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PIP4k γ is a substrate for mTORC1 that maintains basal mTORC1 signaling during starvation

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Abstract

Phosphatidylinositol-5-phosphate 4-kinases (PIP4ks) are a family of lipid kinases that specifically use phosphatidylinositol 5-phosphate (PI-5-P) as a substrate to synthesize phosphatidylinositol 4,5-bisphosphate (PI-4,5-P₂). Suppression of PIP4k function in *Drosophila* results in smaller cells and reduced target of rapamycin complex 1 (TORC1) signaling. Here we showed that the γ isoform of PIP4k stimulated signaling through mammalian TORC1 (mTORC1). Knockdown of PIP4k γ reduced cell mass in cells in which mTORC1 is constitutively activated by Tsc2 deficiency. In Tsc2 null cells mTORC1 activation was partially independent of amino acids or glucose and glutamine. PIP4k γ knockdown inhibited the nutrient-independent activation of mTORC1 in Tsc2 knockdown cells and reduced basal mTORC1 signaling in wild-type cells. PIP4k γ was phosphorylated by mTORC1 and associated with the complex. Phosphorylated PIP4k γ was enriched in light microsomal vesicles, whereas the unphosphorylated form was enriched in heavy microsomal vesicles associated with the Golgi. Furthermore, basal mTORC1 signaling was enhanced by overexpression of unphosphorylated wild-type PIP4k γ or a

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SUPPLEMENTARY MATERIALS:

Figure S1

PIP4k γ knockdown decreases cell size and mTORC1 signaling in serum- or amino acid-starved Tsc2 knockdown cells and identification of mTORC1-dependent phosphorylation sites in PIP4k γ .

Figure S2

Phosphorylation of PIP4k γ does not affect protein stability, dimerization, or associated kinase activity or cellular PI-5-P.

Figure S3

PIP4k γ localizes to cytosolic and Golgi-associated vesicles.

Figure S4

Analysis of the effect of rapamycin on the distribution of PIP4k γ , GM130, or β COP among the four fractions generated by differential centrifugation of BTC6 cells.

AUTHOR CONTRIBUTIONS: A.M.M., D.A.S and L.E.R. designed and performed the experiments, analyzed the data and wrote the manuscript. I.B. performed the PIP4k stability experiments. J.M.A. designed, performed and analyzed the mass spectrometry experiments.

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phosphorylation-defective mutant and decreased by overexpression of a phosphorylation mimetic mutant. Together these results demonstrate that PIP4k γ and mTORC1 interact in a self-regulated feedback loop to maintain low and tightly regulated mTORC1 activation during starvation.

INTRODUCTION

Phosphoinositides (PIs) play critical roles in signaling events that occur in specific membrane compartments. At the plasma membrane, PIs can mediate early growth factor responses that lead to cell proliferation or chemotaxis. At endomembranes PIs can regulate vesicle trafficking events that control endocytosis, exocytosis or autophagy (1). The physiological function of phosphatidylinositol-5-monophosphate (PI-5-P) is unclear. Its discovery followed from the biochemical characterization of the enzyme PIP4k (phosphatidylinositol-5-phosphate 4-kinase), which phosphorylates PI-5-P to generate phosphatidylinositol-4,5-bisphosphate (PI-4,5-P₂) (2).

The PI-5-P pathway contributes only a small fraction of the total PI-4,5-P₂ in cells (3). Thus, it has been postulated that PIP4k's main function is to regulate intracellular PI-5-P and/or generate PI-4,5-P₂ on specific membrane compartments where PI-5-P is present (4). We have reported that PI-5-P is enriched in low-density membrane compartments associated with smooth ER and Golgi, where PIP4k has also been localized (5), suggesting that these are sites of PI-5-P-mediated PI-4,5-P₂ synthesis. PI-4,5-P₂ regulates membrane trafficking events such as vesicle budding, fusion and actin rearrangement (4), but evidence for a physiological role for PI-5-P in these events is scarce (6). Understanding the spatial and temporal regulation of PIP4k will be crucial to establish the physiological function of PI-5-P and of the alternative pathway for PI-4,5-P₂ synthesis.

The PIP4k family is well-conserved from worms to mammals, but absent in yeast (6). Although there is only one gene encoding PIP4k in *Drosophila* and *C. elegans*, three distinct isoforms (α , β and γ) have been identified in mammals. All three isoforms of PIP4k have similar protein structure: a C-terminal kinase domain that is interrupted by an insert region and a N-terminal dimerization domain (7). The α and the β isoforms share 83% identity at the protein level, whereas the γ isoform only shares 60% identity with either one (7). In comparison to α and β , the γ isoform has poor catalytic activity and it has been suggested that it serves as a chaperone for the more active isoforms (8).

Several studies indicate that PIP4k enzymes play a role in cellular signaling events (9–11). All three PIP4k isoforms are phosphorylated in response to extracellular cues (3). The β isoform is phosphorylated by the mitogen-activated protein kinase p38 at Ser³²⁶ in response to stress signals, resulting in attenuation of its kinase activity and subsequent increase in cellular PI-5-P (11). The α isoform is phosphorylated at Thr³⁷⁶ by protein kinase D (PKD), a phosphorylation event that inhibits enzyme activity (12). The γ isoform is phosphorylated in response to growth factors (13), but unlike the α and β isoforms, the effect of phosphorylation and the kinase involved had not been identified.

In the present work, we examined how PIP4k γ regulates signaling downstream of the mammalian target of rapamycin complex 1 (mTORC1). TORC1 is a nutrient-sensing kinase

complex involved in the regulation of protein translation, autophagy and proliferation in response to amino acid, energy and growth factors (14). We found that PIP4 γ is a positive regulator of the mTORC1 signal that controls protein translation. First, we show that knockdown of PIP4 γ reversed the effect of Tsc2 knockdown on cell mass, resulting in smaller cells. Consistently, PIP4 γ knockdown impaired basal mTORC1 activity, as determined by analysis of the phosphorylation of the TORC1 substrates p70S6K (p70S6 kinase) and 4E-BP (eIF4E-binding protein) in starved cells. Furthermore, we demonstrate that PIP4 γ was phosphorylated by mTORC1 in a manner dependent on nutrient availability. Two serine residues in the kinase insert region of PIP4 γ were identified as mTORC1-dependent phosphorylation sites. Using subcellular fractionation, we found that the unphosphorylated form of PIP4 γ predominantly localized in Golgi-associated vesicles, whereas the phosphorylated forms were enriched in cytosolic vesicles. mTORC1 signaling was enhanced by expression of wild-type or a phosphorylation-defective mutant of PIP4 γ and decreased by that of a phosphorylation mimetic mutant. Together these results demonstrate that in its unphosphorylated form PIP4 γ is necessary to maintain basal mTORC1 activity and that upon nutrient stimulation, mTORC1 phosphorylates and shuts down this function of PIP4 γ .

