Supplemental Material:

Supplemental Material and Methods:

Reagents, antibodies, and cell lines: Myc-SKD1 WT and mutant (E235Q) constructs were provided by P. Hanson (Washington University School of Medicine). Eps15WT-FLAG, Eps15Y850F-FLAG and Eps15-E29-eGFP constructs were provided by W. Atwood (Brown University). HA-Rab11WT, HA-Rab11S20V (Rab11 CA), and HA-Rab11S25N (Rab11 DN) constructs were provided by M. Marks (University of Pennsylvania). GFP-HA-PIKfyveWT and GFP-HA-PIKfyve K1831E (PIKfyve DN) constructs were provided by A. Shisheva (Wayne State University School of Medicine). Myc-Rab7WT and Myc-Rab7Q67L (Rab7 CA) constructs were provided by C. Bucci (Universita del Salento, Italy). Rab7T22N (Rab7 DN) and anti-Rab7 antibody was provided by A. Waddinger-Ness (University of New Mexico). Myc-Raptor was purchased from Addgene. Myc-hVps34/hVps15-V5 as described previously (Yan et al., 2009). Anti-GFP antibody was purchased from BabCo. Goat anti-Myc antibody was purchased from Santa Cruz Biotechnology. Anti-pT172 AMPK antibody was purchased from Cell Signaling technology. Anti-Flag antibody, 2-Deoxyglucose, and recombinant human insulin were purchased from Sigma. [3H]-L-Leucine was purchased from Perkin Elmer (Waltham, Ma) and [3H]-2-Deoxyglucose (2-DG) was purchased from Dupont NEN (Newton, MA).

Transferrin Uptake and Recycling: HeLa cells were transfected with either pSUPER-eGFP (vector control) or Eps15-E29-eGFP using TransIT HeLa Monster kit. Cells were placed in PBS with 50 mM HEPES (pH 7.4) containing 2mg/ml Ovalbumin and 20 µg/ml Cy-3 labeled transferrin for 15 min at 37°C. Cells were placed on ice, then immediately rinsed 4 times in ice cold neutral wash buffer (150 mM NaCl, 20 mM HEPES pH 7.4, 1 mM CaCl2, 5 mM KCl, 1 mM MgCl2), followed by two 2 minute washes in ice cold mild acidic buffer (50 mM MES, 280 mM sucrose pH 5.0). Cells were fixed in 4% paraformaldehyde-PBS for 15 min on ice, washed 4 times in neutral wash buffer, mounted onto slides using N-propyl gallate mounting media, and images were taken using a 60x 1.4 NA infinity-corrected optics on a Nikon Eclipse microscope supplemented with a computer-driven Roper cooled CCD camera and operated by IPLab Spectrum software (VayTek).

To measure transferrin recycling, HeLa cells were transfected with either HA-Rab11 WT, HA-Rab11DN, or HA-Rab11CA using TransIT HeLa Monster kit. Cells were placed in serum free RPMI containing 2mg/ml Ovalbumin and 20 µg/ml Cy-3 labeled transferrin for 60 min at 37°C. Cells were washed 3 times in ice cold neutral wash buffer (described above) followed by a 30 min chase in serum free RPMI containing 100 µM desferoxamine mesylate before fixing cells in 4% paraformaldehyde-PBS. Cells were immunostained for HA (for Rab11 expression) and images as described above.

PDGFR Degradation: HeLa cells were cotransfected with human PDGFR-BB and empty vector, Myc-Rab7 WT, Rab7DN, or Myc-Rab7 CA using TransIT HeLa Monster kit. Cells were serum starved 6 hrs then incubated on ice with 20ng/ml PDGF-BB and 5 µg/ml mouse anti-human PDGFR in 1%BSA-L15 for 70 min. Cells were washed with
serum free DMEM, rapidly warmed to 37° C and incubated for 60 min in serum free DMEM. Cells were fixed and immunostained for overexpressed Rab7 and PDGFR.

