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The Late Endosome is Essential for mTORC1 Signaling

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The multisubunit mTORC1 complex integrates signals from growth factors and nutrients to regulate protein synthesis, cell growth, and autophagy. To examine how endocytic trafficking might be involved in nutrient regulation of mTORC1, we perturbed specific endocytic trafficking pathways and measured mTORC1 activity using S6K1 as a readout. When early/late endosomal conversion was blocked by either overexpression of constitutively active Rab5 (Rab5CA) or knockdown of the Rab7 GEF hVps39, insulin- and amino acid–stimulated mTORC1/S6K1 activation were inhibited, and mTOR localized to hybrid early/late endosomes. Inhibition of other stages of endocytic trafficking had no effect on mTORC1. Overexpression of Rheb, which activates mTOR independently of mTOR localization, rescued mTORC1 signaling in cells expressing Rab5CA, whereas hyperactivation of endogenous Rheb in TSC2−/− MEFs did not. These data suggest that integrity of late endosomes is essential for amino acid– and insulin-stimulated mTORC1 signaling and that blocking the early/late endosomal conversion prevents mTOR from interacting with Rheb in the late endosomal compartment.

INTRODUCTION

The mTOR (mammalian target of rapamycin) protein kinase is found in two complexes in eukaryotes: mTORC1, which integrates nutrient and hormonal regulation of protein synthesis and cell growth, and mTORC2, which phosphorylates Akt on Ser473 and plays a role in actin cytoskeletal rearrangements (Sarbassov *et al.*, 2005; Wullschleger *et al.*, 2006). mTORC1 activity is stimulated by growth factors such as insulin and by nutrients such as amino acids and glucose (Harris and Lawrence, 2003; Jaeschke *et al.*, 2004; Sarbassov *et al.*, 2005; Tee and Blenis, 2005; Avruch *et al.*, 2006; Mamane *et al.*, 2006). mTORC1 regulates protein synthesis at least in part through the phosphorylation of two downstream targets, the translational activator S6K1 and the translational inhibitor 4EBP1 (Hay and Sonenberg, 2004). Activation of mTORC1/S6K1 signaling also leads to the inhibitory phosphorylation of IRS-1, initiating a negative feedback loop acting on insulin receptor signaling (Um *et al.*, 2004, 2006; Tremblay *et al.*, 2007) .

There is strong evidence that mTORC1 function is involved in and/or regulated by endosomal trafficking. In *Drosophila melanogaster*, TOR has been shown to positively regulate endocytosis while specifically inhibiting degradation of the amino acid transporter slimfast (Hennig *et al.*, 2006). TOR itself has been localized to endocytic compartments both in yeast and fly (Kunz *et al.*, 2000; Chen and Kaiser, 2003; Wedaman *et al.*, 2003), and more recently an amino acid–driven localization of mTOR to late endosomes was described in mammalian cell culture (Sancak *et al.*, 2008). Furthermore, a direct upstream activator of mTORC1, the GTPase Rheb, alters endosomal morphology when overexpressed (Saito *et al.*, 2005) and localizes to late endosomes (Sancak *et al.*, 2008). The GTPase-activating protein (GAP) for Rheb, the TSC1/2 complex, also has GAP activity toward the early endosomal GTPase, Rab5, implicating it in endosomal trafficking (Xiao *et al.*, 1997).

Given the connections between mTORC1 and endocytic trafficking, we examined whether perturbation of particular stages of endocytic trafficking would alter mTORC1/S6K1 signaling. We find that constitutively activate Rab5 abrogates mTORC1 signaling, whereas perturbation of other stages of endocytic trafficking has no effect on mTORC1 signaling. Constitutively active Rab5 mutants block early to late endosomal conversion (Rink *et al.*, 2005), leading to the formation of hybrid endosomal compartments containing both early and late endosomal markers. We find that mTOR localizes to this hybrid compartment when endosomal conversion is blocked. Blocking endosomal conversion by knockdown of a key member of the HOPS complex, hVps39, also produces merged early/late endosomes (Rink *et al.*, 2005) and inhibits mTORC1 signaling. Our results suggest that mTORC1 signaling requires the integrity of late endosomes.

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Abbreviations used: ECL, enhanced chemiluminescence; 2-DG, 2-deoxyglucose; GAP, GTPase-activating protein; GEF, guanine nucleotide-exchange factor; HEK, human embryonic kidney 293 cells; HOPS, homotypic fusion and vacuole protein sorting; LBPA, lysobisphosphatidic acid; MEF, mouse embryonic fibroblast; mTORC1, mTOR complex 1; MVB, multivesicular bodies.

MATERIALS AND METHODS

Plasmids, Antibodies, and Reagents

HA-S6K cDNA was provided by S. Schreiber (Harvard). Rab5 constructs were provided by M. Zerial (Max Planck Institute of Molecular Cell Biology and

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Genetics). Myc-Rheb was provided by G. Thomas (University of Cincinnati). Anti-Rab5, anti-S6K1, anti-mTOR, and anti-Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti-EEA1 and anti-LAMP-2 antibodies were purchased from BD Biosciences (San Jose, CA). Goat anti-EEA1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-S6 antibody (pS235/236/240/244/247) was provided by D. Fingar (University of Michigan). Mouse anti-HA and anti-Myc antibodies were produced in-house. Rabbit anti-HA was purchased from Rockland Immunochemicals (Gilbertsville, PA). Rabbit anti-Myc antibody was purchased from Immunology Consultants Laboratories (Newberg, OR). Human embryonic kidney (HEK) 293E cells were provided by D. Fingar (University of Michigan). TSC2−/− Mouse embryonic fibroblast (MEF) cells were provided by G. Thomas (University of Cincinnati). Amino acid–free RPMI was purchased from US Biological (Swampscott, MA).

