Cell

Ragulator-Rag Complex Targets mTORC1 to the Lysosomal Surface and Is Necessary for Its Activation by Amino Acids

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DOI 10.1016/j.cell.2010.02.024

SUMMARY

The mTORC1 kinase promotes growth in response to growth factors, energy levels, and amino acids, and its activity is often deregulated in disease. The Rag GTPases interact with mTORC1 and are proposed to activate it in response to amino acids by promoting mTORC1 translocation to a membrane-bound compartment that contains the mTORC1 activator, Rheb. We show that amino acids induce the movement of mTORC1 to lysosomal membranes, where the Rag proteins reside. A complex encoded by the *MAPKSP1*, *ROBLD3*, and *c11orf59* genes, which we term Ragulator, interacts with the Rag GTPases, recruits them to lysosomes, and is essential for mTORC1 activation. Constitutive targeting of mTORC1 to the lysosomal surface is sufficient to render the mTORC1 pathway amino acid insensitive and independent of Rag and Ragulator, but not Rheb, function. Thus, Rag-Ragulator-mediated translocation of mTORC1 to lysosomal membranes is the key event in amino acid signaling to mTORC1.

INTRODUCTION

The multicomponent kinase mTORC1 (mammalian target of rapamycin complex 1) regulates cell growth by coordinating upstream signals from growth factors, intracellular energy levels, and amino acid availability and is deregulated in diseases such as cancer and diabetes (reviewed in Guertin and Sabatini, 2007). The TSC1 and TSC2 proteins form a tumor suppressor complex that transmits growth factor and energy signals to mTORC1 by regulating the GTP-loading state of Rheb, a Ras-related GTP-binding protein. When bound to GTP, Rheb interacts with and activates mTORC1 (reviewed in Laplante and Sabatini, 2009) and appears to be necessary for the activation of mTORC1 by all signals, including amino acid availability. In contrast, TSC1-TSC2 is dispensable for the regulation of mTORC1 by amino acids, and, in cells lacking TSC2, the mTORC1 pathway is sensitive to amino acid starvation but resistant to growth factor withdrawal (Roccio et al., 2006; Smith et al., 2005).

Recently, the Rag GTPases, which are also members of the Ras family of GTP-binding proteins, were shown to be amino acid-specific regulators of the mTORC1 pathway (Kim et al., 2008; Sancak et al., 2008). Mammals express four Rag proteins—RagA, RagB, RagC, and RagD—that form heterodimers consisting of RagA or RagB with RagC or RagD. RagA and RagB, like RagC and RagD, are highly similar to each other and are functionally redundant (Hirose et al., 1998; Sancak et al., 2008; Schürmann et al., 1995; Sekiguchi et al., 2001). Rag heterodimers containing GTP-bound RagB interact with mTORC1, and amino acids induce the mTORC1-Rag interaction by promoting the loading of RagB with GTP, which enables it to directly interact with the raptor component of mTORC1 (Sancak et al., 2008). The activation of the mTORC1 pathway by amino acids correlates with the movement of mTORC1 from an undefined location to a compartment containing Rab7 (Sancak et al., 2008), a marker of both late endosomes and lysosomes (Chavrier et al., 1990; Luzio et al., 2007). How the Rag proteins regulate mTORC1 is unknown, but, in cells expressing a RagB mutant that is constitutively bound to GTP ($RagB^{GTP}$), the mTORC1 pathway is insensitive to amino acid starvation and mTORC1 resides in the Rab7-positive compartment even in the absence of amino acids (Sancak et al., 2008). We previously proposed that amino acids promote the translocation of mTORC1—in a Rag-dependent fashion—to the surface of an endomembrane compartment, where mTORC1 can find its well-known activator, Rheb. Here, we show that the lysosomal surface is the compartment where the Rag proteins reside and to which mTORC1 moves in response to amino acids. We identify the trimeric Ragulator protein complex as a new component of the mTORC1 pathway that interacts with the Rag GTPases, is

essential for localizing them and mTORC1 to the lysosomal surface, and is necessary for the activation of the mTORC1 pathway by amino acids. In addition, by expressing in cells a modified raptor protein that targets mTORC1 to the lysosomal surface, we provide evidence that supports our model of mTORC1 pathway activation by amino acids.

RESULTS

Amino Acids Cause the Translocation of mTORC1 to Lysosomal Membranes, Where the Rag GTPases Are Already Present

To better define the compartment to which mTORC1 moves upon amino acid stimulation, we costained human cells with antibodies to endogenous mTOR, raptor, or RagC, as well as to various endomembrane markers (data not shown). This revealed that in the presence, but not in the absence, of amino acids, mTOR and raptor colocalized with LAMP2 (Figures 1A and 1B), a well-characterized lysosomal marker (reviewed in Eskelinen, 2006). Amino acid stimulation also resulted in an appreciable increase in the average size of lysosomes, which, as determined by live-cell imaging, was most likely caused by lysosome-lysosome fusion (R.Z., unpublished data). The amino acid-induced movement of mTOR to the LAMP2-positive compartment depends on the Rag GTPases, as it was eliminated by the RNA interference (RNAi)-mediated coknockdown of RagA and RagB (Figures S1A and S1B available online). Endogenous RagC also colocalized extensively with LAMP2, but, unlike mTORC1, this colocalization was unaffected by amino acid availability (Figure 1C). Consistent with amino acids not regulating the interaction between RagC and RagA or RagB (Figure 1D), an antibody that recognizes RagA and RagB stained lysosomes in both amino acid-starved and replete cells (Figure 1E). Lastly, GFP-tagged wild-type and GTP-bound mutants of RagB ($RagB^{GTP}$) and RagD ($RagD^{GTP}$) behaved identically to their endogenous counterparts (Figures 1F and 1G). Thus, amino acids stimulate the translocation of mTORC1 to the lysosomal surface, where the Rag GTPases reside irrespective of their GTP-loaded states or amino acid availability. Given that mTORC1 interacts with the Rag heterodimers in an amino acid-dependent fashion (Sancak et al., 2008), the mTORC1 and Rag localization data are consistent with the Rag GTPases serving as an amino acid-regulated docking site for mTORC1 on lysosomes.

The Translocation of mTORC1 to Lysosomes Does Not Depend on Growth Factors, Rheb, or mTORC1 Activity

The movement of mTORC1 to lysosomes is a specific response to amino acids. In wild-type mouse embryonic fibroblasts (MEFs), amino acids promoted the translocation of mTORC1 to lysosomes even when cells were cultured in the absence of serum (Figure S1C), a condition in which mTORC1 signaling, as detected by phosphorylated S6K1, is not active (Figure S1D). Conversely, in the absence of amino acids, neither serum stimulation nor constitutive activation of Rheb caused by the loss of TSC2 led to the lysosomal translocation of mTORC1 (Figure S1C). In both wild-type and TSC2 null MEFs, RNAi-mediated suppression of Rheb1 expression inhibited mTORC1 activation by amino acids (Figure S1E) but did not interfere with the amino acid-induced movement of mTOR to lysosomes (Figure S1F). Thus, the amino acid-induced translocation of mTORC1 to the lysosomal surface occurs independently of mTORC1 activity and does not require TSC2, Rheb, or growth factors.

