry, Z. Wei, G.W. Wong, J. Zhang, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 14509–14514.
[25] S. Dadke, J. Chernoff, J. Biol. Chem. 278 (2003) 40607–40611.
[26] L.-S. Servitja, S. Campbell, A. Claing, Cell. Signal. 25 (2013) 1813–1819.
[27] S. Guo, J. Endocrinol. 220 (2014) T1–T23.
[28] T.G. Kutateladze, Prog. Lipid Res. 46 (2007) 315–327.
[29] Y. Ono, H. Nakanishi, M. Nishimura, M. Kazizaki, K. Takahashi, M. Miyahara, K. Satoh-Horikawa, K. Mandai, Y. Takai, Oncogene 19 (2000) 3050–3058.
[30] D. Bridges, J.T. Ma, S. Park, K. Inoki, L.S. Weisman, A.R. Saitiel, Mol. Biol. Cell 23 (2012) 2955–2962.
[31] P.J. Roberts, N. Mitin, P.J. Keller, E.J. Chenette, J.P. Madigan, R.O. Currin, A.D. Cox, O. Wilson, P. Kirschmeier, D.J. Der, J. Biol. Chem. 283 (2008) 25150–25163.
[32] D.S. Hirsch, Y. Shen, W.J. Wu, Cancer Res. 66 (2006) 3523–3530.
[33] J.C. Obenauer, L.C. Cantley, M.B. Yaffe, Nucleic Acid Res. 31 (2003) 3635–3641.
[34] B. Magnuson, B. Ekim, D.C. Fingar, Biochem. J. 441 (2012) 1–21.
[35] A. Saci, L.C. Cantley, C.L. Carpenter, Mol. Cell 42 (2011) 50–61.
[36] M. Vojtechova, J. Tureckova, D. Kucerova, E. Sloncova, J. Vachtenheim, Z. Tuhackova, Neoplasia 10 (2008) 99–107.
[37] S.S. Martin, T. Haruta, A.J. Morris, A. Klippel, L.T. Williams, J.M. Olefsky, J. Biol. Chem. 271 (1996) 17605–17608.