Cell Culture, Transfections, and Immunoprecipitations

HEK293E cells grown in DMEM/10% FBS were transfected with the indicated constructs using Fugene 6 or Fugene HD reagents (Roche, Indianapolis, IN) as described by the manufacturer. After an overnight incubation in serum-free media, cells were stimulated without or with 1 μM recombinant human insulin for 30 min. Cells were rinsed in cold PBS and then lysed in 120 mM NaCl, 20 mM Tris (pH 7.5), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% NP-40, containing EDTA-free Protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktails 1 + 2 (Sigma, St. Louis, MO). Proteins were immunoprecipitated from cleared lysates and analyzed by Western blot. Blots were visualized using ECL (Amersham Pharmacia, Piscataway, NJ) or the Li-Cor Odyssey imaging system (Lincoln, NE).

For amino acid–signaling experiments, transfected HEK293T cells were incubated for 50 min in amino acid– and serum-free RPMI and then stimulated without or with 1× complete amino acid solution plus glutamine for 10 min. Lysis, immunoprecipitation, and Western blot procedures were performed as described above. In experiments using TSC2−/− MEF cells, cells grown in 10% FBS-DMEM were transfected using TransIT HeLa Monster transfection kit (Mirus, Madison, WI) according to the manufacturer's instructions. Lysis, immunoprecipitation, and Western blot procedures were performed as described above. In experiments evaluating phosphorylation of endogenous S6K1, cells were transfected, treated as above, and lysed directly in SDS sample buffer. Western blot procedures were performed as described above.

Immunofluorescence Experiments

HeLa cells were plated onto acid-washed uncoated glass coverslips. The following day, cells were transfected with the indicated constructs using TransIT HeLa Monster kit (Mirus) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were left untreated or were amino acid—starved and refed as described above, and fixed at 37°C in 4% paraformaldehyde-PBS for 10 min. Coverslips were rinsed one time in PBS and then permeabilized in 0.2% Triton X-100 PBS for 15 min at room temperature. Coverslips were then washed one time in 0.1 M glycine-PBS, incubated in 0.1 M glycine-PBS for 10 min at room temperature, and blocked in PBS containing 1% BSA/1% FBS before being incubated in primary and secondary antibodies as indicated. Coverslips were mounted on slides using *N*-propyl gallate media (0.11 g *N*-propyl gallate in PBS containing 50% glycerol). Images were taken using either a 60× 1.4 NA infinity-corrected optics on a Nikon Eclipse microscope (Melville, NY) supplemented with a computer-driven Roper cooled CCD camera (Tucson, AZ) and operated by IPLab Spectrum software (VayTek, Fairfield, IA) or a Bio-Rad Radiance 2000 Scanning Laser Confocal microscope (Richmond, CA) using 488-, 568-, and 637-nm laser lines and Scion Image software (Frederick, MD) as indicated in figure legends.

hVps39 Knockdown

HeLa cells were transfected with 140 nM Smart Pool siRNA (Dharmacon, Boulder, CO) targeting either Luciferase or hVps39, using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A knockdown efficiency of 80% was confirmed by quantitative RT-PCR. HeLa cells were serum-starved overnight and stimulated without or with 1 μM human insulin for 30 min before direct lysis in SDS sample buffer. Whole cell lysates were probed by Western blot with phospho-T389 pS6K1, total S6K1, and GAPDH antibodies, followed by visualization using HRP secondary antibodies and ECL. For immunofluorescence experiments, hVps39 knockdown cells were starved, stimulated, fixed, and processed as described above. The cells were stained using a cocktail of goat anti-EEA1, mouse anti-LAMP-2, and either rabbit anti-phospho-S6 (pS235/236/240/244/247) or rabbit anti-mTOR antibodies overnight at 4°C. After washing, slips were incubated sequentially with donkey anti-goat Alexa-fluor 546 antibody, followed by goat anti-mouse Alexa-fluor 488 antibody, and goat anti-rabbit Alexa-fluor 647 antibody.

Statistics

Quantification of Western blots was performed using the Li-Cor Odyssey imaging system (HA-S6K1 experiments in HEK293E and HEK293T cells) and ImageJ software (http://rsb.info.nih.gov/ij/; TSC2−/− MEF experiments and hVps39 knockdown experiments). Quantification of phospho-S6 immunofluorescence was performed using ImageJ software. All statistics were performed in Excel (Microsoft, Redmond, WA) using Student's *t* tests to analyze statistical significance.

RESULTS

Overexpression of Constitutively Active Rab5 Blocks Activation of mTORC1/S6K1

Numerous recent studies have suggested a connection between mTORC1 signaling with endosomes (Kunz *et al.*, 2000; Chen and Kaiser, 2003; Wedaman *et al.*, 2003; Hennig *et al.*, 2006; Sancak *et al.*, 2008). Rab5 plays a major regulatory role in endosomal trafficking (Bucci *et al.*, 1992; Stenmark *et al.*, 1994; Zerial and McBride, 2001; Murray *et al.*, 2002). We therefore examined whether mutants of Rab5 would affect insulin and amino acid stimulation of mTORC1. As previously described (Stenmark *et al.*, 1994), overexpression of Rab5WT caused a slight enlargement of early endosomes, whereas overexpression of Rab5Q79L, a GTPase-deficient mutant of Rab5 (henceforth referred to as Rab5CA), caused a striking enlargement of endosomes (data not shown). Also as described (Stenmark *et al.*, 1994), overexpression of a GTP-binding defective Rab5 mutant, Rab5S34N (henceforth referred to as Rab5DN), inhibited the early endosomal localization of EEA1 (data not shown). To evaluate effects of Rab5 mutants on mTORC1 signaling, we overexpressed Rab5 cDNAs and measured phosphorylation of HA-S6K1 at T389 as a readout of mTORC1 activity (Hay and Sonenberg, 2004). Rab5DN led to a modest decrease in insulin-stimulated S6K1 activation, whereas Rab5WT significantly inhibited, and Rab5CA completely inhibited insulin-stimulated S6K1 activation (Figure 1, A and B). Stimulation of S6K1 phosphorylation by amino acids was also inhibited by Rab5CA, suggesting an effect on the nutrient arm of the mTORC1 signaling pathway (Figure 1, E and F). However, the inhibition of mTORC1 signaling was not due to a block in the growth factor–signaling arm of the mTORC1 pathway, because no significant effect on insulin-stimulated Myc-Akt T308 phosphorylation was observed (Figure 1, C and D). Furthermore, overexpression of Rab5CA decreased the activation of endogenous pS6K1 by insulin in HEK293E cells, and by amino acids in HEK293T cells (Figure 1G). Taken together, these experiments show that constitutive activation of Rab5 blocks amino acid– and insulin-stimulated mTORC1 signaling.