RESULTS

PIP4 γ stimulates mTORC1 signaling

A cellular response to TORC1 activation is an increase in the rate of protein translation that leads to an increase in cell mass (15). *Drosophila* that express a loss of function mutant of dPIP4k have smaller cells and delayed larval development due to impaired TORC1 signaling (16). To investigate whether PIP4 γ plays a role in mTORC1 signaling in mammalian cells, we used shRNA to stably knock down PIP4 γ expression in HeLa cells in which mTORC1 was constitutively activated due to knockdown of its inhibitor Tsc2 (17, 18). When compared to control cells, serum-starved Tsc2 knockdown cells had increased phosphorylation of S6 (Fig 1A, compare lanes 1 and 2), consistent with Tsc2 knockdown. We examined the effect of PIP4 γ knockdown on cell size by measuring changes in the low angle forward light scatter (FSC-H) with a flow cytometer. Changes in FSC-H are directly proportional to the diameter of the cell. When compared to control cells, serum-starved Tsc2 knockdown cells had a distinct shift to the right in FSC-H (Fig. S1A, upper left panel), indicating increased cell size. Knockdown of Tsc2 resulted in a 5% increase and 24-hour rapamycin treatment resulted in a 3% decrease in mean FSC-H as compared to control cells (Fig. 1B). These are comparable to the previously reported 4% reduction in mean FSC-H in HeLa cells treated with rapamycin for 72 hours (19). Knockdown of PIP4 γ in serum-starved Tsc2 knockdown cells decreased PIP4 γ abundance by 80 to 90% (Fig. 1A) and reduced cell mass, as demonstrated by a shift in FSC-H distribution as compared to control cells (Fig.S1A, bottom right panel) and a 4% reduction in the mean FSC-H (Fig. 1B). This significant reduction in cell size is comparable to the 3% reduction in cell diameter in 293 cells with mTOR knockdown (20). These results suggested that mTORC1 signaling is impaired in PIP4 γ knockdown cells.

To better understand the role of PIP4 γ in mTORC1-mediated regulation of cell mass, we examined whether PIP4 γ knockdown affected mTORC1-mediated signals that regulate protein translation. mTORC1 activation by amino acids, glucose and growth factors leads to phosphorylation of the ribosomal S6 subunit through activation of p70S6K. mTORC1 also phosphorylates the translation inhibitor 4E-BP (eIF4E-binding protein). In its unphosphorylated form, 4E-BP binds to eIF4E (eukaryotic initiation factor 4E) on mRNAs with 5' caps to prevent assembly of the translation initiation complex (21). TORC1-dependent phosphorylation of 4E-BP leads to its dissociation from eIF4E to allow recruitment of eIF4G, formation of the initiation complex and translation initiation. We examined the effect of PIP4 γ knockdown on the phosphorylation of p70S6K and S6 using phospho-specific antibodies, and on the amounts of unphosphorylated 4E-BP using either an antibody specific to the unphosphorylated form of 4E-BP (anti-non-phospho 4E-BP) or m7GTP pulldown assays. m7GTP-sepharose beads can specifically pull down the unphosphorylated form of 4E-BP associated with eIF4E by mimicking CAP-containing mRNA (22). Cells with complete depletion of Tsc2 have high TORC1 activity and are insensitive to serum starvation (18). mTORC1 signaling was slightly decreased by serum starvation in Tsc2 knockdown cells (Fig 1C, compare lanes 1 and 2, and Fig. S1B) which is likely due to residual Tsc2 expression. As expected, short-term treatment of these cells with rapamycin completely inhibited S6 phosphorylation and increased the abundance of unphosphorylated 4E-BP and eIF4E-associated 4E-BP (Fig. 1C), due to inhibition of TORC1. Knockdown of PIP4 γ in serum-starved HeLa cells with Tsc2 knockdown decreased the phosphorylation of S6, p70S6K, and increased the association of 4E-BP with eIF4E and the amount of unphosphorylated 4E-BP (Fig. 1A, Fig. 1C, Fig. 1D and Fig. S1B). These results confirmed that PIP4 γ knockdown decreased signaling downstream of TORC1 and are consistent with the decrease in cell size.

We compared the effect of PIP4 γ knockdown on mTORC1 signaling in cells growing in complete media or in media lacking amino acids or glucose and glutamine. As expected, amino acid starvation of control cells almost completely inhibited the phosphorylation of p70S6K and increased the abundance of unphosphorylated 4E-BP (Fig 1E, lanes 7 and 8, and Fig. 1F, grey bars). In contrast, Tsc2 knockdown cells were only partially sensitive to amino acid starvation (Fig. 1E, compare lanes 2 and 8, and Fig. 1F, compare black bars with grey bars), as previously reported (23–25). Although PIP4 γ knockdown did not affect mTORC1 activity in Tsc2 knockdown cells growing in complete media, it significantly decreased mTORC1 signaling in starved cells (Fig. 1E, compare lanes 2 and 5, Fig. 1F, compare black bars with white bars, Fig. S1B and S1C). Phosphorylation of p70S6K was 2 to 3-fold lower in the Tsc2 and PIP4 γ knockdown cells starved of glucose and glutamine or amino acids, as compared to cells in which only Tsc2 was knocked down. PIP4 γ knockdown in amino acid or serum starved cells also decreased the phosphorylation of S6 and increased the electrophoretic mobility of p70S6K and S6 proteins, as compared to control cells, without affecting the total abundance of these proteins (Fig. 1A, 1C, 1E and S1C). Furthermore, unphosphorylated 4E-BP was 4-fold more abundant in amino acid-starved cells with knockdown of both Tsc2 and PIP4 γ compared to those with knockdown of only Tsc2 (Fig. 1E and F). The basal mTORC1 activity in amino acid-starved cells with knockdown of both Tsc2 and PIP4 γ was similar to that in cells with normal Tsc2

expression (Fig. 1E, compare lanes 5 and 8, and Fig. 1F). These results indicated that PIP4 γ knockdown impaired mTORC1 signaling during starvation.

To confirm that PIP4 γ promotes basal TORC1 signals that regulate protein translation in starved cells, we examined the effect of PIP4 γ knockdown on 4E-BP association with eIF4E in HeLa cells with normal Tsc2 expression. When these cells were starved of glucose and glutamine, unphosphorylated 4E-BP associated with eIF4E (Fig. 1G). Upon nutrient stimulation, 4E-BP dissociated from the complex, allowing eIF4G and eIF4A to be recruited. In starved cells, knockdown of PIP4 γ increased the abundance of unphosphorylated 4E-BP and the ratio of 4E-BP/eIF4G bound to the m7GTP beads, suggesting a decrease in basal mTORC1 activity and a reduction in the formation of active translation initiation complexes (Fig. 1G and H). Together these results demonstrated that PIP4 γ increased basal TORC1 signaling to regulate protein translation and cell mass.

