Mammalian cells respond to nutrient deprivation by inhibiting energy consuming processes, such as proliferation and protein synthesis, and by stimulating catabolic processes, such as autophagy. p70 S6-kinase (S6K1) plays a central role during nutritional regulation of translation. S6K1 is activated by growth factors such as insulin, and by mTOR, which is itself regulated by amino acids. The Class IA PI 3-kinase plays a well-recognized role in the regulation of S6K1. We now present evidence that the class III PI 3-kinase, hVps34, also regulates S6K1, and is a critical component of the nutrient sensing apparatus. Overexpression of hVps34 or the associated hVps15 kinase activates S6K1, and insulin stimulation of S6K1 is blocked by microinjection of inhibitory anti-hVps34 antibodies, overexpression of a FYVE-domain construct that sequesters the hVps34 product PI[3]P, or siRNA-mediated knock-down of hVps34. hVps34 is not part of the insulin input to S6K1, as it is not stimulated by insulin, and inhibition of hVps34 has no effect on phosphorylation of Akt or TSC2 in insulin-stimulated cells. However, hVps34 is inhibited by amino acid or glucose starvation, suggesting that it lies on the nutrient-regulated pathway to S6K1. Consistent with this, hVps34 is also inhibited by activation of the AMP-activated Kinase (AMPK), which inhibits mTOR/S6K1 in glucose-starved cells. hVps34 appears to lie upstream of mTOR, as siRNA knockdown of hVps34 inhibits the phosphorylation of another mTOR substrate, 4EBP1. Our data suggest that hVps34 is a nutrient-regulated lipid kinase that integrates amino acid and glucose inputs to mTOR and S6K1.

p70 S6-kinase (S6K1) regulates protein synthesis, cell size and proliferation in response to cellular nutritional status and hormonal stimulation (1). Regulation of S6K1 by insulin and nutrients involves a complex pathway that includes mTOR as well as the p85/p110 PI 3-kinases and the downstream kinases PDK-1 and Akt. Phosphorylation of S6K1 at Thr389 is regulated by mTOR, which either directly phosphorylates S6K1 or inhibits its dephosphorylation (2,3). Thr389 is present in a C-terminal hydrophobic motif, and its phosphorylation facilitates docking of PDK-1 and phosphorylation of the S6K1 activation loop (4). mTOR activity is regulated by at least three upstream inputs: amino acids, glucose, and growth factors. Amino acids, particularly leucine, regulate the formation of a nutrient sensing complex with Raptor and GβL/LST8 (referred to as TORC1) that
facilitates the recognition of substrates by mTOR under nutrient-replete conditions (5-8). In glucose-starved cells, mTOR is inhibited by the activation of AMPK (9-11). Finally, growth factor stimulation leads to activation of Akt and phosphorylation of the TSC1/TSC2 complex (reviewed in (12-14)). Akt-mediated phosphorylation inhibits the GAP activity of TSC1/TSC2 toward the Rheb GTPase, leading to Rheb activation (15-21). Rheb binds directly to mTOR, a process that is regulated by amino acids (22,23). While both amino acids and Rheb activation are required for mTOR signaling to S6K1, the role played by TSC1/TSC2 during amino acid regulation of mTOR has been controversial (15,16,24).

Unlike the Class I PI 3-kinase, the Class III PI 3-kinase hVps34 has not been previously implicated in regulation of S6K1 by nutrients. Vps34p was first identified in S. cerevisiae by Emr and colleagues, who characterized its role in vesicular trafficking (25,26). The activity of Vps34p requires the presence of an associated protein kinase, Vps15p (26). The mammalian homologue hVps34 has been shown to play a role in multiple vesicular trafficking pathways (27-33). In yeast, Vps34p is required for autophagy (34), which is stimulated in response to nutritional deprivation. Mammalian Vps34 has also been implicated in autophagy (35,36). Moreover, an hVps34-associated protein, beclin 1 (37,38), is required for autophagy in mammalian cells (39,40). Although these data suggest that hVps34 is involved in nutrient-regulated pathways, the possibility that hVps34 itself might be regulated by cellular nutritional state has not been addressed.