The Trimeric Ragulator Complex Interacts with the Rag GTPases and Colocalizes with Them on Lysosomal Membranes

Inspection of the amino acid sequence of the Rag GTPases did not reveal any obvious lipid modification signals that might mediate Rag recruitment to lysosomal membranes. Thus, we pursued the possibility that unknown Rag-interacting proteins are needed to localize the Rag GTPases to lysosomes and play a role in mTORC1 signaling. To identify such proteins, we used protein purification approaches that have led to the discovery of other mTOR pathway components (see the Extended Experimental Procedures). Mass spectrometric analysis of anti-FLAG immunoprecipitates prepared from human HEK293T cells stably expressing FLAG-RagB or FLAG-RagD, but not FLAG-Rap2a, consistently revealed the presence of proteins encoded by the *MAPKSP1*, *ROBLD3*, and *c11orf59* genes (Figure 2A). Furthermore, the same proteins were also detected in immunoprecipitates of endogenous RagC but not control proteins like p53 or tubulin. Previous work indicates that these three small proteins interact with each other, localize to endosomes and lysosomes, and play positive roles in the MAPK pathway (Lunin et al., 2004; Nada et al., 2009; Schaeffer et al., 1998; Teis et al., 2002, 2006; Wunderlich et al., 2001). The proteins encoded by *MAPKSP1*, *ROBLD3*, and *c11orf59* have been called MP1, p14, and p18, respectively, and we use these names throughout this study. For convenience and because MP1, p14, and p18 are Rag and mTORC1 regulators (see below), we refer to the trimeric complex as the "Ragulator."

Orthologs of MP1, p14, and p18 are readily detectable in vertebrates as well as in *Drosophila* (Figure 2A), but extensive database searches did not reveal any potential orthologs in budding or fission yeast. The amino acid sequences of MP1, p14, and p18 reveal little about their function, and other than p14, which has a roadblock domain of unknown function (Koonin and Aravind, 2000), the proteins do not share sequence homology among themselves or with any other proteins in the databases besides their direct orthologs. In particular, they do not share any sequence similarity with the Ego1p or Ego3p, proteins, which interact with Gtr1p and Gtr2p (Dubouloz et al., 2005; Gao and Kaiser, 2006), the orthologs of the Rag proteins in budding yeast (Gao and Kaiser, 2006; Schürmann et al., 1995). The lysosomal localization of p18 requires its lipidation through N-terminal myristoylation and palmitoylation sites, and p18 likely serves as a platform for keeping MP1 and p14 on the lysosomal surface (Nada et al., 2009).

In humans, a mutation that leads to a partial reduction in the expression of p14 causes a pronounced growth defect so that individuals carrying the mutation are below the third percentile in age-adjusted height (Bohn et al., 2007). Furthermore, mice engineered to lack either p14 or p18 die around embryonic day 7–8 and exhibit severe growth retardation (Nada et al., 2009; Teis et al., 2006). Given the major role of the mTORC1

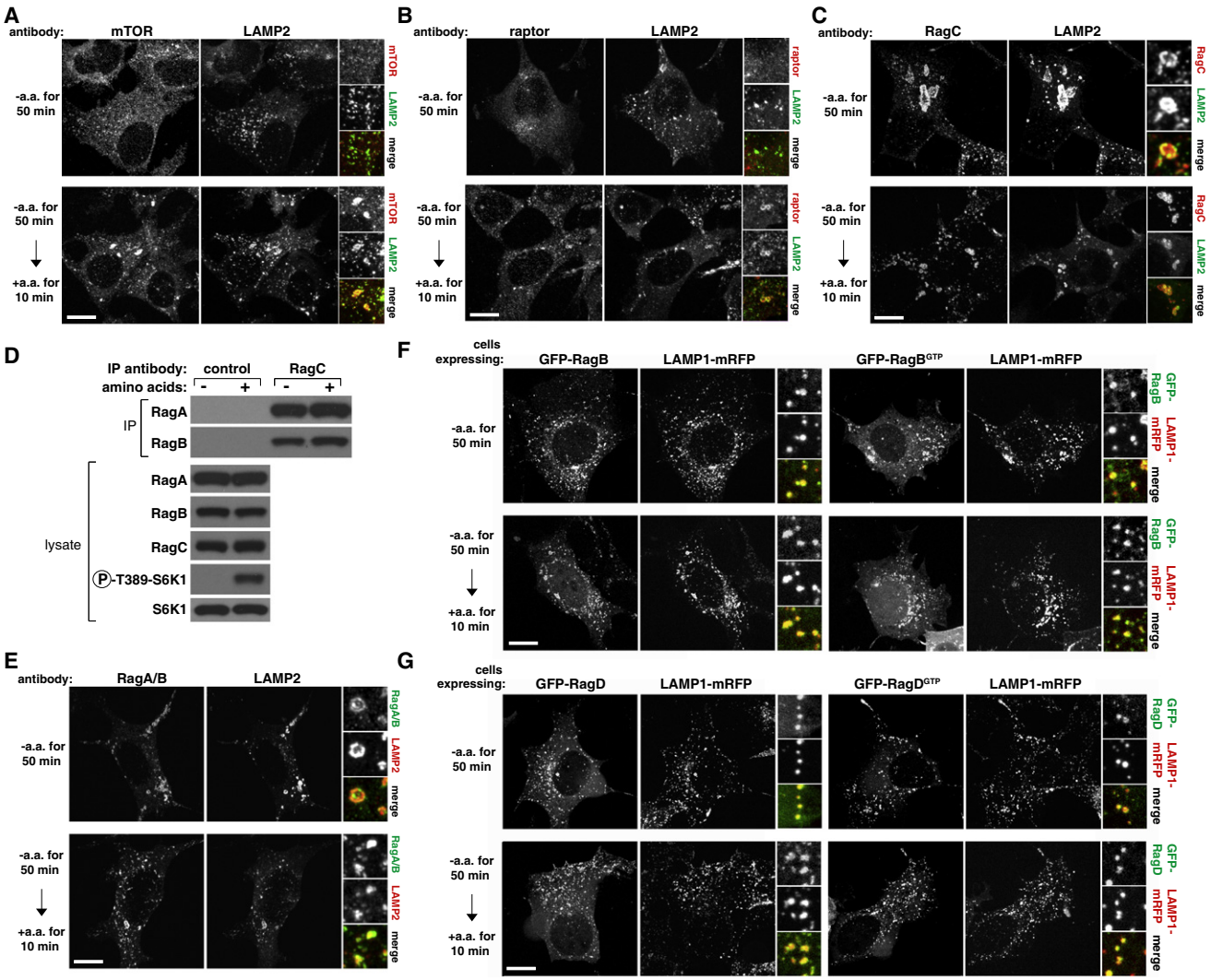

Figure 1. mTORC1 Localizes to Lysosomal Membranes in an Amino Acid-Dependent Fashion while the Rag GTPases Are Constitutively Localized to the Same Compartment

(A) Images of HEK293T cells coimmunostained for lysosomal protein LAMP2 (green) and mTOR (red). Cells were starved of and restimulated with amino acids for the indicated times before processing and imaging.

(B) Images of HEK293T cells coimmunostained for LAMP2 (green) and raptor (red). Cells were treated and processed as in (A).

(C) Images of HEK293T cells coimmunostained for LAMP2 (green) and RagC (red). Cells were treated and processed as in (A).

(D) RagC interacts with RagA and RagB independently of amino acid availability. RagC immunoprecipitates were prepared from HEK293T cells starved or stimulated with amino acids as in (A), and immunoprecipitates and lysates were analyzed by immunoblotting for the indicated proteins.