PIP4 γ is phosphorylated downstream of mTORC1

PIP4 γ exists in multiple phosphorylated forms with distinct electrophoretic mobilities (13, 26). Treatment with phosphatases results in a shift in the PIP4 γ electrophoretic mobility to a single fast-mobility form (13, 26). While examining the abundance of endogenous and exogenous PIP4 γ in HeLa and BTC6 mouse insulinoma cells we observed that, upon glucose and glutamine starvation, the abundance of the slowly migrating forms (upper bands) of PIP4 γ decreased, whereas that of the more quickly migrating form (bottom band) increased (Fig. 2A). Amino acid and glucose starvation were most effective at promoting a shift in PIP4 γ mobility from the upper to the lower form, suggesting that PIP4 γ is phosphorylated by mTORC1 (Fig. S1C). To test this possibility, we compared the electrophoretic mobility of PIP4 γ in wild-type and Tsc2 knockout cells. The ratio of slow/fast PIP4 γ was higher in Tsc2^{-/-} MEFs than in MEFs from wild-type mice (Fig. 2B) and also higher in HeLa cells with Tsc2 knockdown (Fig. 1A, compare lanes 1 and 2). Inhibition of mTORC1 with rapamycin or Ku0063794 resulted in a shift in PIP4 γ mobility from the slow to the fast form (Fig. 2B and C). Changes in PIP4 γ mobility in these cells resembled those in the mobility of p70S6K, a *bona fide* TORC1 substrate (15, 27). In contrast, PIP4 γ mobility was not affected by treatment with staurosporine (Fig. 2B), an AGC kinase inhibitor which inhibits the activity of p70S6K but not that of mTORC1 (28). Thus, PIP4 γ was modified downstream of mTORC1, independently of p70S6K activity.

mTORC1 phosphorylates PIP4 γ at Ser³²⁴ and Ser³²⁸

To identify the amino acid residues phosphorylated downstream of mTORC1, we performed LC-MS/MS analyses on PIP4 γ immunoprecipitated from BTC6 cells treated or not with rapamycin. We identified Ser³²⁸ as a rapamycin-sensitive phosphorylation site (Fig. S1D), which is consistent with the shift in PIP4 γ electrophoretic mobility, given that Ser³²⁸ is followed by a proline residue (Fig. S1E) and the phosphorylation of serine residues adjacent to proline causes substantial shifts in electrophoretic mobility due to proline isomerization (29).

Based on comparison of the PIP4 γ sequence with other mTORC1 substrates (30, 31), we predicted that Ser³²⁴ was an mTORC1-dependent phosphorylation site. This site was not

covered by the LC-MS/MS analyses. To test whether Ser³²⁴ and Ser³²⁸ were phosphorylated by mTORC1, we expressed forms of myc-PIP4k γ that were wild-type or had single S328A or double S324/328A mutations in *Tsc2*^{-/-} MEFs. A form of PIP4k γ with a mutation in the predicted catalytic lysine (K152A) (32) was used to rule out autophosphorylation. Metabolic labeling of these cells with ³²P showed that wild-type PIP4k γ is highly phosphorylated in a rapamycin-sensitive manner (Fig. 2D). Phosphorylation of PIP4k γ was almost completely abolished by the S324/328A double mutation and partially inhibited by the single S328A mutation. To confirm that the more slowly migrating bands were phosphorylated forms of PIP4k γ , we compared the electrophoretic mobility of the different mutants (Fig. 2E and F). While 85–90% of wild-type PIP4k γ migrated with slower mobility (upper band), the double mutant S324/328A migrated as a single faster band (lower band), regardless of whether mTORC1 was active or inhibited by rapamycin treatment. The single S328A mutation partially suppressed the PIP4k γ slow mobility band. These results confirmed that Ser³²⁴ and Ser³²⁸ are mTORC1-dependent phosphorylation sites and that the different electrophoretic mobility bands correspond to PIP4k γ phosphorylated at one or both mTORC1 sites.

Ser³²⁴ and Ser³²⁸ reside within the kinase insert region of PIP4k γ (Fig. S1E). Sequence alignment between the kinase insert regions of all three PIP4k isoforms indicated that Ser³²⁴ is conserved only in the β and γ isoforms and Ser³²⁸ is unique to the γ isoform. Because this region of PIP4k γ is a substrate for the MAPK p38 (11) and PIP4k γ is a substrate for protein kinase D (PKD) (12), we tested the role of these kinases on PIP4k γ phosphorylation. PIP4k γ mobility was not affected by the p38 inhibitor SB203580 or the PKD inhibitor Go6983 (Fig. S1F), demonstrating that mTORC1-mediated phosphorylation of PIP4k γ does not require priming by these kinases.

To address whether mTOR can directly phosphorylate PIP4k γ , we incubated purified GST-PIP4k γ with purified mTOR and [³²P] γ -ATP. This resulted in [³²P]-labeling of a 130 kD band corresponding to the autophosphorylation of recombinant mTOR and a 70 kD band corresponding to the GST-PIP4k γ (Fig. 2G). Addition of the mTOR inhibitor wortmannin (33) abolished the appearance of these bands, thus confirming that the labeled bands were indeed phosphorylation products of mTOR and indicating that PIP4k γ is a substrate for mTOR.

mTORC1 associates with PIP4k γ

The mTORC1 regulatory subunit Raptor associates with many mTORC1 substrates through their TOS (TOR signaling) motif, an interaction that is critical for the phosphorylation of its substrates (21). Rapamycin inhibits mTORC1 partly because it destabilizes the association between mTOR and Raptor (34). To examine whether Raptor interacts with PIP4k γ , we immunoprecipitated Raptor from lysates of *Tsc2*^{-/-} MEFs expressing myc-PIP4k γ . Raptor immunoprecipitates contained PRAS40, an mTORC1 substrate and binding partner, as well as the slow and faster migrating forms of myc-PIP4k γ (Fig. 3A). To show that Raptor associated with endogenous PIP4k γ , we used BTC6 cells because endogenous PIP4k γ is more abundant in these cells than in HeLa cells or MEFs (Fig. 2A). Raptor immunoprecipitates contained a PI-5-P 4-kinase activity, consistent with the association of PIP4k with Raptor (Fig. 3B). To test whether PIP4k γ interacts with mTOR, we

immunoprecipitated myc-PIP4 γ from lysates of *Tsc2*^{-/-} MEFs expressing wild-type or kinase-inactive myc-PIP4 γ . PIP4 γ associated with a protein kinase capable of phosphorylating it, an association that was partially decreased in cells treated with rapamycin (Fig. 3C and D). Rapamycin did not affect myc-PIP4 γ association with Raptor (Fig. 3A), but it partially decreased the association with mTOR (Fig. 3C and D), suggesting that Raptor interacts directly with PIP4 γ . The ability of Ku0063794 to disrupt the interaction between Raptor and endogenous PI-5-P 4-kinase activity (Fig. 3B) was surprising. We speculate that the association of PIP4 γ with mTOR (an enzyme/substrate interaction) strengthens its association with Raptor, but in the presence of the catalytic inhibitor Ku0063794 (35) this complex is disrupted. Together, these results show that PIP4 γ can associate with mTORC1. Since the TOS motif is not easily identifiable through sequence analysis, deletion studies will be necessary to confirm that PIP4 γ has a TOS motif.