**Nutrient Uptake Assays:** To measure leucine uptake, Hela cells grown in 6-well plates were washed and then incubated in warm uptake buffer (10 nM L-Leucine, 135 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 10 mM Glucose, 2 mM Glutamine, 20 mM HEPES (pH 7.5)) at 37° C for 5 minutes. To each well, buffer containing 1 μCi of [3H]-L-Leucine and either 50 μM cold L-Leucine or 10 mM cold L-Leucine (for non-specific uptake) was added. At various times, uptake was terminated by four quick washes in ice-cold PBS followed by 10 minute solubilization at 37° C in 0.5 ml/well 0.1 N NaOH. Uptake was quantified by liquid scintillation counting. Specific uptake was calculated by subtracting non-specific uptake at each time point. To evaluate effect of Rab5 CA on leucine uptake, Hela cells grown in 6-well plates were transfected with empty vector or Rab5CA using TransIT HeLa Monster kit, and uptake during a 1 min incubation was measured as described above. The Micro-BCA assay kit (Pierce Biotechnology, Waltham, MA) was used to calculate total amount of protein. Parallel plates were used for whole cell lysate collection and western blotting to determine Rab5 expression levels and effect of Rab5 CA on amino acid stimulated endogenous pS6K1.

To measure glucose uptake, Hela cells grown on 6-well plates were washed 2 times in PBS and once in 0.1% BSA/DMEM, and incubated with 2 ml/well 0.1% BSA/DMEM for 2 hrs at 37° C. Cells were then incubated in warm KRPH buffer (5 mM Na2HPO4 (pH 7.4), 20 mM HEPES (pH 7.4), 1 mM MgSO4, 136 mM NaCl, 1% BSA, 1 mM CaCl2, 4.7 mM KCl) for 20 mins at 37° C. The cells were placed on ice and the buffer replaced with 1 ml/well ice-cold KRPH containing 1 μCi [3H]-2-DG and 1 mM cold 2-DG on ice. After various times, uptake was terminated by the addition of 1 ml/well KRPH containing 20 μM Cytochalasin B, followed by three quick washes in ice-cold PBS and 10 minute solubilization at 37° C in 0.5 ml/well 0.1 N NaOH. Uptake was quantified by liquid scintillation counting. To evaluate effect of Rab5 CA on glucose uptake, Hela cells grown in 6-well plates were transfected with empty vector or Rab5CA using TransIT HeLa Monster kit, and uptake during a 5 min incubation was measured as described above. The Micro-BCA assay kit (Pierce Biotechnology) was used to calculate total amount of protein. Parallel plates were used for whole cell lysate collection and western blotting to determine Rab5 expression levels and effect of Rab5 CA on insulin stimulated endogenous pS6K1.

**Myc-Raptor and endogenous mTOR coimmunoprecipitation:** HEK293T cells grown in 10 cm tissue culture dishes were cotransfected with Myc-Raptor and empty vector or Rab5CA using Fugene HD. Cells were washed with PBS and lysed in CHAPS lysis buffer as described (Kim et al., 2002). Anti-Myc immunoprecipitates and whole cell lysates were used for western blot analysis of endogenous mTOR, Myc-Raptor, and Rab5CA levels.
Supplemental Figures:

**Supplemental Figure 1: Inhibition of clathrin-dependent endocytosis, endocytic recycling, or endosome to TGN recycling does not affect insulin-stimulated S6K1 activation.**

A) Overexpression of Eps15-E29 blocks internalization of Cy3-labelled transferrin. To inhibit clathrin mediated endocytosis, we used dominant negative Eps15 constructs (Querbes et al., 2004). Eps15 is involved in the assembly of AP-2 and clathrin on the plasma membrane during the formation of clathrin coated pits, and is required for clathrin-dependent endocytosis (van Delft et al., 1997; Benmerah et al., 1998). Deletion of amino acids 95-295, which eliminates the EH2 domain of Eps15 (Eps15-ΔE95), blocks both constitutive and ligand-stimulated endocytosis, whereas a point mutation (Y850F) blocks only ligand inducible clathrin-dependent endocytosis (Querbes et al., 2004). HeLa cells were transfected with either GFP control or GFP-Eps15-DEH95. Cy3-Tf (20 µg/ml) uptake was carried out for 15 min. Cells were washed and fixed as described in methods. Arrows indicate transfected cells. 5 µm scale bars are shown.

B) Overexpression of Eps15 WT, Eps15Y850F, or Eps15-ΔE95 has no effect on S6K1 activation in insulin-stimulated cells. HEK293E cells were transfected with HA-S6K1 and either empty vector, Eps15 WT-FLAG, Eps15-Y850F-FLAG, or GFP-Eps15-DEH95 constructs as indicated. Serum starved cells were incubated without or with 1 µM human insulin for 30 min. Anti-HA immunoprecipitates or cleared lysates were blotted for pT389-S6K1, total S6K1, FLAG and GFP.