(E) Images of HEK293T cells coimmunostained for RagA/B (green) and LAMP2 (red). Cells were treated, processed, and imaged as in (A).

(F) GFP-RagB and GFP-RagB^{GTP} colocalize with coexpressed LAMP1-mRFP independently of amino acid availability. HEK293T cells transfected with the indicated cDNAs were treated and processed as in (A).

(G) GFP-RagD and GFP-RagD^{GTP} colocalize with coexpressed LAMP1-mRFP independently of amino acid availability. HEK293T cells transfected with the indicated cDNAs were treated and processed as in (A).

In all images, insets show selected fields that were magnified five times and their overlays. Scale bars represent 10 μm. See also Figure S1.

pathway in growth control, these loss-of-function phenotypes were of interest to us.

As an initial step in verifying our mass spectrometric identification of MP1, p14, and p18 as Rag-interacting proteins, we coexpressed them along with RagB and RagD in HEK293T cells and found that the Ragulator, but not the control Rap2A protein, coimmunoprecipitated both Rag GTPases but not the metap2 protein that has the same molecular weight as tagged RagB (Figure 2B). Furthermore, when coexpressed with a RagB mutant (RagB^{GTP}) that binds constitutively to GTP, the Ragulator coimmunoprecipitated the mTORC1 components raptor and mTOR (Figure 2C), consistent with the GTP-loading of RagB promoting the interaction of the Rag heterodimers with mTORC1 (Sancak et al., 2008). Furthermore, endogenous RagA, RagB, and RagC

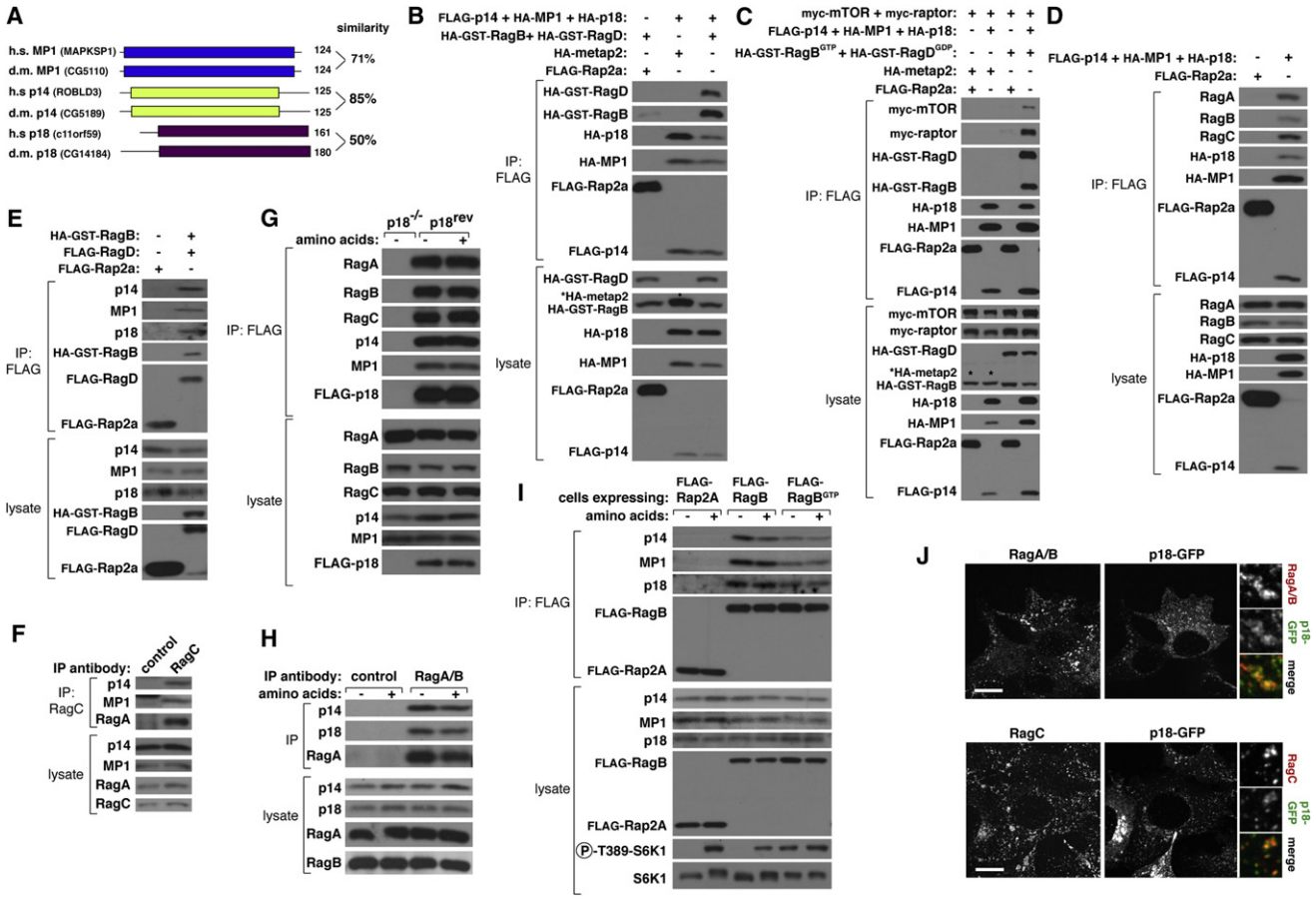

Figure 2. The Trimeric Ragulator Complex Interacts and Colocalizes with the Rag GTPases

(A) Schematic amino acid sequence alignment of human MP1, p14, and p18 and their corresponding *Drosophila* orthologs.

(B) Recombinant epitope-tagged Ragulator coimmunoprecipitates recombinant RagB and RagD. Anti-FLAG immunoprecipitates were prepared from HEK293T cells cotransfected with the indicated cDNAs in expression vectors and cell lysates and immunoprecipitates analyzed by immunoblotting for levels of indicated proteins. The * indicates the band corresponding to the metap2 protein as it has the same apparent molecular weight as HA-GST-RagB.

(C) Recombinant Ragulator coimmunoprecipitates mTORC1 when it is coexpressed with the GTP-bound mutant of RagB. HEK293T cells were cotransfected with the indicated cDNAs in expression vectors and analyzed as in (B). The * indicates the bands corresponding to metap2 as it has the same apparent molecular weight as HA-GST-RagB.

(D) Recombinant Ragulator co-immunoprecipitates endogenous RagA, RagB, and RagC. HEK293T cells were cotransfected with indicated cDNAs in expression vectors and anti-FLAG immunoprecipitates analyzed as in (B).

(E) Recombinant RagB-RagD heterodimers coimmunoprecipitate endogenous p14, MP1, and p18. HEK293T cells were cotransfected with indicated cDNAs in expression vectors and anti-FLAG immunoprecipitates analyzed as in (B).

(F) Endogenous RagC coimmunoprecipitates endogenous p14 and MP1. Anti-RagC immunoprecipitates were prepared from HEK293T cells and analyzed for the levels of the indicated proteins.

(G) Amino acids do not regulate the amounts of endogenous MP1, p14, RagA, or RagB that coimmunoprecipitate with recombinant p18. p18 null cells ($p18^{-/-}$) or p18 null cells stably expressing FLAG-p18 ($p18^{rev}$) were starved for amino acids for 50 min or starved and restimulated with amino acids for 10 min. After in-cell crosslinking, anti-FLAG immunoprecipitates were prepared from cell lysates and analyzed for the levels of the indicated proteins by immunoblotting.