Perturbation of Endocytic Trafficking Does Not Inhibit mTORC1/S6K1 Signaling

We next investigated whether the inhibition of mTORC1/S6K1 by Rab5 was specific or reflected a more general requirement for endosomal trafficking. To test this question, we perturbed particular stages of endosomal trafficking using various constitutively active or dominant negative cDNA constructs (Table 1). Expression of each mutant led to the expected inhibition of receptor internalization, receptor recycling, multivesicular body (MVB) formation, endosome/Golgi trafficking, and late endosome/lysosome fusion (Table 1, Figures S1 and S2). However, none of these mutants had any effect on insulin-stimulated mTORC1/S6K1 signaling (Figures S1 and S2). These data suggest that constitutive activation of Rab5, but not inhibition of upstream or

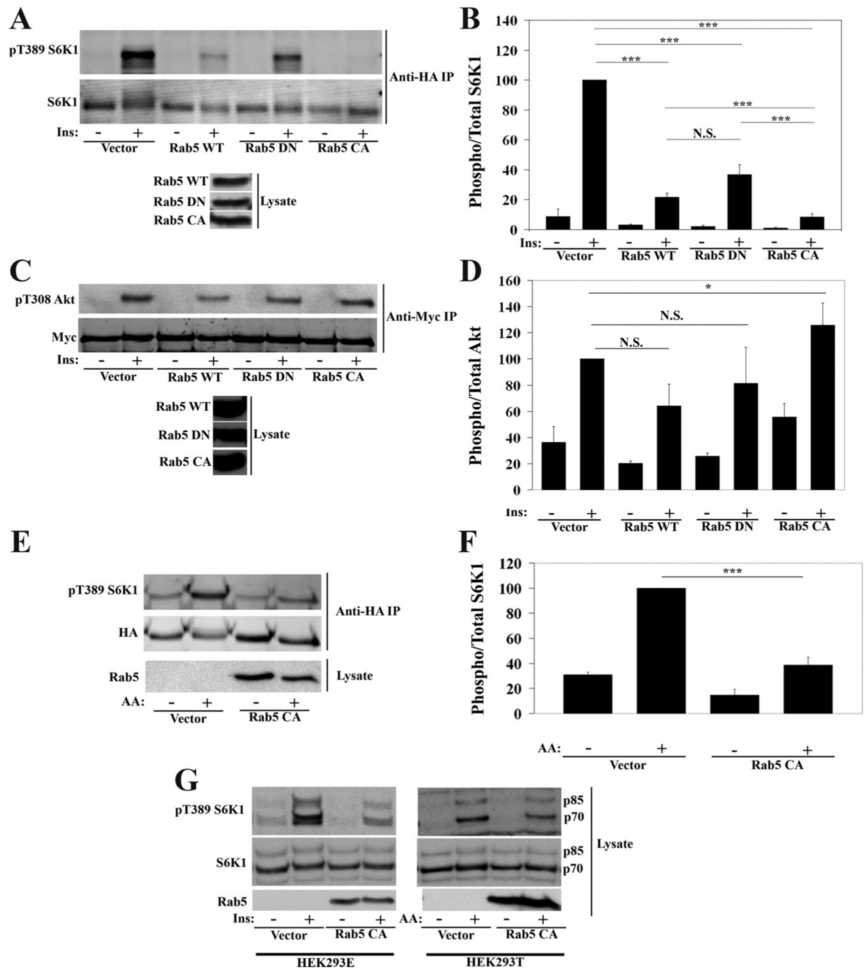

Figure 1. Rab5CA inhibits insulin- and amino acid—stimulated phosphorylation of S6K1. (A) HEK293E cells were transfected with HA-S6K1 and either empty vector, Rab5WT, Rab5DN, or Rab5CA constructs as indicated. Serum-starved cells were incubated without or with 1 μM human insulin for 30 min. Anti-HA immunoprecipitates and cleared lysates were blotted for pT389-S6K1, total S6K1, and Rab5. (B) The ratio of pT389 to total HA-S6K1, normalized to the ratio in vector controls. The data are the mean $\pm$ SEM from seven experiments. ***$p < 0.005$. (C) HEK293E cells were transfected with Myc-Akt and Rab5 constructs as indicated, and stimulated without or with 1 μM insulin for 15 min. Anti-Myc immunoprecipitates as well as cleared lysates were blotted for pT308-Akt, Myc (total Akt), and Rab5. (D) The ratio of pT308 to total Myc-Akt, normalized to the ratio in vector controls. The data are the mean $\pm$ SEM from six experiments. *$p < 0.05$. (E) HEK293T cells were transfected with HA-S6K1 and either empty vector or Rab5CA constructs as indicated. Amino acid–starved cells were incubated without or with 1$\times$ (final) complete amino acid solution for 10 min. Anti-HA immunoprecipitates as well as cleared lysates were blotted for pT389-S6K1, HA (total S6K1), and Rab5. (F) The ratio of pT389 to total HA-S6K1 was calculated as above. The data are the mean $\pm$ SEM from three experiments. ***$p < 0.005$. (G) HEK293E (left) or HEK293T (right) cells were transfected with either empty vector or Rab5CA constructs as indicated. Serum-starved HEK293E and amino acid–starved HEK293T cells were stimulated with insulin or amino acids, respectively, as described above, and whole cell lysates were blotted for endogenous pT389 S6K1, total S6K1, and overexpressed Rab5.

downstream steps in endocytic trafficking, blocks mTORC1 signaling.