Phosphorylation of PIP4 γ increases its affinity for cytoplasmic vesicles

Unlike other mTORC1 substrates, phosphorylation of PIP4 γ did not affect protein stability or associated PI-5-P 4-kinase activity (Fig S2A–C). Furthermore, the phosphorylation state of PIP4 γ did not affect its homo- or heterodimerization or cellular PI-5-P amounts (Fig. S2B–D). Therefore, we set out to determine whether mTORC1-dependent phosphorylation plays a role in the subcellular distribution of PIP4 γ . PIP4 γ localizes to cytoplasmic vesicles and vesicles associated with the Golgi (5, 13, 36). Immunocytochemistry of BTC6 and HeLa cells confirmed that endogenous PIP4 γ localizes to vesicular compartments in these cells (Fig. S3). PIP4 γ -containing vesicles were either spread throughout the cytoplasm, surrounding the nucleus or associated with the Golgi. To examine the phosphorylation state of PIP4 γ in these vesicular compartments in a quantitative manner, we used a differential centrifugation protocol that generates four subcellular fractions enriched in cytosol, light microsomal membranes, heavy microsomal membranes and membranes associated with the nucleus. Immunoblotting for various organelle markers showed that most organelles and membrane compartments, including mitochondria (marker: ATP synthase), plasma membrane (marker: β 1 integrin), ER [marker: protein disulfide isomerase (PDI)], Golgi (marker: GM130) and lysosome (marker: Lamp1), sedimented with the heavy microsomal fraction and the membranes associated with the nucleus (Fig. 3E and S4 A–D). The light microsomal fraction contained intracellular vesicles containing COPI (marker: β -COP) and COPII (marker: Sec23), but virtually no Golgi, ER, mitochondria or plasma membrane. Although PIP4 γ was present in all four fractions, it was enriched in the light microsomal fraction (Fig. 3F) together with β -COP and Sec23 containing vesicles, consistent with the staining of PIP4 γ in small cytoplasmic vesicles (Fig. S3A–V). The light microsomal fraction contained predominantly the phosphorylated form of PIP4 γ , while the heavy microsomal fraction, which includes the Golgi, contained almost exclusively the unphosphorylated form (Fig. 3F, compare lanes 2 and 3 and Fig S4E, compare dark bars with light bars). These data suggested that the distribution of the different phosphorylation states of PIP4 γ is compartmentalized.

To further examine how TORC1-mediated phosphorylation affected PIP4 γ localization, we generated BTC6 cell lines in which the expression of PIP4 γ is under the control of a

doxycycline-inducible promoter. Exogenous wild-type PIP4 γ localized predominantly to the light microsomal fraction, a distribution that was decreased by rapamycin treatment (Fig. 3G and H). While the distribution of a phosphorylation mimetic mutant (S324/328D) resembled that of wild-type PIP4 γ , the phosphorylation-defective mutant S324/328A resembled that of rapamycin-treated cells with decreased localization in the light microsomal fraction (Fig. 3G and H), suggesting that phosphorylation determines whether PIP4 γ concentrates on light vesicles. Upon overexpression, PIP4 γ abundance in the heavy microsomal fraction did not increase proportionally to its overall increase in expression (compare Fig. 3F with 3G), suggesting that localization to this compartment is saturable or limited by a cellular component. Nonetheless, the percentage of PIP4 γ in the heavy microsomal fraction was two-fold higher for the S324/328A mutant than for wild-type (Fig. 3G and H). As expected, the localization of the phosphorylation-defective mutant was not affected by rapamycin (Fig S4G and H). Together, these results demonstrated that unphosphorylated PIP4 γ co-fractionated with the Golgi in the heavy microsomal fraction, whereas phosphorylated PIP4 γ was enriched in the light cytosolic vesicles. This compartmentalization raises the possibility that the phosphorylated and unphosphorylated PIP4 γ could have distinct functions in cells.

Unphosphorylated PIP4 γ increases mTORC1 signaling

The ability of PIP4 γ to regulate mTORC1 was enhanced during nutrient starvation when the abundance of the unphosphorylated form of PIP4 γ was enhanced (Fig. S1C and 2A). These data suggested that phosphorylation inhibits the ability of PIP4 γ to regulate mTORC1. To investigate how the different phosphorylation states of PIP4 γ affect mTORC1 signaling, we expressed wild-type PIP4 γ , phosphorylation-defective (S324/328A) or phosphorylation-mimetic (S324/328D) mutants in HeLa cells using a doxycycline-inducible promoter. The S324/328D mutant protein had the same electrophoretic mobility as the more slowly migrating band of wild-type protein, while the S324/328A had the same electrophoretic mobility as the more rapidly migrating band (Fig. 4A). Unlike wild-type PIP4 γ , the electrophoretic mobility of the mutant proteins was not affected by serum or amino acid starvation. In nutrient-deprived cells expressing wild-type PIP4 γ , the unphosphorylated form of the kinase was predominant over the phosphorylated form and basal mTORC1 signaling was enhanced in a dose responsive manner (Fig 4B and C). Phosphorylation of p70S6K was increased by expression of the S324/328A mutant and decreased by expression of the S324/328D mutant in nutrient-deprived cells (Fig. 4B and C). The effect of mutant PIP4 γ on mTORC1 activity demonstrated that unphosphorylated PIP4 γ promotes basal mTORC1 activation and that phosphorylation counteracts this function of PIP4 γ .

DISCUSSION

mTORC1 signaling regulates many complex cellular processes including cell proliferation, cell size and autophagy. mTORC1-mediated regulation of cell size can be partially attributed to its ability to stimulate protein translation through phosphorylation of p70S6K and 4E-BP. Here we identified PIP4 γ as a substrate for mTORC1 involved in the regulation of cell size. We found that PIP4 γ knockdown impaired mTORC1 signaling in starved cells, but not in

cells growing in complete media, suggesting a specific role for PIP4k γ in maintaining basal mTORC1 signaling. Thoreen and collaborators determined that acute inhibition of mTORC1 significantly suppressed the translation of a subset of mRNAs with 5' terminal oligopyrimidine (TOP) motifs (37). Many components of the translational machinery, such as ribosomes and initiation factors are TOP-containing mRNAs regulated by mTORC1 signaling. We speculate that PIP4k γ -mediated mTORC1 activation is an important mechanism to maintain the basal abundance of the components of the translational machinery necessary to restart translation once ATP and amino acid stores are replenished. PIP4k γ 's long half-life (approximately 10 hrs; Fig S2A) makes it a suitable regulator of basal protein translation during nutrient starvation.