In the present study, we have examined the function and regulation of hVps34 in nutrient sensing in mammalian cells. Based on our previous finding that inhibition of hVps34 blocks insulin-stimulated DNA synthesis (27), we tested whether hVps34 might play a role in insulin activation of S6K1, which is required for the G1-S transition in insulin-stimulated cells (41). We now report that hVps34 is required for insulin stimulation of S6K1. hVps34 is not regulated by insulin, nor does it affect insulin-stimulated phosphorylation of Akt or TSC2. However, hVps34 is inhibited by amino acid or glucose starvation, and by activation of AMPK. These data suggest a novel role for hVps34 in nutrient sensing, and in the integration of signaling from amino acids and glucose to mTOR and S6K1.

Experimental Procedures.

Cell Culture. HepG2 cells and the insulin responsive CHO-derived cell line GRC+LR-73 were cultured as previously described (27,42). MCF-7 cells expressing beclin 1 from a tetracycline-repressible promoter (43) were obtained from Dr. Beth Levine, UT Southwestern Medical Center, Dallas.

Antibodies and inhibitors. Anti-hVps34 antibodies have been previously described (27). Anti-phospho-Akt (Ser473) and anti-phospho-ribosomal S6 (Ser235) antibodies were obtained from Upstate (Lake Placid, NY). Anti-phospho-S6K1 (Thr389 and Thr421/Ser424) and anti-phospho-TSC2 (Ser939) antibodies were obtained from Cell Signaling (Beverly, MA). Rapamycin, oligomycin and AICAR (5-Aminoimidazole-4-carboxamide-1-β-riboside) were purchased from CalBiochem (La Jolla, CA).

Plasmid constructs and transfections. Myc-hVps34 has been previously described (33). Myc-tagged Akt was obtained from Dr. B. F. Hansen, Novo-Nordisk (Denmark). The eGFP-2X-FYVE construct was obtained from Dr. H. Stenmark, Norwegian Radium Hospital, Norway. HA-tagged p70...
S6 kinase was obtained from Dr. S. Schreiber, Harvard University. Constructs were introduced into HEK293T cells using calcium phosphate transfection. Alternatively, plasmids were introduced into GRC+LR-73 cells using Lipofectamine Plus (Invitrogen, Carlsbad, CA). HeLa cells were transfected with siRNA duplexes using Oligofectamine (Invitrogen, Carlsbad, CA).

**hVps34 siRNA experiments.** Cells were transfected with the following RNA duplexes:

- ACUCAACACUGCUAAUUAUU and UAAUUAGCCAGUUGAGUUU;
- AUAGAUAGCUCCCAAUUAUU and UAAUUUGGGAGCUAUCUAUU;
- GAAACAACGUGCUGCUUUU and AAGAGCGAAACCGUUGUUCUU;
- GGAGGCAAAUAUCCAGUUAUU and UAACUGGAUAUUUGCCUCCUU.

Control duplexes were derived from the sequence of luciferase. Cells were harvested after 3 days, and lysates were immunoprecipitated and blotted anti-hVps34 antibodies. Parallel samples were blotted with anti-Akt, S6K1, phospho-Akt, phospho-S6K1 and phospho-TSC2 antibodies as indicated.

**hVps34 activity assay.** Endogenous hVps34 was immunoprecipitated with anti-hVps34 antibodies. Washed immunoprecipitates were incubated for 30 min at 30 °C with 10 μM of the peptide used to raise the antibody (AVVEQIHKFAQYWRK). This incubation releases hVps34 from the antibody, which is inhibitory, and allows its activity to be measured *in vitro* as previously described (27). In control experiments, the specific activity of hVps34 measured under these conditions was identical to that of myc-hVps34 immunoprecipitated by anti-myc antibodies, which are not inhibitory. Alternatively, beclin 1-associated hVps34 activity was measured directly in washed immunoprecipitates of Flag-tagged beclin 1.

**In Vitro p70 S6-kinase Assay.** HEK293T or GRC+LR-73 cells were transfected with HA-S6K1 alone or with myc-hVps34, eGFP, or eGFP-2X-FYVE as indicated. Quiescent cells were incubated in the absence or presence of insulin as indicated, and anti-HA immunoprecipitates were assayed for kinase activity using S6 kinase substrate peptide KRRRLASLAA as described (44).