(H) Amino acids do not affect the amounts of endogenous p14 and p18 that coimmunoprecipitate with endogenous RagA/B. HEK293T cells were treated as in (G), and anti-RagA/B immunoprecipitates were analyzed by immunoblotting for the indicated proteins.

(I) Endogenous Ragulator coimmunoprecipitates with FLAG-RagB independently of amino acid availability and GTP-loading of RagB. HEK293T cells stably expressing FLAG-RagB or FLAG-$RagB^{GTP}$ were starved and restimulated with amino acids as in (G), and anti-FLAG immunoprecipitates were analyzed for the levels of indicated proteins.

(J) The Rag GTPases colocalize with GFP-tagged p18. HEK293T cells were transfected with a cDNA encoding p18-GFP, processed for immunostaining for endogenous RagA/B or RagC, and imaged for the RagA/B (red) or RagC (red) signal as well as for p18-GFP fluorescence (green). Note that not all cells express p18-GFP. In all images, insets show selected fields that were magnified five times and their overlays. Scale bars represent 10 μm.

See also Figure S2.

copurified with recombinant Ragulator (Figure 2D), and endogenous Ragulator components copurified with the recombinant RagB-RagD heterodimer (Figure 2E). Lastly, endogenous p14 and MP1 were present in immunoprecipitates prepared with an antibody directed against endogenous RagC that readily coimmunoprecipitates RagA (Figure 2F).

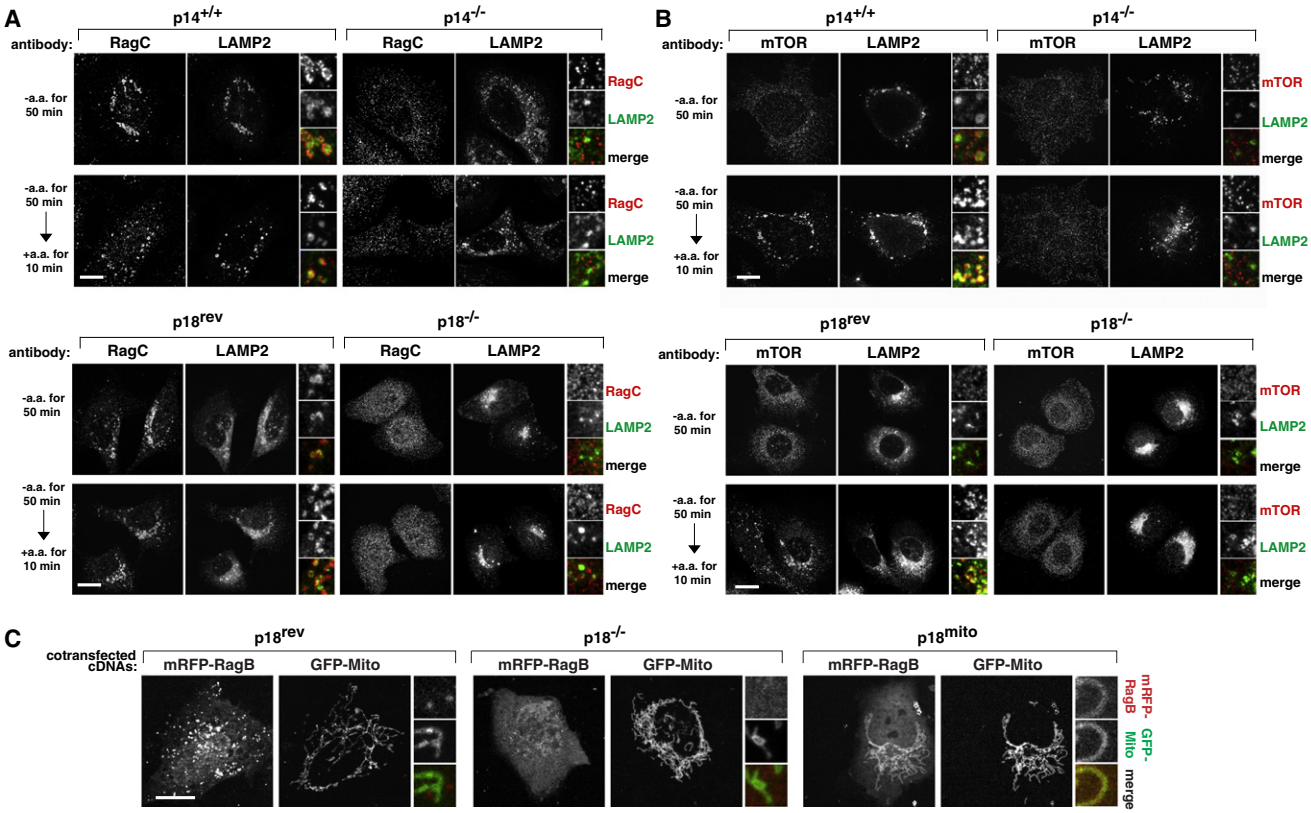

Figure 3. The Ragulator Is Necessary to Localize the Rag GTPases and mTORC1 to Lysosomal Membranes

(A) Images of p14 null or p18 null cells or their respective controls coimmunostained for RagC (red) and LAMP2 (green). Cells were starved of and restimulated with amino acids for the indicated times before processing for the immunofluorescence assay and imaging.

(B) Images of p14 null or p18 null cells or their respective controls coimmunostained for mTOR (red) and LAMP2 (green). Cells were treated and processed as in (A).

(C) Colocalization of mRFP-RagB (red) with GFP-Mito (green) in cells expressing mitochondrially localized p18. p18 null cells ($p18^{-/-}$), or p18 null cells expressing wild-type p18 ($p18^{rev}$) or mitochondrially localized p18 ($p18^{mito}$), were transiently transfected with the indicated cDNAs in expression plasmids and imaged.

In all images, insets show selected fields that were magnified five times and their overlays. Scale bars represent 10 μm. See also Figure S3.

Amino acids did not appreciably regulate the interaction of recombinant p18 with endogenous p14, MP1, or the Rag GTPases (Figure 2G). Similarly, amino acids did not affect the interaction of endogenous Ragulator with endogenous Rag A/B (Figure 2H). The amounts of p14, p18, and MP1 that coimmunoprecipitated with the GTP-bound RagB mutant ($RagB^{GTP}$) were slightly less than with wild-type RagB (Figure 2I). Because mTORC1 pathway activity is high in cells expressing $RagB^{GTP}$ (Sancak et al., 2008), the reduced Ragulator-Rag interaction in these cells may reflect a compensatory mechanism to reduce mTORC1 activity. To test whether the Rag GTPases interact with one or more Ragulator components directly, we performed in vitro binding assays between purified RagB-RagD heterodimers and individual Ragulator proteins. p18 interacted with RagB-RagD in vitro, but not with the Rap2a control protein (Figure S2A). In contrast, we did not detect a direct interaction between either p14 or MP1 and the Rag GTPases (data not shown), suggesting that p18 is the principal Rag-binding subunit of the Ragulator. Lastly, within HEK293T cells, GFP-tagged p18 colocalized with endogenous RagA/B and RagC (Figure 2J). Collectively, these results show that the Ragulator interacts with the Rag GTPases and that a supercomplex consisting of Ragulator, a Rag heterodimer, and mTORC1 can exist within cells.