C) Overexpression of Rab11DN, but not Rab11WT or Rab11CA, leads to decreased endocytic recycling of cy3-labelled transferrin. To inhibit endocytic recycling we used mutants of Rab11, which regulates trafficking of membrane proteins from endosomes and the recycling compartments back to the plasma membrane. Inhibition of Rab11 signaling through a S25N mutation (Rab11DN) leads to a decrease in endocytic recycling and a build up of internalized proteins in endosomes and recycling compartments (Ren et al., 1998). HeLa cells were transfected with wild type and mutant Rab11 constructs as indicated. Cy3-Tf uptake was carried out for 30 min, and the cells were then washed two times and incubated in presence of desferoxamine at 37°C for 30 min. The cells were fixed and immunostained for HA (to visualize Rab11). Arrows indicate transfected cells. 5 µm scale bars are shown.

D) Overexpression of these Rab11 mutants has no effect on activation of S6K1 in insulin-stimulated cells. HEK293E cells were transfected with HA-S6K1 and either empty vector or wild type and mutant Rab11 constructs as indicated. Serum starved cells were incubated without or with 1 µM human insulin for 30 min. Anti-HA immunoprecipitates or cleared lysates were blotted for pT389-S6K1, total S6K1, and HA (Rab11).

E) Overexpression of PIKfyveDN, but not PIKfyveWT, leads to the enlargement of late endosomes. To inhibit endosome to TGN recycling we used a dominant negative mutant of PIKfyve, a PI[3]P-5-kinase. PIKfyve is recruited to endosomes through its FYVE domain, which binds to PI[3]P (Sbrissa et al., 2002). Inhibition of PIKfyve lipid kinase activity through a K1831E mutation (PIKfyve DN) blocks endosome to TGN recycling and leads to a swelling of late endosomes (Ikonomov et al., 2001; Ikonomov et al., 2003; Ikonomov et al., 2009). HeLa cells were transfected with either GFP-HA-PIKfyve-WT or GFP-HA-PIKfyve-DN (PIKfyve K1831E; dominant negative, kinase-deficient) as indicated. Cells were fixed and immunostained for endogenous LAMP-2. Arrows indicate transfected cells. 5µm scale bars are shown.
Overexpression of PIKfyve WT or PIKfyve DN has no effect on insulin stimulation of S6K1. HEK293E cells were transfected with HA-S6K1 and either empty vector, GFP-HA-PIKfyve WT or GFP-HA-PIKfyve DN constructs as indicated. Serum starved cells were incubated without or with 1 µM human insulin for 30 min. Anti-HA immunoprecipitates or cleared lysates were blotted for pT389-S6K1, total S6K1, and HA (PIKfyve).

Supplemental Figure 2: Inhibition of multivesicular body biogenesis or late endosome to lysosome trafficking does not affect insulin-stimulated S6K1 activation.

A) Overexpression of SKD1-EQ, but not SKD1-WT, leads to the enlargement of late endosomes. To inhibit MVB biogenesis, we used a dominant negative mutant of SKD1 (Vps4p in yeast), which associates with late endosomes by binding to ESCRT complexes II and III (Lin et al., 2005). SKD1 is an AAA ATPase, and it causes the disassembly of ESCRT complexes from late endosomes, thereby allowing continual rounds of multivesicular body biogenesis to occur (Babst, 2005). Inhibition of SKD1 ATPase activity through a E235Q mutation (SKD1-EQ) leads to a block in multivesicular body biogenesis, leading to a build up of ubiquitinated proteins at swollen late endosomal membranes (Lin et al., 2005). HeLa cells were transfected with either Myc-SKD1-WT or Myc-SKD1-EQ. 24 hrs after transfection, cells were fixed and immunostained for endogenous LAMP-2 and Myc (to visualize SKD1). Arrows indicate transfected cells. 10 µm scale bars are shown

B) Overexpression of wild type or mutant SKD1 has no effect on insulin stimulation of S6K1. HEK293E cells were transfected with HA-S6K1 and either empty vector, Myc-SKD1-WT, or Myc-SKD1-EQ constructs as indicated. 24 hrs post transfection, serum starved cells were incubated without or with 1 µM human insulin for 30 min. Anti-HA immunoprecipitates and anti-Myc immunoprecipitates were blotted for pT389-S6K1, total S6K1, and Myc (SKD1).