Overexpression of Rheb, But Not Increased Activation of Endogenous Rheb, Rescues mTORC1/S6K1 Signaling in Cells Expressing Rab5CA

To explore the mechanism by which Rab5CA inhibits mTORC1/S6K1 signaling, we first examined whether Rab5CA inhibition of mTORC1 could be due to decreases in nutrient uptake. However, uptake of both leucine and glucose were unaffected by expression of Rab5CA (Supplemental Figure 3). Similarly, expression of Rab5CA did not increase basal activation of AMPK, and in fact led to a slight decrease in AMPK phosphorylation in 2-DG–treated cells (Supplemental Figure 4A). As others have shown, extracellular signal–regulated kinase (ERK) is minimally activated in HEK cells by insulin (Herbert *et al.*, 2000), and we did not detect insulin-stimulated activation of ERK in control or Rab5CA–expressing cells (data not shown). Together with the data on Akt activation, this suggests that Rab5CA inhibition of mTORC1 signaling is not due to changes in upstream signaling. Next we examined whether mTORC1 complex stability or formation was altered by overexpression of Rab5CA. However, the ability of endogenous mTOR to co-immunoprecipitate with overexpressed Myc-Raptor was unaffected by expression of Rab5CA (Supplemental Figure 4B). Finally, given the role Rab5 plays in recruiting hVps34 to early endosomes (Murray *et al.*, 2002) and the role of hVps34

Table 1. General perturbation of endocytic trafficking does not affect mTORC1/S6K1 signaling

cDNA construct	Effect on vesicular trafficking	Supplemental figure no.
Eps15Y850F-FLAG, GFP-Eps15ΔEH95 (Querbes *et al.*, 2004)	Inhibition of Clathrin Mediated Endocytosis	Sl. A-B.
HA-Rab11 WT, HA-Rab11S25N(DN) HA-Rab11S20V(CA) (Ren *et al.*, 1998)	Perturbation of Recycling Compartment	Sl. C-D.
PIKfyveDN-HA-GFP (Ikonomov *et al.*, 2001)	Inhibition of Endosome to TGN Recycling	Sl. E-F.
Myc-SKD1E235Q (Lin *et al.*, 2005)	Inhibition of Multivesicular Body Biogenesis	S2. A-B.
Myc-Rab7 WT, Rab7T22N, Myc-Rab7Q67L (Vitelli *et al.*, 1997)	Perturbation of Late Endosome-Lysosome Fusion	S2. C-D.

Specific cDNA constructs were employed to perturb particular stages of endocytic trafficking as indicated. Supplemental figure location is listed in the table. No effect on insulin-stimulated phosphorylation of S6K1 was observed with any of the indicated constructs.

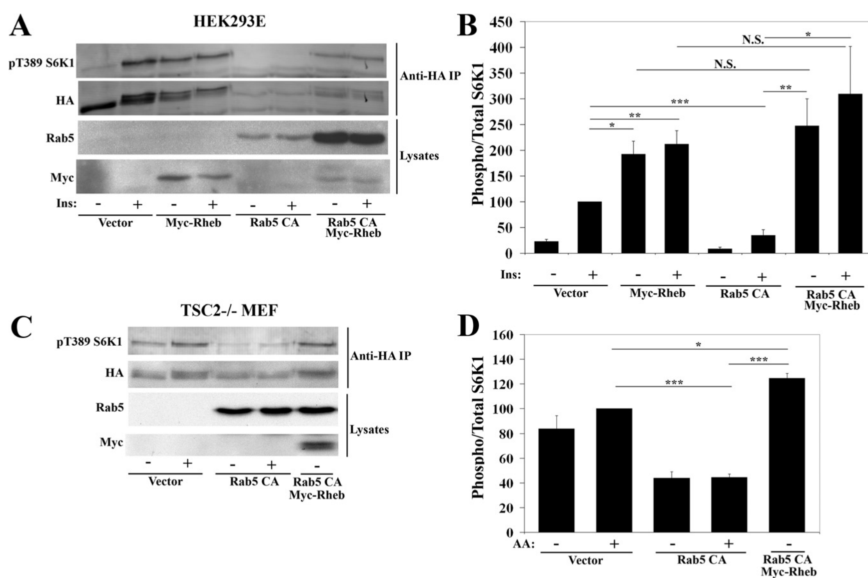

Figure 2. Overexpression of Rheb, but not hyperactivation of endogenous Rheb, rescues insulin- and amino acid–stimulated S6K1 activation in Rab5CA-overexpressing cells. (A) HEK239E cells were transfected with HA-S6K1 and combinations of empty vector, Rheb, and Rab5CA, as indicated. Serum-starved cells 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 and HA (total S6K1). (B) The graph shows the ratio of pT389/total HA-S6K1 calculated as in Figure 1. The data are the mean ± SEM from four experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$. (C) TSC2−/− MEFs were transfected with HA-S6K1 and combinations of empty vector, Rheb, and Rab5CA as indicated. Amino acid–starved cells were incubated without or with 1× (final) complete amino acid solution for 10 min. Anti-HA immunoprecipitates or cleared lysates from each condition were blotted for pT389-S6K1 and HA (total S6K1). (D) The graph shows the ratio of pT389/total HA-S6K1 calculated as in Figure 1. The data are the mean ± SD from two experiments. $*p < 0.05$, $***p < 0.005$.

in mTORC1 signaling in cultured mammalian cells (Byfield *et al.*, 2005; Nobukuni *et al.*, 2005), we explored the possibility that Rab5CA was sequestering hVps34 away from its site of action in mTORC1 signaling. However, overexpression of hVps34 along with hVps15 in a bicistronic vector (Yan *et al.*, 2009) did not rescue the inhibition of mTOR signaling by Rab5CA (Supplemental Figure 4C).