**Microinjection.** GRC+LR-73 cells were grown on polylysine-coated glass coverslips. Quiescent cells were microinjected as previously described (27) using antibodies mixed with Oregon Green dextran conjugate (Molecular Probes, Eugene, OR) to a final concentration of 3 mg/ml antibody in PBS, pH 7.4. Cells were allowed to recover for 2 h, then incubated in the absence or presence of 1 μM insulin for 30 min. Cells were fixed, permeabilized with 1% Triton X-100, and blocked with Renaissance blocking reagent (NEN Dupont, Boston, MA). Cells were stained with sheep anti-pSer235-S6 antibody followed by Cy3 anti-sheep antibody, or with mouse anti-pSer473-Akt (Ser473) followed by Cy3 anti-mouse antibodies.

**Imaging and fluorescence quantitation.** Images were collected on a Nikon Eclipse 400 upright microscope using either 60x 1.4 NA or 100x 1.25 NA oil immersion objectives and a Roper Cool-Snap cooled CCD camera. Fluorescent images of individual microinjected cells (30-50 cells per condition) were traced, and the total fluorescence intensity was measured using NIH Image. Data were expressed as a percentage of the fluorescence observed in insulin-stimulated control cells.

**Results and Discussion**

To investigate the potential role of hVps34 in nutrient sensing, we tested whether overexpression of hVps34 could activate S6K1. In HEK 293T cells,
overexpression of myc-hVps34 lead to a 2-fold increase in HA-S6K1 activity, similar to what was seen in control cells stimulated with insulin (Fig. 1A). Furthermore, overexpression of either hVps34 or the associated hVps15 protein kinase in the insulin-responsive CHO line GRC+LR-73 (42) lead to an increase in phosphorylation of S6K1 at Thr389 (Fig 1B), which correlates with S6K1 activation (45). These data show that increased expression of hVps34 or hVps15 activates S6K1.

To determine whether hVps34 is required for regulation of S6K1 by insulin, we measured S6K1 activity in insulin-stimulated cells overexpressing an eGFP-2XFYVE construct. Low-level expression of this construct is useful as a marker for PI[3]P-containing membranes (46). However, we found that high level expression of the 2X-FYVE domain leads to sequestration of PI[3]P and disruption of PI[3]P-dependent signaling, as indicated the loss of EEA1 endosomal targeting (Fig 1C). Similarly, expression of eGFP-2XFYVE, but not eGFP, caused a significant reduction in insulin stimulation of HA-S6K1 (Fig. 1D). Basal HA-S6K1 activity was not affected. These data suggest that the hVps34 product, PI[3]P, is required for activation of S6K1 by insulin.

To directly address the requirement for hVps34 during insulin activation of S6K1, we used previously described antibodies that specifically inhibit hVps34 activity in microinjected cells; these antibodies have been used to characterize hVps34-dependent signaling in a number of systems (27-33). S6K1 activation was analyzed by immunofluorescence in GRC+LR-73 cells, using anti-pSer235-S6 antibodies to detect S6K1 phosphorylation of the ribosomal S6 subunit (Fig. 1E). GRC+LR-73 cells were microinjected with control IgG or anti-hVps34 antibody, incubated in the absence or presence of 1 μM insulin for 10 min, and then fixed and stained. Quantitative imaging of anti-phospho-S6 staining showed that insulin activation of S6K1 was blocked in cells injected with anti-hVps34 antibodies, but not in cells injected with control IgG (Fig. 1F). We also used siRNA to reduce hVps34 expression in HeLa cells (Fig. 1G). Transfection of HeLa cells with siRNA targeting hVps34, but not control siRNA, lead to an almost complete loss of hVps34 expression. Whereas total S6K1 expression was minimally affected, hVps34 siRNA-treated cells showed a marked decrease in insulin-stimulated phosphorylation of S6K1 at Thr389. Similar results were obtained with a second siRNA duplex (data not shown). Finally, to determine if hVps34 is required for insulin activation of S6K1 in a more physiological cell type, we co-transfected the insulin-responsive hepatoma line HepG2 with S6K1 plus eGFP or the eGFP-2XFYVE construct. Insulin caused a 2-fold stimulation of S6K1 activity in HepG2 cells expressing eGFP, which was abolished in cells expressing the 2X-FYVE construct (Fig. 1H). S6K1 expression was unaffected (data not shown). Overall, these data show that both hVps34 and its product, PI[3]P, are required for insulin-stimulated activation of S6K1.