We demonstrated that the ratio between the phosphorylated and unphosphorylated forms of PIP4k γ depends on the activity of mTORC1. In starved cells, PIP4k γ is predominantly in the unphosphorylated state and participates in the activation of mTORC1. When ATP and amino acids are abundant and mTORC1 is fully active, phosphorylated PIP4k γ becomes the predominant form, which cannot activate mTORC1 (Fig. 4D). This negative feedback loop could be a mechanism for keeping basal mTORC1 activity low and tightly regulated. However, it is possible that in cancer cells this negative feedback loop is disrupted, allowing them to grow in low nutrients. PIP4k γ 's role in activating mTORC1 in nutrient deprived cells seems contrary to the role of LKB1 (liver kinase B1), a tumor suppressor kinase that inhibits mTORC1 activity through phosphorylation of AMPK (AMP-dependent kinase) when ATP concentrations are low (39). However, it is unlikely that PIP4k γ -mediated activation of mTORC1 in its basal state is mediated by inhibition of AMPK signaling, given that HeLa cells are deficient in LKB (40) and have impaired inactivation of mTORC1 in response AMPK activation (41).

Based on the ability of mTORC1 to phosphorylate PIP4ks in vitro and the sequence similarity of the mTORC1-dependent phosphorylation sites on PIP4k γ to those in other mTORC1 substrates, we propose that PIP4k γ is a direct substrate for mTORC1. The two mTORC1-dependent phosphorylation sites that we identified reside in proximity to each other in the kinase insert region of PIP4k γ . This region of the protein is highly disordered and protrudes away from the rest of the enzyme (7). Thus, it is not completely surprising that phosphorylation by mTORC1 did not affect the low intrinsic lipid kinase activity of PIP4k γ or the activity of the associated α and β isoforms. Instead, we found that unphosphorylated PIP4k γ is preferentially localized at the Golgi, while the phosphorylated form is preferentially at light cytoplasmic vesicles. The α , β and γ isoforms of PIP4k can form heterodimers (Fig S2B and (7, 42) (Thorsell et al., unpublished structure PDB entry 2GK9) and PIP4k γ has been suggested to serve as a chaperone for the more active α and β isoforms (36). Therefore, the subcellular localization of PIP4k γ is likely to determine sites of active PI-5-P consumption and PI-4,5-P₂ synthesis. Since PI-4,5-P₂ participates in actin rearrangement and membrane trafficking events such as vesicle budding, docking and fusion (43), we propose that the PI-5-P 4-kinase activity associated with PIP4k γ plays a role in the trafficking of a population of Golgi-derived vesicles. We envision that PIP4k γ cycles between the Golgi, cytoplasmic vesicles and the cytosol. In our model, unphosphorylated PIP4k γ exits the Golgi in Golgi-derived vesicles, where it becomes phosphorylated by mTORC1. Upon mTORC1 inhibition, de-phosphorylated PIP4k γ dissociates from these

vesicles into the cytosol, where it may be recruited back to the Golgi. Live cell imaging of PIP4 γ will help confirm this model.

The mechanism by which PIP4 γ regulates basal mTORC1 activity is not entirely clear. Activation of the small GTPase Rheb by inhibition of Tsc2 (a GTPase activating protein for Rheb) is indispensable for mTORC1 activation in response to all stimuli (25). In addition, it is critical that mTOR is transported to intracellular membrane compartments where Rheb is present (44, 45). In fact, amino acid-dependent activation of mTORC1 occurs at the surface of the lysosomes (46). However, it is likely that additional sites of mTORC1 activation exist, given that Rheb resides in various intracellular compartments (25, 45). Phosphoinositide kinases can control mTOR localization and activity. For example, the phosphatidylinositol-3-phosphate 5-kinase PIKFYVE and the Class II phosphatidylinositol 3-kinase PI3K-CII α activate mTORC1 through the generation of PI-3,5-P₂ at the plasma membrane (47) and the endosomal Class III phosphatidylinositol 3-kinase hVps34 activates mTORC1 in a nutrient-dependent manner (48). The subcellular distribution of PIP4 γ suggests a role for the PI-5-P pathway in the synthesis of PI-4,5-P₂ for Golgi-mediated intracellular membrane trafficking. Furthermore, our results show that PIP4 γ can associate with mTORC1, which is also found at the Golgi (49). Thus, we propose that during nutrient starvation, PIP4 γ facilitates mTORC1 transport to sites where active Rheb is present. Future studies will seek to identify the mechanisms by which PIP4 γ and the PI-5-P pathway for PI-4,5-P₂ synthesis contribute to vesicle trafficking events that modulate mTORC1 signaling.

MATERIALS AND METHODS

Cell lines, maintenance and manipulations

HeLa and BTC6 cells were from ATCC. MEF cells were a gift from Dr. Brendan Manning (Harvard Medical School). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, CellGro Mediatech, Inc) supplemented with 10% fetal bovine serum (FBS). Transfections were performed using TransPassD1 (NEB) or Lipofectamine plus (Invitrogen) according to the manufacturer's protocols. For stable expression of recombinant PIP4 γ and knockdowns we used retroviral and lentiviral delivery systems. Viruses were generated by transiently transfecting 293T cells with the appropriate expression plasmid and packing plasmids. Stable knockdown cells were generated by infection with pSuper.retro.puro and/or pReSi-derived retrovirus carrying the target sequence for PIP4 γ (shPIP4 γ) or a control sequence (shControl or C2), which contained the target sequences with 4–6 bases mismatched, or with pMSCV carrying the target sequence for Tsc2 (shTsc2) or luciferase (C1), as a control. Transient knockdowns were obtained by transfection with 125 pmol of the siRNA targeting PIP4 γ , PIP4 γ or a control sequence (see above) using Lipofectamine 2000 (Invitrogen). For transient expression of myc-PIP4 γ , we used pCMV vector. For stable expression of untagged PIP4 γ , we used pLXSN (high expression) and pLVX.Tight.puro tet-inducible (Clontech) vectors. Doxycycline was used at 0.007 to 0.5 μ g/ml. For nutrient starvation experiments, cells were kept in DMEM deprived of glucose and glutamine (Gibco) for 3 to 5 hrs. For amino acid starvation experiments, cells were kept in Dulbecco PBS supplemented with CaCl₂, MgCl₂, sodium pyruvate and glucose for 60 to

90 minutes. For nutrient deprivation experiments, cells were collected 72 hrs after addition of fresh media. For nutrient re-stimulation experiments, 25 mM glucose and 4 mM L-glutamine were added to the medium. Rapamycin was used at 20nM, staurosporin at 0.1 μ M, SB203580 at 1 μ M, Go6976 at 0.5 μ M, Go6983 at 0.25 μ M and Ku0063794 at 1 μ M. Cells were treated with these inhibitors for 2 to 4 hrs, with the exception of the cell size experiments in which cells were treated with rapamycin for 24 hrs.