Overexpression of Rheb activates mTORC1 independently of amino acids (Long *et al.*, 2005a,b), presumably by overcoming the requirement for the amino acid–induced translocation of mTORC1 to late endosomes (Sancak *et al.*, 2008). We therefore tested whether Rheb overexpression would relieve the inhibition of mTORC1 by Rab5CA. Interestingly, Rheb overexpression led to constitutive activation of S6K1 in both control and Rab5CA-expressing cells (Figure 2, A and B). We next tested whether activation of endogenous Rheb could reverse the effects of Rab5CA. We overexpressed Rab5CA in TSC2−/− MEFs, a cell line in which the loss of Rheb-GAP activity leads to constitutively elevated levels of activated, GTP-bound Rheb (Garami *et al.*, 2003). Overexpression of Rab5CA was still able to inhibit amino acid–stimulated mTORC1/S6K1 in TSC2−/− MEFs, whereas cooverexpression of Myc-Rheb with Rab5CA in TSC2−/− MEFs blocked the inhibition of mTORC1/S6K1 signaling (Figure 2, C and D). These results suggest that Rab5CA does not act at the level of Rheb activation, but rather interferes with mTORC1–Rheb interactions.

Rab5CA Alters mTORC1 Localization and Leads to Mixing of Early and Late Endosomes

Rheb binding to mTORC1 activates mTORC1 (Patel *et al.*, 2003; Saucedo *et al.*, 2003; Long *et al.*, 2005a), and this interaction has been suggested to occur on late endosomes (Sancak *et al.*, 2008). Zerial and coworkers have shown that late endosomes arise from the conversion of early endosomes (Rink *et al.*, 2005), a process that requires the sequential activation of Rab5, recruitment of the HOPS complex, recruitment and activation of Rab7, and inactivation and removal of Rab5. The process is disrupted by either the expression of Rab5CA or the knockdown of hVps39, a component of the HOPS complex that is a Rab7 guanine exchange factor (GEF). Both of these manipulations lead to the formation of enlarged vesicles with a mixture of early and late endosomal markers (Rink *et al.*, 2005).

To address whether expression of Rab5CA alters mTORC1 targeting to late endosomes, we examined mTOR localization under amino acid–starved or amino-stimulated conditions in HeLa cells. As described (Sancak *et al.*, 2008), mTOR is observed in a diffuse cytosolic staining pattern in amino acid–starved cells. After a brief amino acid stimulation, mTOR moves to a late endocytic compartment that contains LAMP-2 (Figure 3A, left-hand panels). However, in the presence of Rab5CA, mTOR is found in enlarged vesicles that contain LAMP-2 but look quite distinct from the normal mTOR localization pattern in amino acid–stimulated cells (Figure 3A, right-hand panels). A fraction of mTOR is found associated with these enlarged LAMP–positive vesicles even under amino acid–starved conditions, which is in contrast to amino acid–starved control cells, where mTOR is not found colocalized with LAMP-2. Notably, when mTOR localization was compared with that of the early endosomal marker EEA1, little to no colocalization was observed between mTOR and EEA1 in control cells (Figure 3B, left-hand panels). However, in cells expressing Rab5CA, mTOR was also found in enlarged vesicles that contain EEA1 (Figure 3B, right-hand panels). To determine whether this was due to a mixing of early and late endosomes, as opposed to a less stringent targeting of mTOR, we costained Rab5CA-expressing cells for EEA1, LAMP-2, and mTOR. Overexpression of Rab5CA leads to the formation of hybrid endosomes that contain markers for both early and late endosomes (Figure 3C). Under amino acid–stimulated conditions in Rab5CA expressing cells, mTOR is found in these hybrid endosomes (Figure 3D). These data suggest that hybrid early/late endosomes cannot support mTOR signaling.

Knockdown of hVps39 Blocks Early/Late Endosome Conversion and Inhibits mTORC1 Signaling

We used an alternative approach to blocking early/late endosome conversion, by knocking down hVps39 in HeLa cells using a pool of four small interfering RNA (siRNA) oligonucleotides. Knockdown was 80% efficient as determined by real time PCR (data not shown). Knockdown of hVps39 led to the formation of hybrid endosomes that were