C) Overexpression of Rab7 DN, but not Rab7 WT or Rab7 CA, leads to decreased lysosomal degradation of transfected PDGF receptors. To inhibit late endosome to lysosome fusion we used the dominant negative Rab7-T22N mutant (Rab7 DN). This construct leads to a block of late endosome-lysosome fusion and a build up in late endosomes of proteins that were targeted for degradation, such as growth factor receptors (Vitelli et al., 1997). HeLa cells were transfected with human PDGFR and either empty vector, Myc-Rab7WT, Rab7DN, or Myc-Rab7CA as indicated. Serum starved cells were incubated on ice with 20ng/ml PDGF-BB and 5 µg/ml mouse anti-human PDGFR, washed, then rapidly warmed to 37°C and incubated for 60 min. Cells were fixed and immunostained for overexpressed Rab7 and PDGFR. 5 µm scale bars are shown.

D) Overexpression of Rab7 WT, Rab7 DN or Rab7 CA has no effect on insulin-stimulated mTORC1/S6K1 signaling. HEK293E cells were transfected with HA-S6K1 and either empty vector, Myc-Rab7WT, Rab7DN, or Myc-Rab7CA constructs as indicated. Serum starved cells were incubated without or with 1 µM human insulin for 30 min. Anti-HA immunoprecipitates or cleared lysates were blotted for pT389-S6K1, total S6K1, and Rab7.

Supplemental Figure 3: Overexpression of Rab5 CA does not inhibit leucine or glucose uptake. A) Hela cells grown in 6-well plates were washed and incubated with
[³H]-Leucine for various times as described in methods. 0.1 N NaOH solubilized cells were measured by scintillation counting for [³H]-Leucine uptake. Graph represents specific leucine uptake in pmoles/well at the indicated times. Data represent mean +/- S.E.M. of 4 replicates. B) Hela cells grown in 6-well plates transiently transfected with either empty vector or Rab5 CA constructs were washed and incubated with [³H]-Leucine for 1 minute. Graph represents specific leucine uptake in pmoles/mg. Data represent mean +/- S.E.M. of 4 replicates. C) A parallel plate of Hela cells transfected at same time and conditions as in (B), were incubated in amino acid free media for 50 min then stimulated without or with 1X (final) complete amino acid solution for 30 min. Whole cell lysates were blotted for endogenous pT389 S6K1, Total S6K1, and overexpressed Rab5 as indicated. D) Hela cells grown in 6-well plates were washed and incubated with [³H]-2-DG for various times as described in methods. 0.1 N NaOH solubilized cells were measured by scintillation counting for [³H]-2-DG uptake. Graph represents specific glucose uptake in pmoles/well at the indicated times. Data represent mean +/- S.E.M. of 4 replicates. E) Hela cells grown in 6-well plates transiently transfected with either empty vector or Rab5 CA constructs were washed and incubated with [³H]-2-DG for 5 minutes. Graph represents specific activity of leucine uptake in pmoles/mg. Data represent mean +/- S.E.M. of 6 replicates. F) A parallel plate of Hela cells transfected at same time and conditions as in (E) were serum starved overnight then stimulated without or with 1µM human Insulin for 30 min. Whole cell lysates were blotted for endogenous pT389 S6K1, Total S6K1, and overexpressed Rab5 as indicated.

Supplemental Figure 4: Overexpression of Rab5CA does not inhibit mTORC1 stability or upstream signaling to mTORC1, and cannot be rescued by overexpression hVps34/hVps15. A) HEK293E cells were transfected with either empty vector or Rab5 CA as indicated. Cells were either serum starved overnight then stimulated without or with 1 µM human insulin for 30 minutes, or serum starved for 45 minutes then treated with 25 mM 2-DG for 15 minutes. Whole cell lysates were probed for endogenous pT172 AMPK, pT389 S6K1, pT308 Akt, GAPDH, and overexpressed Rab5 as indicated. B) HEK293T cells were transfected with Myc-Raptor and either empty vector or Rab5CA as indicated. Cells were lysed in CHAPS lysis buffer and anti-Myc immunoprecipitates or cleared lysates from each condition were blotted with the indicated antibodies. C) HEK239E cells were transfected with HA-S6K1 and with combinations of empty vector, Myc-hVps34/hVps15-V5 and Rab5CA, as indicated. Serum starved cells were incubated without or with 1 µM human insulin for 30 min. Anti-HA immunoprecipitates or cleared lysates from each condition were blotted for pT389-S6K1, total S6K1, V5, Myc, and Rab5 as indicated.