The insulin- and amino acid-regulated inputs to S6K1 may be distinct. To determine the mechanism by which hVps34 signals to S6K1, we first measured hVps34 activity in insulin stimulated cells. We were unable to detect significant increases in endogenous hVps34 activity after insulin stimulation (Fig. 2A). We next examined the requirement for hVps34 during insulin stimulated activation of Akt, which is the
In mammalian cells, PI[3]P can be converted to PI[3,4,5]P3 by the action of Class I PIP kinases (47, 48). This pathway could provide a plausible mechanism for the role of hVps34 during regulation of S6K1, as it could lead to production of PI[3,4,5]P3, activation of Akt and inhibition of TSC1/TSC2. However, when GRC+LR-73 cells were microinjected with control IgG or inhibitory anti-hVps34 antibody, we observed no effects on insulin-stimulated Akt activation, as measured by immunofluorescence staining with anti-pSer473-AKT antibodies. (Fig. 2B). Similarly, insulin-stimulated Akt phosphorylation at Ser473 was unaffected by siRNA knock-down of hVps34 in HeLa cells (Fig. 2C). Finally, we looked directly at insulin-stimulated phosphorylation of TSC2 at Ser939; siRNA knock-down of hVps34 had no effect on phosphorylation of TSC2, using two distinct siRNA duplexes (Fig. 2D, 2E). Thus, the effect of hVps34 inhibition on S6K1 is independent of the Akt/TSC2 pathway.

S6K1 is also regulated by amino acids, which are required for the activity of mTOR (1, 2). We therefore tested whether hVps34 was involved in the amino acid-dependent input to S6K1. Insulin stimulated HA-S6K1 activity and endogenous hVps34 activity were both inhibited in HEK293T cells deprived of amino acids (Fig. 3A). Similar results were obtained in GRC+LR-73 cells (Fig. 3B), where inhibition of hVps34 by amino acid starvation was maximal by 30 min. This decrease occurred without any change in the abundance of hVps34 protein (inset).

In yeast and mammalian cells, hVps34 forms complexes with the autophagy-related protein Apg6p/beclin 1 (34, 38), and a previous report suggested that beclin 1-associated hVps34 is transiently activated during the induction of autophagy by withdrawal of amino acids (49). To study the regulation of beclin 1-associated hVps34 in an established autophagy-competent cell line, we measured hVps34 activity in anti-flag immunoprecipitates from MCF-7 cells expressing an inducible flag-tagged beclin 1 (43). We found that beclin 1-associated hVps34 activity was inhibited by amino acid deprivation (Figure 3C); the amount of beclin-associated hVps34 did not change appreciably (inset).

mTOR activation of S6K1 is blocked by both amino acid and glucose starvation (5). This latter regulation may be due at least in part to AMPK, which is activated by glucose deprivation in mammalian cells and yeast (9), and which inhibits mTOR/S6K1 through an activating phosphorylation of the TSC1/TSC2 complex, or via an inhibitory phosphorylation of mTOR (10, 11). To determine if hVps34 is also regulated by glucose, we measured hVps34 activity in cells deprived of glucose, amino acids, or both. Inhibition of hVps34 by glucose starvation was similar to that seen during amino acid starvation (Fig. 4A); removal of both nutrients did not cause additional inhibition of hVps34. To examine the potential role of AMPK in this inhibition, GRC+LR-73 cells were treated for 30 min with the AMPK activator AICAR. hVps34 activity was significantly inhibited in AICAR-treated cells, suggesting that hVps34 is negatively regulated by the AMPK-mediated response to nutrient deprivation (Figure 4B). Similar results were observed after treatment of cells with oligomycin, which also activates AMPK (Fig. 4C). AMPK-mediated inhibition of hVps34 is independent of effects on mTOR, since hVps34 activity is unaffected by treatment of cells with rapamycin, which inhibits TORC1 (Fig. 4D).
These data suggest that mTOR and hVps34 show similar regulation by amino acids, glucose and AMPK. Figure 4D additionally suggests that hVps34 is not downstream from the rapamycin-sensitive TORC1 complex, which is involved in nutrient-related signaling (5-8). We therefore tested whether hVps34 might be upstream of mTOR signaling. Interestingly, siRNA knock-down of hVps34 inhibits insulin stimulation of Thr389 phosphorylation, but does not abolish the insulin-stimulated reduction in the electrophoretic mobility of S6K1 (Fig. 1G, middle panel). Similarly, overexpression of hVps34 or hVps15 causes an increase in S6K1 Thr389 phosphorylation, but does not cause a gel-shift (Fig. 1B). These finding are reminiscent of data from Swiss 3T3 cells treated with rapamycin for short times, where significant loss of S6K1 activity and Thr389 phosphorylation occurs prior to loss of the serum-induced gel shift (50). To test whether inhibition of mTOR leads to preferential loss of Thr389 phosphorylation in HeLa cells, we treated cells with low-dose rapamycin for short times prior to insulin stimulation (Fig. 5A). We found that phosphorylation of Thr389 was diminished by a 1 min preincubation with rapamycin, and abolished by a 5 min pre-incubation. In contrast, the insulin-stimulated gel shift was unaffected by a 1 min pretreatment, and was still apparent after a 10 min pre-treatment. Thus, the specific effects of hVps34 on Thr389 phosphorylation are consistent with a specific regulation of S6K1 phosphorylation by mTOR. We also examined phosphorylation of S6K1 at Thr421/Ser424, a putative Erk site (51). In control cells, we observed significant basal Thr421/Ser424 phosphorylation, with a decrease in electrophoretic mobility of the phosphorylated protein upon insulin stimulation (Fig. 5B). After knock-down of hVps34 expression using four distinct siRNA duplexes, we saw no effect on Thr421/Ser424 phosphorylation or the insulin-stimulated gel shift, suggesting that these phosphorylation sites are not regulated by hVps34.