Immunoprecipitation and western blot analysis

Total protein lysates were obtained by lysing cells in buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% Triton-X100 and 10% glycerol as well as protease (Sigma) and phosphatase inhibitors (1 mM sodium orthovanadate, 2 mg/ml sodium fluoride and 2 mg/ml β -glycerophosphate). Lysates were centrifuged at 14,000 $\times$ g to remove the insoluble fraction and were normalized based on total protein content measured by Bradford Assay (BioRad). For immunoprecipitations, antibody was added to the cell lysate and incubated for 2 hrs at 4°C. Protein A or protein G-sepharose beads (Amersham) were then added to the lysates and incubated for one hour at 4°C. Lysates were centrifuged and sepharose beads were washed three times in lysis buffer. Total cellular protein or immunoprecipitates were mixed with SDS-loading buffer, boiled for 5 min and separated by 10%-SDS-PAGE or by 4–20%-SDS-PAGE (Bio-Rad). For separation of the phosphorylated forms of PIP4k and p70S6k, electrophoresis was carried out on a 15cm gel. Proteins were transferred onto nitrocellulose membrane, which was blocked with 5% milk in Tris-buffered saline (TBS) plus 1 mM sodium orthovanadate. Membranes were probed overnight at 4°C with the appropriate primary antibody. After washing, membranes were incubated for 60 min at room temperature with secondary antibodies conjugated to IR680 (Rockland and Molecular Probes) or IR800 (Rockland). The membranes were washed in TBS-Tween and bound antibodies were detected and quantified using the Odyssey Infrared Imaging System (LICOR). Antibodies against tubulin were from BD Transduction Laboratories. Antibodies against phosphorylated p70S6k, total p70S6k, phosphorylated S6, total S6, total and unphosphorylated 4E-BP, Raptor, eIF4G, and eIF4E were from Cell Signaling Technology. Raptor antibody for immunoprecipitation was from Millipore. Antibody against PIP4k γ was a gift from Dr. M. Chao or purchased from Cell Signaling Technologies, and antibody against PIP4k γ was raised in rabbits against a GST-PIP4k γ N-terminal fusion protein.

Measurements of unphosphorylated 4E-BP

Protein lysates were prepared as described above. The abundance of unphosphorylated 4E-BP was measured by western blot of total lysates using an antibody specific to the unphosphorylated form of 4E-BP (anti-non-phospho 4E-BP), which recognizes the mTORC1-dependent target site in 4E-BP (Thr⁴⁶) only when in its unphosphorylated form (Cell Signaling Technologies). The amounts of eIF4E-associated 4E-BP were measured using m7GTP-sepharose beads in a pulldown assay. In this assay, protein lysates were normalized for protein content and incubated with m7GTP beads (GE Healthcare) for 3 hrs. The beads were washed in lysis buffer and the associated proteins were analyzed by western blot using antibody against 4E-BP, eIF4E or eIF4G (Cell Signaling Technologies).

***In vivo* phosphorylation assay**

Tsc2^{-/-} MEFs were transiently transfected with myc-PIP4kγ, WT or mutants, then labeled with 0.2 mCi/ml of inorganic [³²P] (Perkin-Elmer) in phosphate-free DMEM supplemented with 10% dialyzed FBS for 3 hrs prior to lysis. Exogenous PIP4kγ was immunoprecipitated using anti-myc antibody (Millipore), as described above. After electrophoresis, gels were stained with coomassie, dried and exposed to film.

PI-5-P 4-kinase assay

Immunoprecipitates were incubated with 200 μM PI-5-P/phosphatidylserine (1:5) and 1 μCi [³²P]-GTP (Perkin-Elmer) in 30 mM Hepes pH 7.0, 1 mM EGTA, 10 mM MgCl₂ for 10 min at room temperature. Under these conditions, PIP4k activity assay is linear and therefore quantitative. Lipids were extracted with HCl:chloroform: methanol and separated by TLC as described (50).

Subcellular fractionation by differential centrifugation

Cells were washed in PBS, rinsed with cytosol buffer (0.2 M sucrose; 25 mM HEPES, pH 7; 125 mM potassium acetate; 1 mM dithiothreitol; 1 mM sodium orthovanadate; 2 mg/ml sodium fluoride; 2 mg/ml β-glycerophosphate; 1 mM phenanthroline; 1 mM benzamidine and protease inhibitor cocktail from Sigma) and scraped from the dish. The cell suspension was passed through a 29 1/2 gauge needle 12 times and centrifuged at 100×g for 10 min to obtain a pellet containing nuclei, nuclei-associated membranes and unbroken cells (approximately 5 % of total cells, as determined by Trypan Blue staining). The supernatant from this spin (microsomal fraction) was centrifuged at 16,000×g for 30 min to pellet the heavy microsomes. The supernatant from this step was further centrifuged for 45 min at 400,000×g in a TL100 centrifuge using a TLA120.2 rotor (Beckman) to separate the light microsomes from the cytosol. All pellets were re-suspended in cytosol buffer containing 1% Triton X100 and centrifuged at 14,000×g for 10 min (in the case of the nuclear and heavy microsomal pellet) to remove any triton-insoluble material. Fractions were normalized for cell number, mixed with SDS-containing loading buffer and resolved by SDS-PAGE. Western blot analysis of the different fractions used antibodies against the following organelle markers: anti-GM130 (BD Biosciences) for Golgi, anti-KDEL (Stressgen) for endoplasmic reticulum, anti-β-COP (ABR or Sigma) for COPI vesicles, anti-Sec23 (Santa Cruz Biotechnology) for COPII vesicles, anti-Lamp1 (Cell Signaling Technologies) for lysosomes and anti-β1-integrin (BD Biosciences) for plasma membrane.

Immunostaining

Cells were plated on 25mm glass coverslips, cultured for 1–2 days and fixed in cold methanol or 4% paraformaldehyde at 4°C for 10 minutes, followed by treatment with 0.2% Triton/PBS. Coverslips were blocked in saline buffer containing 10% donkey serum for 1 hr, incubated overnight at 4°C with primary antibody and then incubated for 1 hr at room temperature in donkey anti-IgG antibody conjugated with Cy2, Cy3 or Cy5 and DAPI. Coverslips were mounted in Fluoromount-G (Southern Biotech) and cells were imaged using a Leica SP5 confocal microscope.

Metabolic labeling of phosphoinositides

Cells were metabolically labeled at 37°C with 10 μ Ci/ml [3 H]inositol (ARC) for 24–72 hrs in inositol-free DMEM supplemented with dialyzed fetal calf serum (Gibco) and 4 mM L-glutamine (Invitrogen).

HPLC method for phosphoinositide analysis

Cellular phosphoinositides were extracted and deacylated as described (50). Deacylated lipids were separated by anionic-exchange HPLC (Agilent 1200) using two partisphere SAX columns (Whatman) in tandem and a four-step gradient of ammonium phosphate pH 6.0 (1 mM to 4 mM over 60 min; 4 mM to 15 mM over 5 min; 15 mM isocratic for 20 min and 15 mM to 65 mM over 25 min). Radiolabeled eluates were detected by an online flow scintillation analyzer (Perkin-Elmer) and quantified using ProFSA software (Perkin-Elmer).

mTOR kinase assay and PIP4 γ associated kinase assay

For Figure 2G, GST-PIP4 γ and GST-PIP4 γ were expressed in bacteria and purified using GSH-sepharose beads. Recombinant mTOR was purchased from Calbiochem. For Figure 3C, myc-PIP4 γ was immunoprecipitated with an anti-myc antibody, as described above. Purified PIP4 γ and/or mTOR or immunoprecipitates were incubated for 10 min in kinase buffer (50 mM Tris pH7.5, 100 mM NaCl, 10 mM MnCl₂, 1 mM DTT, 10 μ M ATP) with 10 μ Ci of [32]P γ ATP (Perkin Elmer) at 37°C. Wortmannin was used at 1 μ M to inhibit mTOR. The reaction was stopped by addition of SDS-containing loading buffer and subjected to SDS-PAGE. The gel was dried and the radioactivity was detected using a Storm phosphorimager and film.