Ragulator Localizes the Rag Proteins to the Lysosomal Surface and Is Necessary for the Amino Acid-Dependent Recruitment of mTORC1

Because the Rag GTPases interact with Ragulator and given the function of p18 in localizing MP1 and p14 to lysosomes (Nada et al., 2009), it seemed possible that the Ragulator is necessary for localizing the Rag proteins to the lysosomal surface. Indeed, in cells lacking p14 or p18 (Nada et al., 2009; Teis et al., 2006), endogenous RagC was localized in small puncta throughout the cytoplasm of the cells rather than to lysosomes (Figure 3A), the morphology of which was not obviously affected by the loss of either protein. In contrast, in $p14^{+/+}$ cells or p18 null cells reconstituted with wild-type p18 ($p18^{rev}$), RagC constitutively colocalized with the LAMP2 lysosomal marker (Figure 3A). Analogous results were obtained in HEK293T cells with an RNAi-mediated reduction in MP1 expression (Figure S3A). Consistent

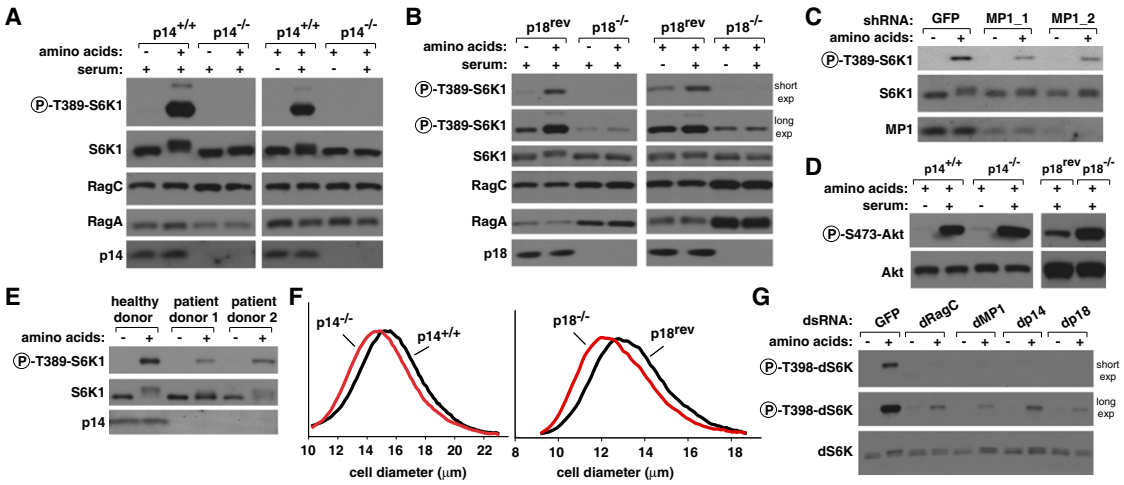

Figure 4. Ragulator Null and -Depleted Cells are Highly Deficient in the Activation of mTORC1 Signaling by Amino Acids

(A) p14 is necessary for the activation of the mTORC1 pathway by amino acids and serum. p14 null or control cells were starved of amino acids or serum for 50 min, or starved and restimulated with amino acids or serum for 10 min. Immunoblot analyses were used to measure the levels of the indicated proteins and phosphorylation states.

(B) p18 is necessary for the activation of the mTORC1 pathway by amino acids and serum. p18 null or control cells were treated and analyzed as in (A).

(C) Partial knockdown of MP1 blunts mTORC1 pathway activation by amino acids. HEK293T cells expressing a control shRNA or two distinct shRNAs targeting MP1 were starved for amino acids for 50 min, or starved and stimulated with amino acids for 10 min and analyzed as in (A).

(D) p14 and p18 are not necessary for mTORC2 pathway activity. p14 null or control cells were starved for serum, or starved and then restimulated with serum as in (A). p18 null or control cells were grown in complete media. Cell lysates were prepared and analyzed by immunobloting for the levels of Akt1 and Akt phosphorylation at the S473 site phosphorylated by mTORC2.

(E) Decreased p14 expression impairs amino acid-induced mTORC1 activation in human cells. Cells derived from patients with lower p14 expression or healthy individuals were treated and analyzed as in (A).

(F) Cells lacking Ragulator are smaller than control cells. Cell size distributions of p14 null or p18 null cells are overlaid with those from corresponding control cells.

(G) Ragulator function is conserved in *Drosophila* cells. *Drosophila* S2 cells were transfected with a control dsRNA, or dsRNAs targeting dRagC, dMP1, dp14, or dp18, starved of amino acids for 90 min, or starved and restimulated with amino acids for 30 min. Levels of indicated proteins and phosphorylation states were analyzed by immunobloting.

See also Figure S4.

with the essential role of the Rag proteins in the translocation of mTORC1 to the lysosomal surface (Figure S1), in cells lacking p14 or p18 or in HEK293T cells with p14, p18, or MP1 knockdowns, amino acids failed to induce lysosomal recruitment of mTOR, which was found throughout the cytoplasm in both amino acid-starved and -stimulated cells (Figure 3B, Figures S3B and S3D). Thus, all Ragulator subunits are required for lysosomal targeting of the Rag GTPases and mTORC1.

To determine whether Ragulator is sufficient to control the intracellular localization of the Rag proteins, it was necessary to target Ragulator to a location that is distinct from the lysosomal surface. As p18 binds both p14 and MP1 and is necessary for targeting them to the lysosomal surface (Nada et al., 2009), we chose to manipulate the intracellular localization of p18. To accomplish this, we generated a variant of p18, called $p18^{mito}$, which lacks its N-terminal lipidation sites but is fused at its C terminus to the transmembrane region of OMP25, which is sufficient to target heterologous proteins to the mitochondrial surface (Nemoto and De Camilli, 1999). When expressed in p18 null cells, $p18^{mito}$ was associated with mitochondria, as verified by colocalization with the established mitochondrial protein Cytochrome c (Figure S3E). Remarkably, in the p18 null cells expressing $p18^{mito}$, RFP-tagged RagB colocalized with the mitochondrial marker GFP-mito (Figure 3C). In contrast, RFP-RagB did not colocalize with GFP-mito in p18 null cells ($p18^{-/-}$) or $p18^{rev}$ cells and instead was present in a cytoplasmic or lysosomal pattern, respectively (Figure 3C). In cells expressing $p18^{mito}$, mTORC1 activity remained very low and mTOR was not recruited to the mitochondria (Figures S3E and S3F), likely because the mitochondrial surface does not contain the machinery necessary to load the Rag GTPases with the appropriate nucleotides. These results indicate that the location of p18 is sufficient to define that of the Rag proteins and are consistent with Ragulator serving as a constitutive docking site on lysosomes for the Rag heterodimers, which, in amino acid-replete cells, have an analogous function for mTORC1.