Finally, as an additional measure of mTOR activity, we examined the insulin-stimulated phosphorylation of the translational inhibitor 4EBP1 (Fig. 5C). Insulin caused a clear increase in phosphorylation of 4EBP1 at Ser65, which is the major mTOR-regulated site (52). Insulin-stimulated phosphorylation of 4EBP1 at Ser65 was markedly inhibited by cells treated with two distinct siRNA duplexes targeting hVps34. These data show that mTOR phosphorylation of both S6K1 and 4EBP1 requires hVps34.

Taken together, our data suggest that hVps34 plays an unappreciated role during nutrient sensing and activation of S6K1 (Fig. 5D). Overexpression of hVps34 activates S6K1, and sequestration of hVps34 products, or direct inhibition of hVps34 activity or expression, blocks insulin-stimulated S6K1 activity. Another group has recently made similar observations (T. Nobukuni, M. Joaquin, M. Roccio, S.G. Dann, S.Y. Kim, P. Gulati, F. Natt, J.L. Bos, F.J.T. Zwartkruis, and G. Thomas, submitted). hVps34 is not itself regulated by insulin, consistent with previous measurements of PI[3]P levels in insulin stimulated CHO cells (53). Moreover, inhibition of hVps34 does not interfere with insulin activation of Akt, or with insulin-stimulated phosphorylation of TSC2. However, hVps34 activity is markedly depressed during amino acid or glucose starvation. Given that amino acid and growth factor stimulation may represent independent inputs to S6K1 (5,15,24), our data places hVps34 as a component of the
amino acid-regulated arm of S6K1 regulation. hVps34 is also inhibited by glucose deprivation, presumably via the activation of AMPK. These data suggest that hVps34 may integrate signals from both amino acid- and glucose-sensing systems in the cell.

Where does hVps34 fit into the amino acid regulatory pathway? While not conclusive, our data suggests that hVps34 acts at the level of mTOR. hVps34 does not appear to be downstream of the rapamycin-sensitive TORC1 complex, since hVps34 activity is unaffected by treatment of cells with rapamycin. We cannot rule out the possibility that TORC2 could regulate hVps34. Could hVps34 signal through mTOR? Like mTOR, hVps34 is inhibited by amino acid or glucose starvation, and by activation of AMPK. Furthermore, siRNA knock-down of hVps34 preferentially inhibits phosphorylation of the mTOR-dependent Thr389 site in S6K1, and also inhibits insulin-stimulated phosphorylation of 4EBP1. These data are consistent with hVps34 regulation of mTOR. While the mechanism of a potential hVps34-mTOR interaction is not clear, hVps34 associates with the Ser/Thr kinase hVps15 (26,33,54). The domain structure of hVps15 is strikingly similar to that of Raptor, with central HEAT and C-terminal WD-40 repeats (5). GβL/LST8, which associates with mTOR, also contains multiple WD-40 repeats (6). The finding of similar protein-protein interaction domains in these proteins may suggest a common mechanism of association or regulation.