Supplemental Figure 5: Overexpression of SKD1 EQ or Rab7 DN do not lead to early/late endosomal mixing and do not alter amino acid stimulated mTOR localization to late endosomes. A) Hela cells transfected with SKD1 EQ or Rab7 DN as indicated were fixed and immunostained for Myc (SKD1 EQ only) or Rab7 (Rab7 DN only) and EEA1 and LAMP2. EEA1 and LAMP-2 color merges are displayed. Arrows indicate transfected cells. 5µm scale bars are shown. B) Hela cells transfected with SKD1
EQ or Rab7 DN as indicated were incubated in amino acid free media for 50 min then stimulated without or with 1X (final) complete amino acid solution for 10 min. Cells were fixed and immunostained for Myc (SKD1 EQ only) or Rab7 (Rab7 DN only) and endogenous mTOR and LAMP-2 as indicated. ROIs for mTOR and LAMP-2 are displayed at higher magnification. Arrows indicate transfected cells. 5 μm scale bars, or 2 μm scale bars in the high magnification ROIs are shown.

Supplemental References

Babst, M. (2005). A protein's final ESCRT. Traffic 6, 2-9.

Benmerah, A., Lamaze, C., Begue, B., Schmid, S.L., Dautry-Varsat, A., and Cerf-Bensussan, N. (1998). AP-2/Eps15 interaction is required for receptor-mediated endocytosis. J Cell Biol 140, 1055-1062.

Ikonomov, O.C., Fligger, J., Sbrissa, D., Dondapati, R., Mlak, K., Deeb, R., and Shisheva, A. (2009). Kinesin adapter JLP links PIKfyve to microtubule-based endosome-to-trans-Golgi network traffic of furin. J Biol Chem 284, 3750-3761.

Ikonomov, O.C., Sbrissa, D., Mlak, K., Deeb, R., Fligger, J., Soans, A., Finley, R.L., Jr., and Shisheva, A. (2003). Active PIKfyve associates with and promotes the membrane attachment of the late endosome-to-trans-Golgi network transport factor Rab9 effector p40. J Biol Chem 278, 50863-50871.

Ikonomov, O.C., Sbrissa, D., and Shisheva, A. (2001). Mammalian cell morphology and endocytic membrane homeostasis require enzymatically active phosphoinositide 5-kinase PIKfyve. J Biol Chem 276, 26141-26147.

Kim, D.H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 110, 163-175.

Lin, Y., Kimpler, L.A., Naismith, T.V., Lauer, J.M., and Hanson, P.I. (2005). Interaction of the mammalian endosomal sorting complex required for transport (ESCRT) III protein hSnf7-1 with itself, membranes, and the AAA+ ATPase SKD1. J Biol Chem 280, 12799-12809.
Querbes, W., Benmerah, A., Tosoni, D., Di Fiore, P.P., and Atwood, W.J. (2004). A JC virus-induced signal is required for infection of glial cells by a clathrin- and eps15-dependent pathway. J Virol 78, 250-256.

Ren, M., Xu, G., Zeng, J., De Lemos-Chiarandini, C., Adesnik, M., and Sabatini, D.D. (1998). Hydrolysis of GTP on rab11 is required for the direct delivery of transferrin from the pericentriolar recycling compartment to the cell surface but not from sorting endosomes. Proc Natl Acad Sci U S A 95, 6187-6192.

Sbrissa, D., Ikonomov, O.C., and Shisheva, A. (2002). Phosphatidylinositol 3-phosphate-interacting domains in PIKfyve. Binding specificity and role in PIKfyve. Endomenbrane localization. J Biol Chem 277, 6073-6079.

van Delft, S., Schumacher, C., Hage, W., Verkleij, A.J., and van Bergen en Henegouwen, P.M. (1997). Association and colocalization of Eps15 with adaptor protein-2 and clathrin. J Cell Biol 136, 811-821.

Vitelli, R., Santillo, M., Lattero, D., Chiariello, M., Bifulco, M., Bruni, C.B., and Bucci, C. (1997). Role of the small GTPase Rab7 in the late endocytic pathway. J Biol Chem 272, 4391-4397.

Yan, Y., Flinn, R.J., Wu, H., Schnur, R.S., and Backer, J.M. (2009). hVps15, but not Ca2+/CaM, is required for the activity and regulation of hVps34 in mammalian cells. Biochem J 417, 747-755.