Ragulator Is Necessary for TORC1 Activation by Amino Acids in Mammalian and *Drosophila* Cells

We employed the cells lacking p14 or p18 to determine whether Ragulator is necessary for mTORC1 activation by amino acids. Strikingly, in both p14 and p18 null cells, but not in control cells, amino acids were incapable of activating the mTORC1 pathway as detected by the phosphorylation of S6K1 (Figures 4A and 4B) and 4E-BP1 (Figure S4A). Similarly, cells derived from patients with a homozygous mutation in the p14 gene that causes a reduction in p14 expression (Bohn et al., 2007) showed a defect in amino acid-induced mTORC1 activation compared to cells

derived from a healthy donor (Figure 4E). In addition, autophagy, a process normally inhibited by mTORC1, was activated in p14 null cells, as detected by an increase compared to in control cells in the size and number of GFP-LC3-II puncta (Figures S4B and S4C). mTORC1 activity was also suppressed in HEK293T cells with RNAi-induced reductions in p14, p18, or MP1 levels (Figure 4C, Figure S3C). Consistent with the known requirement of amino acids and Rag function for growth factors to activate mTORC1 (Sancak et al., 2008), serum was also incapable of activating the mTORC1 pathway in cells null for p14 or p18 (Figures 4A and 4B). In contrast, no defect was observed in the level of S473 phosphorylation of Akt (Figure 4D). In fact, Akt phosphoylation was slightly higher in the p14 null and p18 null cells than in controls cells, which likely results from the lack of the well-appreciated inhibitory input from mTORC1 to the PI3K pathway in these cells (reviewed in Manning, 2004). As mTORC2 is the growth factor-regulated S473 kinase of Akt (Sarbassov et al., 2005), these results also indicate that the Ragulator does not play a detectable positive role in mTORC2 signaling. Interestingly, in the p18 null cells the expression of RagA and RagC was higher than in control cells (Figure 4B), suggesting that feedback signals in these cells may be trying to overcome the defect in mTORC1 activity by boosting Rag expression or that Ragulator also negatively controls Rag GTPase levels. Consistent with p18, p14, and MP1 forming a complex, the expression or stability of the Ragulator proteins seems to be coregulated because in cells that lack p14, p18 protein levels are also reduced, and, similarly, in cells that lack p18, p14 protein levels are also low (Figure S2B). A well-known function of the mTORC1 pathway is the positive regulation of cell growth, so that inhibition of the pathway leads to a reduction in cell size (reviewed in Laplante and Sabatini, 2009). Consistent with Ragulator being a positive component of the mTORC1 pathway, the p14 and p18 null cells were smaller in size than their respective controls (Figure 4F).

Many components of the TORC1 pathway, such as the Rag proteins, have conserved roles in mammalian and *Drosophila* cells (Kim et al., 2008; Sancak et al., 2008). RNAi-inducing double-stranded RNAs (dsRNAs) that target the *Drosophila* orthologs of MP1 (CG5110), p14 (CG5189), and p18 (CG14184) were as effective at blocking amino acid-stimulated activation of dTORC1 in *Drosophila* S2 cells as dsRNAs targeting dRagC (Figure 4G). Our loss-of-function experiments indicate that Ragulator is a component of the TORC1 pathway that, like the Rag GTPases, is essential for amino acids to activate TORC1 signaling in mammalian and *Drosophila* cells.

Forced Targeting of mTORC1 to the Lysosomal Surface Eliminates the Amino Acid Sensitivity of the mTORC1 Pathway

The findings we have presented so far are consistent with the amino acid-induced movement of mTORC1 to the lysosomal surface being necessary for the activation of mTORC1 by amino acids. To test whether the placement of mTORC1 on lysosomal membranes is sufficient to mimic the amino acid input to mTORC1, it was necessary to force mTORC1 onto these membranes in the absence of amino acids. To accomplish this, we expressed in HEK293T cells modified raptor proteins that consist of epitope-tagged raptor fused to the intracellular targeting signals of Rheb1 or Rap1b, small GTPases that localize, in part, to the lysosomal surface (Pizon et al., 1994; Saito et al., 2005; Sancak et al., 2008). Because the targeting signals of these proteins are in their C-terminal tails, we added the last 15 or 17 amino acids of Rheb1 or Rap1b, respectively, to the C terminus of raptor (Figure 5A). For simplicity, we refer to these fusion proteins as raptor-Rheb15 and raptor-Rap1b17. As a control, we generated a raptor fusion protein that lacks the CAAX box of the Rheb1 targeting signal (raptor-Rheb15ΔCAAX) and so cannot associate with membranes (Buerger et al., 2006; Clark et al., 1997; Takahashi et al., 2005).

When expressed in cells together with myc-mTOR, raptor-Rheb15 and raptor-Rap1b17 localized to lysosomes in the presence or absence of amino acids, as judged by costaining with LAMP2 (Figure 5B). In contrast, raptor-Rheb15ΔCAAX behaved like wild-type raptor and localized to lysosomes only upon amino acid stimulation (Figure 5B). In all cases, the localization of the coexpressed myc-mTOR mirrored that of the wild-type or altered forms of raptor, indicating that C-terminal modifications of raptor do not perturb its interaction with mTOR (Figure 5C), which was confirmed in coimmunoprecipitation experiments (Figure S5A).

Remarkably, transient expression of raptor-Rheb15 or raptor-Rap1b17 in HEK293T cells was sufficient to render the mTORC1 pathway, as judged by the phosphorylation of S6K1, resistant to amino acid starvation (Figure 6A). In contrast, the expression of wild-type raptor or raptor-Rheb15ΔCAAX did not affect the amino acid sensitivity of the pathway (Figure 6A). In HEK293E cells, the expression of raptor-Rheb15 made S6K1 phosphorylation insensitive to amino acid starvation but did not affect its regulation by insulin (Figure 6B). Thus, lysosomal targeting of mTORC1 can substitute for the amino acid, but not growth factor, input to mTORC1. This is consistent with previous work showing that growth factors signal to mTORC1 in large part through the TSC1-TSC2-Rheb axis (reviewed in Laplante and Sabatini, 2009), and not through the Rag GTPases (Sancak et al., 2008).

To verify the effects of lysosomally targeted mTORC1 in a more physiological setting than that achieved by transient complementary DNA (cDNA) expression, we generated HEK293T cell lines stably expressing FLAG-tagged raptor-Rheb15 or wild-type raptor. In cells expressing the lysosomally targeted but not wild-type raptor, mTOR was always associated with lysosomes, irrespective of amino acids (Figure 6C). As with the transient expression of raptor-Rheb15, its stable expression rendered the mTORC1 pathway fully resistant to amino acid starvation (Figure 6D). Furthermore, under normal growth conditions, these cells had an increase in mTORC1 activity and were larger than controls (Figure 6E).

We next examined whether the targeting of mTORC1 to membranes other than lysosomal membranes could also eliminate the amino acid sensitivity of the mTORC1 pathway. This was not the case because although the stable expression of a raptor variant consisting of raptor fused to the last 25 amino acids of H-Ras (raptor-HRas25) (Figure 5A, Figure S5B) was sufficient to target a fraction of cellular mTOR to the plasma membrane (Figure 6C), it did not render the mTORC1 pathway resistant to amino acid starvation (Figure 6D).