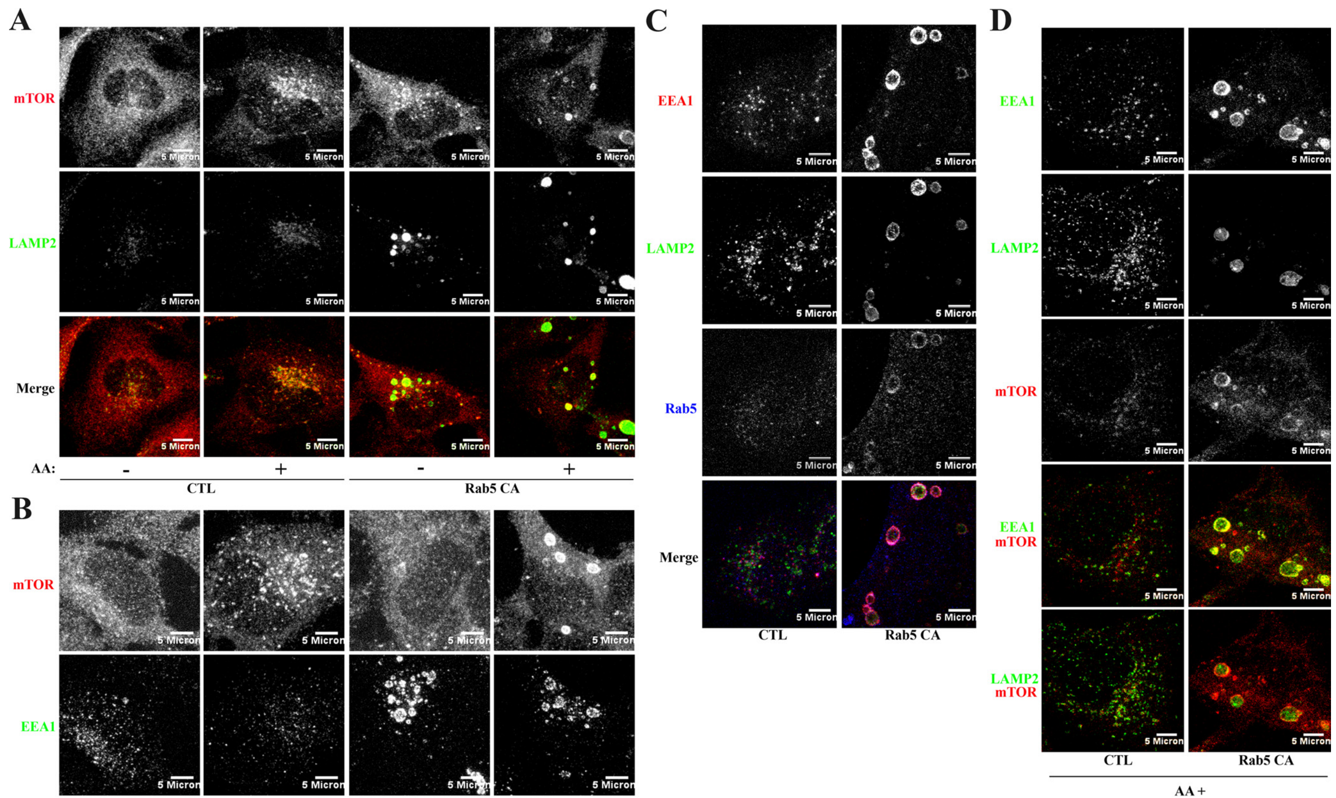

Figure 3. Overexpression of Rab5CA produces hybrid early/late endosomes and alters amino acid–stimulated mTOR localization. (A) Control HeLa cells or cells transfected with Rab5CA were incubated in amino acid–free media for 50 min and then stimulated without or with 1× (final) complete amino acid solution for 10 min. Cells were fixed and immunostained for endogenous mTOR and LAMP-2 as indicated and analyzed by laser scanning confocal microscopy. Scale bars, 5 μm. (B) Control HeLa cells or cells transfected with Rab5CA were treated as in A, but were immunostained for endogenous EEA1 and mTOR. Scale bars, 5 μm. (C) HeLa cells transfected with either empty vector or Rab5CA were fixed and immunostained for Rab5, LAMP-2, and EEA1 as indicated and analyzed by laser scanning confocal microscopy. Scale bars, 5 μm. (D) Control HeLa cells or cells transfected with Rab5CA were treated as in A except that cells were immunostained for EEA1, LAMP-2, and mTOR as indicated and analyzed by laser scanning confocal microscopy. Color merges between mTOR and EEA1 and mTOR and LAMP-2 are displayed. Scale bars, 5 μm.

smaller than those produced by Rab5CA but still showed colocalization of EEA1 and LAMP-2 (Figure 4A). When HeLa cells were serum-starved, stimulated with insulin, and immunostained for phosphorylated ribosomal S6, the hVps39 knockdown caused a near complete inhibition of insulin-stimulated S6 phosphorylation (Figure 4, B and C). Similarly, insulin-stimulated phosphorylation of S6K1 at T389 was significantly inhibited in the hVps39 knockdown cells (Figure 4, D and E). Similar to overexpression of Rab5CA, knockdown of hVps39 also led to localization of mTOR at hybrid endosomes (Figure 4F). Thus, blocking early/late endosome conversion by knockdown of hVps39 inhibits mTORC1/S6K1 signaling, suggesting that late endosomes are specifically required for mTORC1 signaling.

Dominant Negative Mutants of Rab7 and SKD1 Do Not Disrupt Early/Late Endosomal Conversion or mTOR Targeting

Given the requirement for a normal late endosomal compartment in mTORC1 signaling, it was surprising that mutants of Rab7 and SKD1, which disrupt trafficking through the late endosomal compartment (Fujita *et al.*, 2003; Vanlandingham and Ceresa, 2009), do not inhibit mTORC1. However, we find that mTORC1 localization to late endosomes in unaffected by SKD1-EQ or Rab7DN and that these mutants do not cause early/late endosomal mixing (Supplemental Figure 5). This suggests that the composition of the late endosomal compartment, rather than its sorting functions, is required for mTORC1 signaling. This would be consistent with the hypothesis that the late endosome serves as a scaffold for mTORC1 signaling.