Flow cytometric analysis of cell size

For cell size experiments, HeLa cells were seeded in 6-well plates at a density of 1.5×10^5 cells/well and cultured overnight. The next day, media was replaced with serum free medium with or without 20 nM rapamycin as a control. Following 24hr treatment, cells were processed for flow cytometry. Briefly, cells were lifted from the plate by trypsinization, centrifuged and re-suspended in 0.3ml of PBS and fixed overnight at 4°C in 70% cold ethanol. Fixed cells were centrifuged at 1000 rpm for 5 mins, washed once with PBS and then incubated at 37°C for 1 hr in RNase A (0.2 mg/ml in PBS). Propidium iodide was added to the cells at a concentration of 10 μ g/ml immediately before analysis. DNA content and cell size were assessed using a BD FACS calibur flow cytometer. Data was analyzed using Cell Quest software. Single cells were selected using FL2-width versus FL2-area dot plot and 10,000 cells were acquired. To measure relative cell size, mean forward scatter (FSC-H) of cells in G1 phase of the cell cycle population was determined.

LC/MS/MS Tandem mass spectrometry

For all mass spectrometry (MS) experiments, PIP4 γ immunoprecipitates were separated using SDS-PAGE, the gel was stained with Coomassie blue, and the PIP4 γ band was excised. Samples were subjected to reduction with dithiothreitol, alkylation with iodoacetamide, and in-gel digestion with trypsin or chymotrypsin overnight at pH 8.3, followed by reversed-phase microcapillary/tandem mass spectrometry (LC-MS/MS). LC-

MS/MS was performed using an Easy-nLC nanoflow HPLC (Thermo Fisher Scientific) with a self-packed 75 μm id $\times$ 15 cm C₁₈ column connected to a hybrid linear ion trap LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) in the data-dependent acquisition and positive ion mode at 300 nL/min. MS/MS spectra collected through collision induced dissociation in the ion trap were searched against the concatenated target and decoy (reversed) Swiss-Prot and PIP4k γ single entry protein databases using Sequest with differential modifications for Ser/Thr/Tyr phosphorylation (+79.97) and the sample processing artifacts Met oxidation (+15.99), deamidation of Asn and Gln (+0.984) and Cys alkylation (+57.029) as affixed modification. Phosphorylated and unphosphorylated peptide sequences were identified if they initially passed the following Sequest scoring thresholds against the target database: 1+ ions, Xcorr 2.0 Sf 0.4, P 5; 2+ ions, Xcorr 2.0, Sf 0.4, P 5; 3+ ions, Xcorr 2.60, Sf 0.4, P 5 against the target protein database. Passing MS/MS spectra were manually inspected to be sure that all b- and y- fragment ions aligned with the assigned sequence and modification sites. Determination of the exact sites of phosphorylation was aided using *Fuzzylons* and *GraphMod* software (Proteomics Browser Software suite, Harvard University). False discovery rates (FDR) of peptide hits were estimated below 1.2% based on reversed database hits.

Statistical Analysis

Bar graphs show the mean and standard error of the mean (SEM). P values were calculated using paired two-tailed student's test (T test) of the data before normalization (Fig. 1B and 1D) or of the data normalized against a third set of data used as reference (Fig. 1F, 1H, 3D, 4C and S2D). For distribution analysis, P values were calculated using two-sample equal variance, two-tailed T test (Fig 2F, 6H and S4). P values less than or equal to 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. PIP4k γ knockdown cells have reduced cell size and impaired basal mTORC1 signaling
(A) Western blot analysis of serum-starved HeLa cells showing the effect of Tsc2 and PIP4k γ shRNAs on PIP4k γ abundance and S6 phosphorylation. N = 3 independent experiments. **B**, Bar graph showing the mean FSC-H of each population relative to the shTsc2 cells (mean and SEM). N=4 independent biological replicates. NS, indicates that differences are not statistically significant, P=0.25; * indicates that differences are statistically significant, P=0.0475. The ordinate scale was adjusted to encompass the window of change in cell size achieved by mTORC1 activation. **C**, m7GTP pulldown assay showing the association

between 4E-BP and eIF4E in HeLa cells expressing the Tsc2 shRNA (shTsc2) and either control (shControl) or PIP4 γ shRNA (shPIP4 γ). Cells were serum-starved or treated with rapamycin (R) as indicated. Also shown are the phosphorylation of S6 and the abundance of unphosphorylated 4E-BP and PIP4 γ in total protein lysates. N=3 independent experiments. **D**, Bar graphs showing the phosphorylation of p70S6K and the abundance of unphosphorylated 4E-BP in serum-starved HeLa cells expressing the Tsc2 shRNA and either control (black bars) or PIP4 γ (open bars) shRNA. Shown are the mean and SEM of the data corrected for loading and calculated relative to shControl. N = 3 independent biological replicates. *P=0.0015, **P=0.044. **E**, Western blot analysis showing the effect of PIP4 γ knockdown on the abundance of unphosphorylated 4E-BP and the phosphorylation of p70S6K in Tsc2 knockdown cells untreated (u), starved for amino acids (aa) or starved for glucose and glutamine (g/g). Also shown is the effect of amino acid starvation on mTORC1 signaling in cells with endogenous wild-type Tsc2 (shControl). N=3 independent experiments. **F**, Bar graph showing the phosphorylation of p70S6K and the abundance of unphosphorylated 4E-BP bands in cells with only Tsc2 knockdown (black bars) or with Tsc2 and PIP4 γ knockdown (open bars), untreated (u), starved for amino acids (aa) or glucose and glutamine (g/g). Grey bars show control HeLa cells for comparison. Shown are the mean and SEM of the data corrected for loading and calculated relative to untreated shControl. N= 3 independent biological replicates. NS indicates that differences are not statistically significant; *P=0.0006, **P=0.002, ***P=0.0088. **G**, Western-blot analysis of m7GTP-beads pulldown assay showing the amounts of eIF4E-associated 4E-BP, eIF4A or eIF4G in control HeLa (shControl) or PIP4 γ knockdown cells (shPIP4 γ) starved and restimulated with glucose and glutamine (g/g) for the times indicated. Also shown are the abundance of unphosphorylated 4E-BP, eIF4G and eIF4E in the lysates. N = 3 independent experiments. **H**, Bar graph showing the relative amounts of eIF4E-associated 4E-BP in starved or glucose and glutamine (g/g) stimulated shControl (black bars) or shPIP4 γ (open bars) HeLa cells. Shown are the mean and SEM of the ratio between eIF4E-associated 4E-BP over eIF4E-associated eIF4G, calculated relative to shControl stimulated with g/g. N=3 independent biological replicates. *P=0.023.