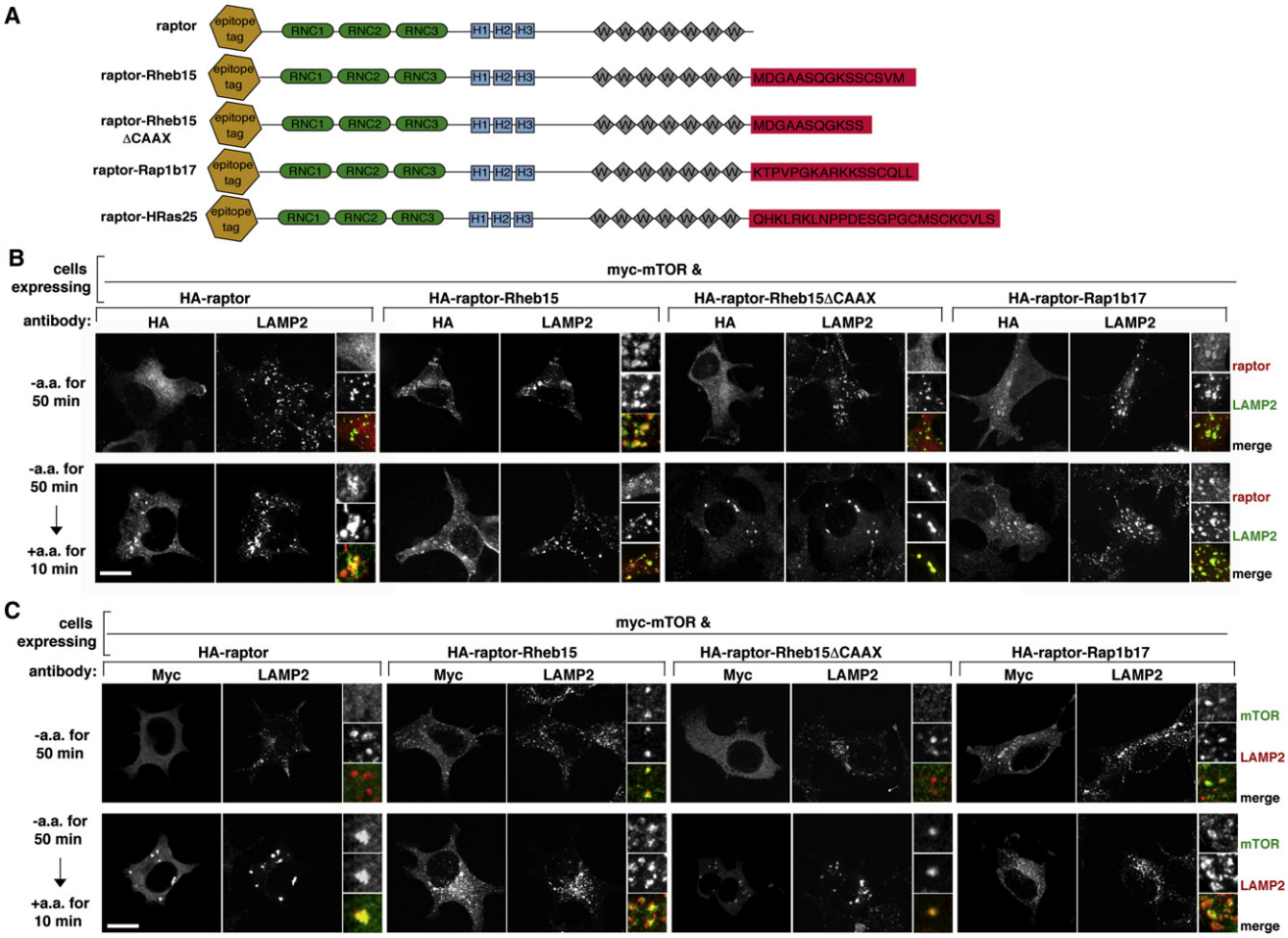

Figure 5. In Cells Expressing Raptor Variants Fused to the Targeting Signals of Rheb1 or Rap1b, mTORC1 Localizes to Lysosomal Membranes in an Amino Acid-Independent Fashion

(A) Schematic of raptor fusion proteins that target mTORC1 to lysosomal membranes (raptor-Rheb15; raptor-Rap1b17) or to the plasma membrane (Raptor-HRas25) as well as proteins used as controls (wild-type raptor; raptor-Rheb15ΔCAAX).

(B) Images of amino acid starved or replete cells expressing lysosomally targeted or control HA-tagged raptor proteins and coimmunostained for the HA epitope (red) and endogenous LAMP2 (green). HEK293T cells were transfected with the indicated cDNAs, starved of and restimulated with amino acids for the indicated times, and processed in the immunofluorescence assay.

(C) Images of amino acid starved or replete cells coexpressing myc-mTOR and the indicated raptor fusion proteins and coimmunostained for the myc epitope (green) and endogenous LAMP2 (red). HEK293T cells were co-transfected with the indicated cDNAs and treated and processed as in (B).

In all images, insets show selected fields that were magnified five times and their overlays. Scale bars represent 10 μm.

Forced Targeting of mTORC1 to the Lysosomal Surface Eliminates the Requirement in mTORC1 Signaling for Rag and Ragulator, but Not Rheb, Function

The ability to constitutively localize mTORC1 to lysosomal membranes enabled us to probe in more detail the role of the Rag and Rheb GTPases, as well as Ragulator, in the activation of mTORC1 by amino acids. We hypothesized that if the major role of the Rag GTPases is to allow mTORC1 to localize to lysosomes, then in cells that express raptor-Rheb15, mTORC1 activity should be independent of Rag function. Indeed, while in control cells the RNAi-mediated knockdown of both RagA and RagB strongly blunted the activation of mTORC1 by amino acids, it did not reduce the amino acid-insensitive mTORC1 activity observed in raptor-Rheb15-expressing cells (Figure 7A). As an additional approach to inhibit Rag function, we exploited the fact that coexpression of a GDP-bound RagB mutant ($RagB^{GDP}$) and a GTP-bound RagD mutant ($RagD^{GTP}$) eliminates mTORC1 pathway activity within cells (Kim et al., 2008; Sancak et al., 2008). Expression of $RagB^{GDP}$-$RagD^{GTP}$ completely prevented mTORC1 activation by amino acids in control cells but had no effect on the amino acid-insensitive mTORC1 activity of cells expressing raptor-Rheb15 (Figure 7B).

If the main function of Ragulator in the mTORC1 pathway is to localize the Rag GTPases to the lysosomes, then it should be possible to reactivate the mTORC1 pathway in Ragulator null cells by expressing raptor-Rheb15. Remarkably, the stable expression of raptor-Rheb15, but not wild-type raptor, in p14 or p18 null cells reactivated mTORC1 signaling and made it