DISCUSSION

In this study, we have characterized the role of endocytic trafficking in mTORC1 signaling and described how blocking a particular endocytic step, the early/late endosomal transition, affects the amino acid–sensing branch of mTORC1 signaling. When early/late endosome conversion is blocked by either overexpression of Rab5CA or knockdown of hVps39, hybrid endosomes are formed that contain markers for both early and late endosomes. mTOR localizes to these hybrid endosomes under amino acid–replete conditions, but does not signal to downstream effectors. Because overexpression of Rheb, but not hyperactivation of endogenous Rheb, can rescue the inhibition of mTORC1 by Rab5CA, we infer that the local-

ization of mTOR to these hybrid endosomes decreases the interaction of mTOR with endogenous Rheb (Figure 5).

There is strong evidence implicating an evolutionarily conserved role for endocytic trafficking in TOR signaling. Work in *D. melanogaster* has linked dTOR to the endocytic system, revealing both general and specific roles for dTOR in endocytosis (Hennig *et al.*, 2006). In mammalian cells, the Rag GTPases have been implicated in the amino acid input to mTOR signaling (Kim *et al.*, 2008; Sancak *et al.*, 2008). In yeast, the Rag homologues Gtr1p and Gtr2p are required for the nutrient-driven movement of amino acid permeases from endosomes to the plasma membrane (Gao and Kaiser, 2006). Furthermore, a vacuole-associated complex containing Grt2p, Ego1p, and Ego3p are required for reinitiation of proliferation after rapamycin-induced senescence (Dubouloz *et al.*, 2005). A subsequent study linked the yeast HOPS complex to this same process (Zurita-Martinez *et al.*, 2007). Binda *et al.* (2009) have recently published that in yeast, Vps39 acts as a Rag GEF whose activity contributes to TOR activation. We cannot rule out such a role for mammalian hVps39. However, if hVps39 knockdown led to a loss of Rag activation, then we would have expected to see a disruption of mTOR localization. Instead, we still observe mTOR localization to the hybrid early/late endosomes in hVps39 knockdown cells, but mTOR does not signal to S6K1. Given that Rag is required for mTOR endosomal localization (Sancak *et al.*, 2008), this would suggest that Rag GTP loading is preserved in hVps39 knockdown cells.

Rab5 is a pivotal player in early/late endosomal conversion. Rab5 cycles between the cytosol and early endosomes, with GDP-bound Rab5 found mostly in the cytosol and GTP-bound Rab5 found predominantly on early endosomal membranes (Stenmark *et al.*, 1994; Ullrich *et al.*, 1994). GTP-bound Rab5 recruits numerous effector proteins that serve multiple functions in endocytic trafficking, including EEA1, which is required for homotypic early endosome fusion, and the class C Vps/HOPS complex, which recruits and activates Rab7 (Wurmser *et al.*, 2000; Rink *et al.*, 2005). The distinct identity and function of early versus late endosomes is in part defined by whether they contain Rab5 or Rab7 on their membranes. Thus, a key step during early/late endosomal conversion is both the recruitment of Rab7 and the extraction of Rab5 from the early endosomal membrane. Rab5CA apparently blocks early/late endosome conversion through the failure of the constitutively GTP-bound Rab5 to be extracted from the early endosomal membrane (Rink *et al.*, 2005). Knockdown of hVps39, a member of the HOPS complex and a GEF for Rab7, also leads to a block in early/late endosomal conversion by inhibiting the recruitment and activation of Rab7. Zerial and coworkers (Rink *et al.*, 2005) speculate that during early to late endosomal conversion, a negative feedback loop takes place, wherein recruitment and GTP-loading of Rab7 by hVps39 leads to decreased Rab5 activation, most likely through a Rab7 effector protein with GAP activity toward Rab5. This deactivation of Rab5 allows the subsequent extraction of Rab5 from endosomal membranes.

Although the precise mechanism by which the early/late endosomal conversion facilitates amino acid–stimulated mTORC1 signaling remains unclear, we suggest that blocking endosomal conversion decreases the ability of Rheb to interact with mTOR. Endogenous Rheb may be localized to late endosomes, whereas overexpression of Rheb leads to its mislocalization to other compartments (Sancak *et al.*, 2008). This explains why overexpressed Rheb activates mTOR in the absence of amino acids; presumably the Rheb-mTOR interaction is driven by mass action in this case, and no longer requires the amino acid–stimulated movement of