It is also possible that the role of hVps34 in the activation of S6K1 is related to its role in the endocytic system. hVps34 is regulated at least in part by Rab5-directed endosomal targeting (28,33). Although TSC2 has GAP activity towards Rab5 in vitro (55), siRNA knockdown of Rab5 does not affect activation of Drosophila S6K in S2 cells (17). However, a recent study showed that overexpression of Rheb-eGFP constructs produce large Rab7/9-positive vesicles (56), suggesting a link between Rheb and endocytic trafficking. The formation of enlarged vesicles in Rheb-expressing cells was insensitive to rapamycin but was blocked by LY294002; these data may reflect the involvement of an undetermined PI 3-kinase, but could also be explained by signaling from the TORC2 complex (57,58). Finally, a link between mTOR and the endocytic system is suggested by a study showing that siRNA knock-down of mTOR inhibits clathrin mediated endocytosis at multiple steps (59).

How is the activity of hVps34 regulated during amino acid and glucose deprivation? In yeast, Vps34p is presumed to be regulated by Vps15p-mediated phosphorylation, based on data showing that the activity of Vps34p requires the presence of an active copy of the Vps15p kinase (26). If changes in hVps15-mediated phosphorylation of hVps34 are involved during nutrient deprivation, it remains to be determined how hVps15 itself is regulated. A second alternative is suggested by our finding that hVps34 is inhibited by activators of AMPK. Of known mammalian kinases, the Vps15 kinase domain is in fact most similar to the kinase domain of AMPK (48% similar over the first 140 residues of the kinase domain by NCBI BLAST), and hVps34 has a number of potential phosphorylation sites that fit the AMPK consensus sequence (60,61). It is therefore possible that hVps34 may be an AMPK substrate. While the activation of AMPK may explain the regulation of hVps34 by glucose, it would not provide a mechanism for inhibition of hVps34 during amino acid starvation.
The inhibition of hVps34 by amino acid withdrawal is surprising, given its presumed role as a positive effector of autophagy in mammalian cells (34,36). hVps34 is associated with at least two distinct protein complexes in yeast (34), and it is possible that an autophagy-related pool of hVps34 might be regulated differently. Autophagy-related hVps34 would presumably be associated with Apg6/beclin 1, which is complexed with Vps34 and is required for autophagy in yeast and mammals (34,39,40). However, we find that beclin 1-associated hVps34 is also inhibited by amino acid withdrawal. Some of this apparent complexity in the regulation of hVps34 may result from differences in yeast versus mammalian cells. For example, AMPK, whose activation inhibits hVps34, is required for autophagy in yeast (62) yet inhibits autophagy in mammalian cells (63). Furthermore, mammalian cells contain PI 3-kinases not present in yeast, such as the Class II PI 3-kinases, that could produce PI[3]P to support autophagy despite inhibition of hVps34. For example, insulin-stimulated increases in PI[3]P levels have been observed in 3T3-L1 adipocytes but are resistant to low-dose wortmannin, suggesting the activity of a Class II PI 3-kinase (64). Finally, the residual hVps34 activity in starved cells may be sufficient to support autophagy. In this regard, the requirement for Vps34 during autophagy in yeast has been demonstrated in Vps34 null strains, in which PI[3]P production is completely eliminated.

Interestingly, recent studies in Drosophila have shown that dS6K, like hVps34, is a positive effector of autophagy that is inhibited by amino acid starvation, a condition that promotes autophagy (65). The authors suggest that inhibition of dS6K by amino acid starvation might set limits on autophagy, minimizing potential cellular injury by excessive autophagy during prolonged periods of starvation. A similar rationale might explain the inhibition of hVps34 by amino acid withdrawal.
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Abbreviations: AMPK: AMP-activated protein kinase; S6K1: p70 S6 kinase; BSA: bovine serum albumin; CHO: Chinese hamster ovary; FBS: fetal bovine serum; PBS: phosphate buffered saline; mTOR: mammalian Target of Rapamycin; TSC1/2: Tuberous Sclerosis Complex 1/2; TCA: trichloroacetic acid.
Figure Legends