Fig. 2. mTORC1 phosphorylates PIP4 γ

A, Western blot analysis showing changes in the electrophoretic mobility of endogenous and myc-PIP4 γ from BTC6 or HeLa cells transiently transfected with empty vector or myc-PIP4 γ and left untreated (U) or starved of glucose and glutamine (g/g). Also shown is the Ponceau staining of the membrane prior to western blotting. N>3 independent experiments. **B**, Western blot analysis of endogenous PIP4 γ , p70S6K and phosphorylated S6 in wild-type (WT) or *Tsc2*^{-/-} MEFs left untreated (U) or treated with rapamycin (R) or staurosporine (Stp). N=3 independent experiments. **C**, Western blot analysis of endogenous PIP4 γ and p70S6K from BTC6 cells left untreated (U) or treated with rapamycin (R) or Ku0063794 (Ku). N = 3 independent experiments. **D**, Autoradiography and coomassie stain

of anti-myc IP from [32 P] -labeled *Tsc2*^{-/-} MEFs expressing wild-type or mutant myc-PIP4k γ and treated with rapamycin (R) or not (U). N = 2 independent experiments. **E**, Western blot of PIP4k γ from total cell lysates or from anti-myc IP of *Tsc2*^{-/-} MEFs expressing myc-PIP4k γ wild-type or mutants and treated with rapamycin (R) or not (U) with rapamycin, as indicated. Grp78 was used as a loading control. N >3 independent experiments. **F**, Bar graph showing the mean and SEM of the ratio of upper (dark grey) to lower (light grey) form of PIP4k γ from *Tsc2*^{-/-} MEFs expressing myc-PIP4k γ wild-type or mutants and treated with rapamycin (R) or not (U). N=3 independent biological replicates. * indicates that the differences are statistically significant, as compared to WT PIP4k γ (upper or lower form) expressed in untreated cells, P<0.0002. **G**, Phosphorylation of PIP4k by mTOR *in vitro*. Autoradiograph image of *in vitro* kinase assays using [32 P] γ ATP and PIP4k and/or purified recombinant mTOR, as indicated, showing [32 P] incorporation into GST-PIP4k γ , wild-type (WT) or mutant (S328A) or PIP4k γ and mTOR autophosphorylation. Wortmanin was used to inhibit mTOR activity. N = 2 independent experiments.

Fig. 3. PIP4k γ associates with mTORC1 and is enriched in cytoplasmic vesicles

A, Western blot analysis of PIP4k γ and Raptor after Raptor immunoprecipitation from lysates of *Tsc2*^{-/-} MEFs expressing empty vector or myc-PIP4k γ and treated or not with rapamycin, as indicated. PRAS40 was used as a loading control. Lane 4 contained beads and lysate only, as a negative control. N = 3 independent experiments. **B**, TLC showing the [³²P]-PI-4,5-P₂ product of the PI-5-P 4-kinase associated with Raptor IP or control (beads and lysate only) immunoprecipitate, from BTC6 cells treated or not with Ku0063794 (Ku). Graph shows the values from the quantification of the [³²P]-PI-4,5-P₂ spot from two

independent experiments calculated relative to control (beads and lysate only). **C**, Autoradiography showing [^{32}P]-myc-PIP4 γ labeled by an associated kinase from *Tsc2*^{-/-} MEFs expressing wild-type PIP4 γ or the K152A mutant and treated or not with rapamycin. Also shown is the coomassie stain of the PIP4 γ bands. N= 5 independent experiments. **D**, Bar graph showing the mean and SEM of the quantification of the [^{32}P]-labeled PIP4 γ band corrected for the amount of PIP4 γ in the coomassie stain and calculated relative to cells expressing K152A mutant. N=5 independent biological replicates. * indicates that the differences are statistically significant, P=0.024. **E**, Western blot analysis showing the subcellular distribution of the mitochondria marker ATP synthase, the plasma membrane marker β 1-integrin, the ER marker PDI, the Golgi marker GM130, the lysosomal marker Lamp1, the COPI-containing vesicle marker β COP and the COPII-containing vesicle marker Sec23, among light microsomal (L), heavy microsomal (H) and nuclear-associated (Nu) fractions and cytosol from BTC6 cells. Nuclear associated fractions also contained approximately 5% of unbroken cells. **F–H**, Western blot analysis and quantification of the subcellular distribution of PIP4 γ . The bar graphs show the mean and SEM of the percentage of PIP4 γ in each fraction, relative to the sum of the post-nuclear fractions. **F**, Distribution of endogenous PIP4 γ in BTC6 cells. N=4 independent biological replicates. **G**, Western blot showing the distribution of doxycycline-inducible PIP4 γ WT, S324/328A (AA) or S324/328D (DD) mutants expressed in BTC6 cells treated with rapamycin (R) or not. N=3 independent biological replicates. **H**, Bar graph showing the distribution of doxycycline-inducible PIP4 γ expressed in BTC6 cells treated with rapamycin or not. N=3 independent biological replicates. * indicates that the data is statistically different from the WT untreated, P=0.05. ** indicates that the data is statistically different from the WT, P=0.03. NS indicates that the data is not statistically different from the WT, P=0.23.

Fig. 4. Unphosphorylated PIP4k γ increases basal mTORC1 signaling

A, Western blot analysis showing the abundance and electrophoretic mobility of doxycycline-inducible PIP4k γ expressed in HeLa cells before and after treatment with low or high doxycycline (DOX), as indicated. PIP4k γ wild-type, phosphorylation defective (S324/328A, AA) or phosphorylation mimetic (S324/328D, DD) mutants were left untreated (U) or starved for serum (S) or amino acids (aa). N>3 independent experiments. Also shown is the Ponceau staining of the membrane prior to western blotting. **B**, Western blot analysis showing the effect of increasing doses of doxycycline on basal phosphorylation of p70S6K

in nutrient-deprived HeLa cells expressing wild-type PIP4k γ (WT), phosphorylation defective (S324/328A, AA) or phosphorylation mimetic (S324/328D, DD) mutants. N=3 independent experiments. **C**, Bar graph showing the quantification of the phospho-p70S6K bands from HeLa cells expressing PIP4k γ wild-type or mutants at 2 fold the amount of endogenous. Shown are the mean and SEM of the data corrected for loading and calculated relative to no doxycycline control. N=3 independent biological replicates. * indicates that the differences are statistically significant, P=0.014. **D**, Model illustrating the negative feedback loop between mTORC1 and PIP4k. PIP4k activates mTORC1 signaling in the dephosphorylated state, when cells are nutrient deprived. As a result, mTORC1 phosphorylates PIP4k γ to shut down this pathway.