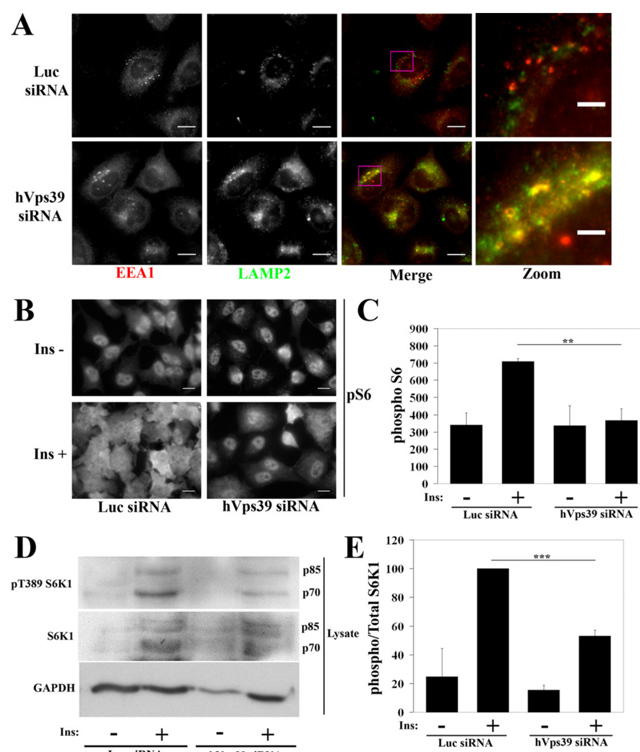

Figure 4. Knockdown of hVps39 causes early/late endosomal mixing and inhibits insulin-stimulated phosphorylation of S6K1 and S6. (A) HeLa cells were transfected with either luciferase or hVps39 siRNA oligonucleotides and grown for 72 h before fixation. Cells were immunostained for endogenous LAMP-2 and EEA1 as indicated. Scale bars, 20 μm; scale bars in the high-magnification ROIs (Zoom) 5 μm. (B) HeLa cells transfected and grown as above were serum-starved and incubated without or with 1 μM human insulin for 30 min before fixation and immunostaining for phospho-S6 (pS235/236/240/244/247). Scale bars, 20 μm. (C) Total phospho-S6 fluorescence intensity was measured in 50 cells per condition in two separate experiments and is displayed as the mean ± SD. $^{**}p < 0.01$. (D) Luciferase or hVps39 siRNA-treated HeLa cells treated as above were lysed in SDS sample buffer, and whole cell lysates for each condition were blotted for pT389 S6K1, total S6K1, and GAPDH as indicated. (E) The graph shows quantification of the ratio of phospho/total S6K1, normalized to the ratio in luciferase controls. The data are the mean ± SD from two experiments. $^{***}p < 0.005$. (F) Luciferase or hVps39 siRNA-treated HeLa cells were incubated in amino acid–free media for 50 min then stimulated without or with 1× (final) complete amino acid solution for 10 min. Cells were fixed and immunostained for endogenous mTOR, EEA1, and LAMP-2 as indicated and analyzed by laser scanning confocal microscopy. Color merges between mTOR and EEA1 and mTOR and LAMP-2 are shown. Scale bars, 10 μm.

mTOR to late endosomes. Our Rheb overexpression data are consistent with this hypothesis, because mTOR signaling in Rab5CA-expressing cells is not rescued by constitutive hyperactivation of endogenous Rheb in TSC2−/− MEFs, but is rescued by overexpression of Rheb. These data suggest that overexpressed Rheb can interact with mTOR in the hybrid endosomes formed in Rab5CA-expressing cells, whereas endogenous Rheb cannot. Unfortunately, attempts to measure the binding of endogenous mTOR and Rheb has been unsuccessful even in control cells, and the localization of endogenous Rheb by fractionation or immunofluorescence has not yet been achieved, as has been described elsewhere (Buerger *et al.*, 2006; Sancak *et al.*, 2008). During the preparation of this article, a study describing the localization of

F

mTOR

EEA1

LAMP2

mTOR
EEA1

mTOR
LAMP2

AA: - + - +

Luc siRNA hVps39 siRNA

Figure 4. (**continued**).

GFP-Rheb to the endoplasmic reticulum (ER) and Golgi was published (Hanker *et al.*, 2009). However, overexpression of Rheb activates mTORC1 signaling even in the absence of amino acid–stimulated mTORC1 translocation (Sancak *et al.*, 2008), suggesting that the localization of GFP-Rheb may not mimic that of endogenous Rheb.

Our results indicate that some distinct characteristic of late endosomes is vital for mTORC1 signaling, presumably

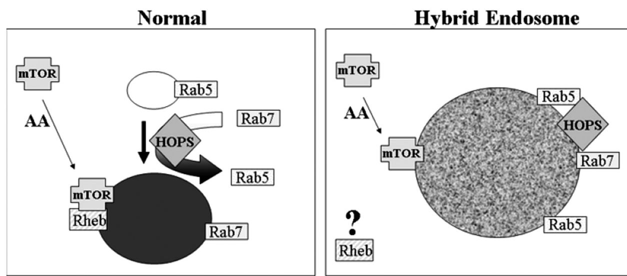

Figure 5. Model of endosome conversion and mTORC1 signaling. The model shows a normal early-to-late endosome conversion (left) and a mixed early/late endocytic compartment (right). In order for mTOR to be properly activated in the late endocytic compartment in control cells (left), early endosomes must first undergo endosome conversion through the normal cycling of Rab5 and Rab7 and through the action of the HOPS complex, which contains hVps39. Amino acids stimulate the localization of mTOR to late endosomes, where it is proposed to interact with Rheb (Sancak *et al.*, 2008). If early/late endosome conversion is blocked (right), either by overexpression of Rab5CA or knockdown of hVps39, then amino acids stimulate the localization of mTOR to a mixed endocytic compartment that does not contain Rheb. Under these conditions, mTORC1 is not activated.

related to the targeting of Rheb. One key distinction between early and late endosomes is their luminal pH, with endosomes maintaining a lower intraluminal pH than early endosomes (Murphy *et al.*, 1984; Yamashiro and Maxfield, 1987a,b). Changes in intraluminal pH can affect the recruitment and activation of membrane associated GTPases. For example, endosomal recruitment of the small GTPase Arf6 and its GEF, ARNO, is blocked by inhibition of the vacuolar ATPase and subsequent increase in intraluminal pH (Maranda *et al.*, 2001). Similar results have been observed for βCOP and Arf1 binding to early endosomes (Gu and Gruenberg, 2000). Another distinction between early and late endosomes is their lipid compositions. Late but not early endosomes contain the lipid lysobisphosphatidic acid (LBPA; Matsuo *et al.*, 2004). LBPA helps regulate late endosomal cholesterol levels through an associated protein Alix (Chevallier *et al.*, 2008). Late endosomal cholesterol levels are lower than in the plasma membrane or recycling compartment but higher than in the ER (Mesmin and Maxfield, 2009). GTPase association with distinct membranes depends in part on the lipid composition of the target membrane (ten Klooster and Hordijk, 2007). Future work will address whether mTORC1 requires the distinct intraluminal pH and lipid composition of late endosomes for productive interactions with Rheb and for signaling to downstream effectors.

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