Figure 1. hVps34 is required for insulin activation of p70 S6-kinase. (A). HEK 293T cells were transfected with HA-tagged S6K1 plus control vector or myc-tagged hVps34 (hVps34 overexpression approximately 10-fold over endogenous). S6K1 activity in anti-HA immunoprecipitates was measured. The data is representative of 3 separate experiments. Blots showing HA-S6K1 and myc-hVps34 expression are shown in inset. (B) GRC+LR-73 cells were transfected with HA-S6K1 plus vector, hVps34 (approximately 8-12-fold overexpression) or hVps15. Cells were lysed and anti-HA immunoprecipitates were blotted with anti-S6K1 or anti-pThr389-S6K1 antibodies. (C). GRC+LR-73 cells were transfected with eGFP-2xFYVE, fixed and stained with anti-EEA1 antibodies and Cy3-labeled secondary antibodies. Left panel: EEA1. Right panel: eGFP-FYVE. (D). GRC+LR-73 cells were transfected with HA-S6K1 plus eGFP or eGFP-2XFYVE. The cells were quiesced and then incubated in the absence or presence of 1 μM insulin for 30 min. S6K1 activity in anti-HA immunoprecipitates was determined as described. The data is representative of 3 separate experiments. (E). Quiescent GRC+LR-73 cells were stimulated with 1 μM insulin for 30 min. The cells were fixed and stained with anti-pSer235-S6 antibody as described. (F). Quiescent GRC+LR-73 cells were microinjected with Oregon Green dextran and control IgG or anti-hVps34 antibody as indicated. The cells were incubated in the absence or presence of 1 μM insulin for 30 min, fixed, and stained with anti-pSer235-S6 antibody as described. Digital images were collected and cell-associated fluorescence was quantitated using NIH image. The data is pooled from 3 separate experiments. (G). HeLa cells were transfected with siRNA duplexes targeting hVps34, or control duplexes. After 3 days, the cells were incubated in the absence or presence of 1 μM insulin for 30 min. Lysates were immunoprecipitated and blotted with anti-hVps34 antibodies, or blotted directly with anti-S6K1 and pThr389-S6K1 antibodies. (H). HepG2 cells were transfected with HA-S6K1 plus eGFP or eGFP-2XFYVE, and S6K1 activity was assayed as in (D).

Figure 2. hVps34 is not regulated by insulin and is not required for insulin stimulated phosphorylation of Akt or TSC2. (A) Anti-hVps34 immunoprecipitates from unstimulated or insulin-stimulated (1 μM, 30 min) GRC+LR-73 cells were assayed for lipid kinase activity as described. (B). Quiescent GRC+LR-73 cells were microinjected with Oregon Green dextran and control IgG or anti-hVps34 antibody. The cells were incubated in the absence or presence of 1 μM insulin for 30 min, fixed, and stained with anti-pSer473-Akt antibodies as described. Digital images were collected and cell-associated fluorescence was quantitated with NIH image. (C). siRNA-transfected HeLa cell lysates from Figure 1G were blotted with anti-pSer473Akt and anti-Akt antibodies. The blot of hVps34 expression levels, from Fig. 1G, is shown again for reference. (D). Cells were transfected with hVps34 or control siRNA for 3 days; (a) and (b) indicate two distinct siRNA duplexes. The cells were then incubated in the absence or presence of insulin for 30 min, lysed, and blotted with anti-pSer939TSC2 antibodies or anti-hVps34 antibodies. (E). Anti-pSer939TSC2 blots from control or hVps34 siRNA-transfected cells were scanned and quantitated using NIH image. The data quantified in (A) and (B) is the mean ± S.E.M. from 3-4 independent experiments. Data in (E) is the mean ± SEM from 4 experiments using two different siRNA duplexes targeting hVps34.
Figure 3. Inhibition of hVps34 by amino acid deprivation. (A) HEK 293T cells transfected with HA-S6K1 were amino acid starved for various times. S6K1 activity in anti-HA immunoprecipitates and endogenous hVps34 activity in anti-hVps34 immunoprecipitates was determined. (B) GRC+LR-73 cells were amino acid starved for various times, and endogenous hVps34 activity in anti-hVps34 immunoprecipitates was determined. Inset: Parallel anti-hVps34 immunoprecipitates from the same experiment were blotted with anti-hVps34 antibody. (C) MCF-7 cells stably expressing a tetracycline-repressible flag-beclin construct were induced by tetracycline withdrawal. Cells were amino acid starved for various times and hVps34 activity in anti-flag immunoprecipitates was determined. Inset: Duplicate samples from the same experiment were blotted with anti-hVps34 antibody and quantitated by densitometry (mean ± standard deviation of duplicates). All other quantitative data (A, B, and C) is presented as the mean ± S.E.M., and is representative of 2-3 independent experiments.

Figure 4. Inhibition of hVps34 by glucose deprivation and activation of AMPK. (A) GRC+LR-73 cells were incubated for 2 h in PBS in the absence or presence of 1X MEM amino acids and 25 mM glucose as indicated. Endogenous anti-hVps34 activity was determined as above. (B). GRC+LR-73 cells were incubated in the presence of 0.5 mM AICAR or carrier for 30 min. Endogenous hVps34 activity was determined as above. (C). GRC+LR-73 cells were incubated in the presence of 0.2 ng/ml oligomycin or carrier for 10 min. Endogenous hVps34 activity was determined as above. (D). HEK293T cells were incubated in 20 nM rapamycin for 30 min. Endogenous hVps34 activity was determined as above. Quantitative data is presented as the mean ± S.E.M. from 2-3 independent experiments (A-C), or are representative of 3 separate experiments (D).

Figure 5. hVps34 is required for mTOR-dependent signaling. (A). HeLa cells were treated with 1 nM rapamycin for varying times, washed and then stimulated with insulin for 30 min. The cells were lysed and blotted with anti-pThr389-S6K1 or anti-S6K1 antibodies. (B). HeLa cells were transfected with control or hVps34 siRNA duplexes. After 3 days, the cells were incubated in the absence or presence of insulin for 30 min, lysed and blotted with anti-pThr421/Ser242-S6K1 or anti-hVps34 antibody. (C) siRNA-transfected HeLa cell lysates from Figure 2D were blotted with anti-pSer65-4EBP1 antibodies. The blot of hVps34 expression levels is shown again for reference. (D) The role of hVps34 during regulation of S6K1 by glucose and amino acids. hVps34 is inhibited by both glucose and amino acid starvation, and could represent the nutrient-regulated input to mTOR. Alternatively, hVps34 could signal to S6K1 by another mechanism. Nutrient regulation of hVps34 may be mediated in part by AMPK, which is activated in glucose-starved cells.
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Figure 1.

A. 

B. 

C. 

D. 

E. 

F. 

G. 

H. 

siRNA:

|   | Ctl | hVps34 |
|---|-----|--------|
|   |     |        |

| P-T389 S6K1 |
|---|--------|
|   |        |

| S6K1   |
|---|-----|
|    |     |

| hVps34 |
|---|-----|
|     |     |

insulin
Figure 2.

A. hVps34 IP kinase assay

Activity (% of Control)

- + Insulin

B. Anti-P-Akt immunofluorescence

Fluorescence (% of Maximum)

- + + + Insulin

Control IgG Anti-hVps34

C. Anti-P-Akt blot

siRNA: Ctl hVps34

P-S473-Akt

Akt

hVps34

- + - + Insulin

D. Anti-P-TSC2 blot

siRNA: Ctl hVps34 (a) hVps34 (b)

P-S939-TSC2

- + - + - + Insulin

hVps34

Ctl (a) (b)

E. % of Control

- + - + Insulin

siRNA: Ctl hVps34
Figure 3.
Figure 4.

A. hVps34 activity

B. AICAR

C. Oligomycin

D. Rapamycin
Figure 5.

A. P-T389

|   | S6K1  |
|---|-------|
| - |       |
| + |       |
| + |       |
| - |       |
| + |       |
| - |       |
| + |       |

Insulin

0 1 5 10 15 20 Rapamycin (min)

B.

siRNA: Ctl  hVps34  hVps34  hVps34  hVps34
(a) (b) (c) (d)

P-T421/S424 S6K1

C. siRNA: Ctl  hVps34  hVps34
(a) (d)

P-S65 4EBP1

Insulin

siRNA: Ctl  hVps34
(a) (d)

D.

\[ \text{hVps34/hVps15} \]

\[ \text{mTOR/Raptor} \]

\[ \text{S6K1} \]

\[ \text{AMPK} \]

\[ \text{Akt} \]

\[ \text{TSC1/2} \]

\[ \text{Rheb(GTP)} \]

\[ \text{Insulin} \]

\[ \text{↓AA} \]

\[ \text{↓Glucose} \]
hVps34 is a Nutrient-regulated lipid kinase required for activation of p70 S6-kinase
Maya P. Byfield, James T. Murray and Jonathan M. Backer

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