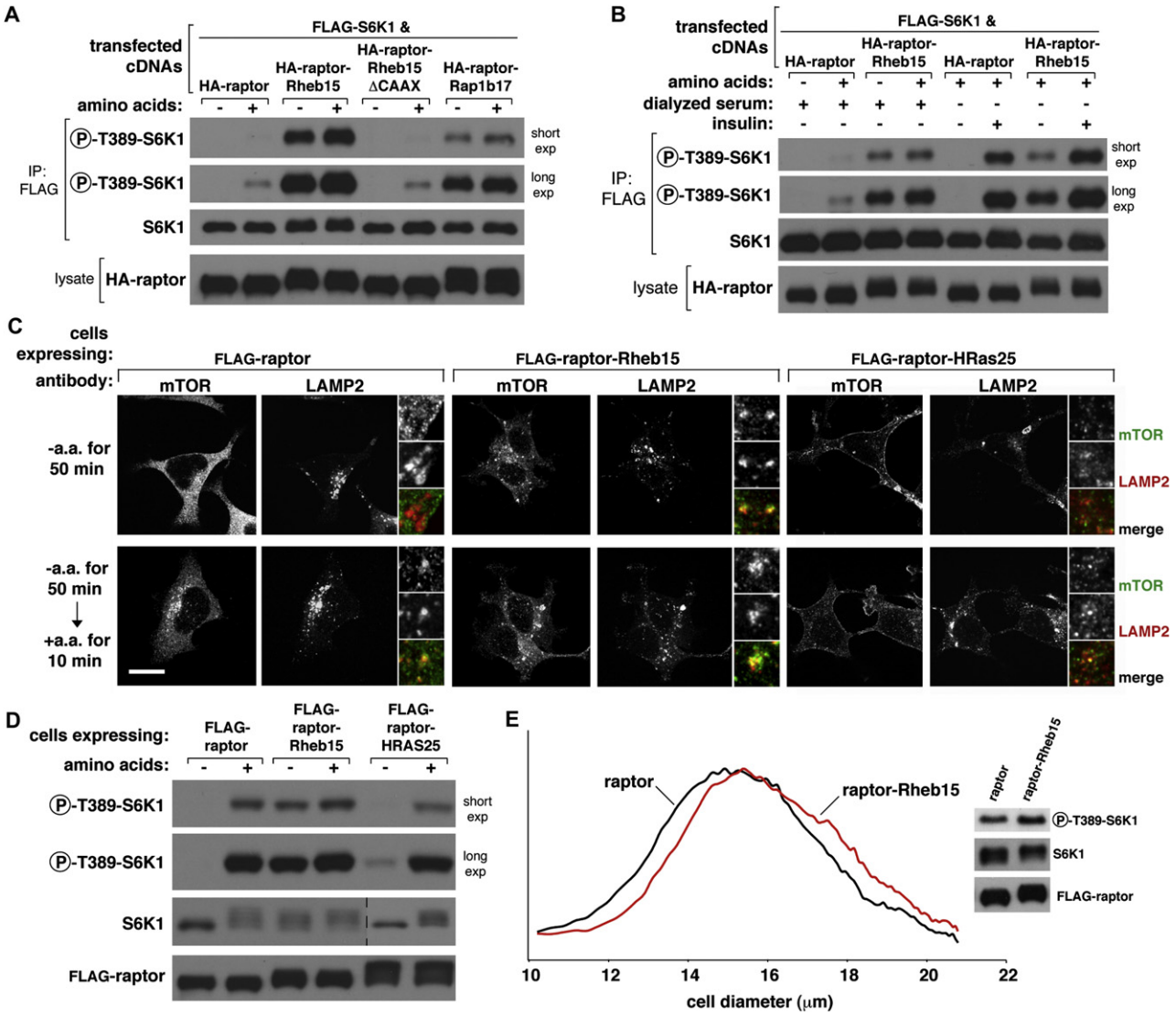

Figure 6. Constitutive Association of Raptor with Lysosomal Membranes, but Not the Plasma Membrane, Is Sufficient to Make the mTORC1 Pathway Insensitive to Amino Acid Starvation

(A) The mTORC1 pathway is not sensitive to amino acid starvation in cells that express lysosomally targeted but not control raptor proteins. HEK293T cells were cotransfected with the indicated cDNA expression plasmids and starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min. Cell lysates and anti-FLAG-S6K1 immunoprecipitates were analyzed by immunobloting for the levels of the indicated proteins and phosphorylation states.

(B) The mTORC1 pathway is sensitive to serum starvation and insulin stimulation in cells that express lysosomally targeted as well as control raptor proteins. HEK293E cells were cotransfected with the indicated cDNA expression plasmids, starved of amino acids for 50 min, or starved and restimulated with amino acids for 10 min. Duplicate cultures were starved of serum for 50 min or starved and stimulated with insulin for 10 min. Cell lysates and anti-FLAG-S6K1 immunoprecipitates were analyzed by immunobloting for the levels of the indicated proteins and phosphorylation states.

(C) Images of cells stably expressing FLAG-raptor, FLAG-raptor-Rheb15, or FLAG-raptor-HRas25 and coimmunostained for endogenous mTOR (green) and endogenous LAMP2 (red). HEK293T cells stably expressing the indicated proteins were treated as in (A) for the indicated times before processing in the immunofluorescence assay. In all images, insets show selected fields that were magnified five times and their overlays. The scale bar represents 10 μm.

(D) Targeting of mTORC1 to the lysosomal but not the plasma membrane makes the mTORC1 pathway insensitive to amino acid starvation. HEK293T cells stably expressing FLAG-raptor, FLAG-raptor-Rheb15, or FLAG-raptor-HRas25 were treated as in (A) and analyzed by immunoblotting for the levels of the indicated proteins and phosphorylation states.

(E) Targeting of mTORC1 to the lysosomal membrane increases cell size and pathway activity in cells under normal growth conditions. Cell size distributions of cells that stably express FLAG-raptor or FLAG-raptor-rheb15 as well as immunoblot analyses of the mTORC1 pathway in the same cells are shown.

insensitive to amino acid deprivation (Figures 7C and 7D). Furthermore, expression of raptor-Rheb15 in the p18 null cells was sufficient to increase their size (Figure 7E). In contrast to the results observed with the Rag GTPases and Ragulator, RNAi-mediated suppression of Rheb1 blocked amino acid-induced mTORC1 activation in cells expressing raptor-Rheb15 to the same extent as it did in control cells (Figure 7F).

To test whether the presence of mTORC1 and Rheb on the same membrane compartment is sufficient to render the mTORC1 pathway insensitive to amino acid levels, we generated cells in which mTORC1 and Rheb are both present on the plasma membrane. To accomplish this, we prepared a Rheb1 variant, called Rheb1-HRas25, that localizes to the plasma-membrane (Figure S5C) because it contains the C-terminal 25 amino acids of H-Ras instead of the normal Rheb1 localization signal. When Rheb1-HRas25 was stably coexpressed with raptor-HRas25, but not wild-type raptor, the mTORC1 pathway became insensitive to amino acid starvation (Figure 7G). Importantly, mTORC1 signaling remained amino acid-sensitive in cells in which either Rheb or mTORC1, but not both, was targeted to the plasma membrane (Figure 7G).

DISCUSSION

Our findings, together with previous work showing that Rheb is required for amino acids to activate the mTORC1 pathway (Roccio et al., 2006; Smith et al., 2005) and can localize to late endosomes/lysosomes (Saito et al., 2005; Sancak et al., 2008), is consistent with a model in which amino acids induce mTORC1 to associate with the endomembrane system of the cell and thus allow it to encounter its activator Rheb. In this model, the essential role of the Ragulator-Rag complex is to serve as an amino acid-regulated docking site for mTORC1 on lysosomal membranes (see schematic in Figure 7H). The proposed link between the Rag and Rheb GTPases in the regulation of the mTORC1 pathway provides an explanation for why activation of mTORC1 occurs only when activators of both Rheb (e.g., growth factors and energy) and the Rags (i.e., amino acids) are available. For technical reasons (Buerger et al., 2006; Sancak et al., 2008), it has not been possible to determine the intracellular localization of endogenous Rheb, and work using overexpressed GFP-tagged Rheb1 has placed it on various endomembrane compartments, including endosomes and lysosomes (Buerger et al., 2006; Saito et al., 2005; Sancak et al., 2008; Takahashi et al., 2005). Our results suggest that at some point in its life cycle, Rheb must traverse the lysosomal surface in order to encounter mTORC1, and so in our model we have chosen to place Rheb on this compartment (Figure 7H). However, at any given time only a small fraction of cellular Rheb may actually be on the lysosomal surface, or, alternatively, some of the mTORC1 within the cell may move to a nonlysosomal endomembrane compartment that also contains Rheb. These issues will only be answered once a definitive location for endogenous Rheb can be determined.

The trimeric p14, p18, and MP1 protein complex, which we call Ragulator, is a Rag-interacting complex that is essential for amino acid signaling to mTORC1 and represents an additional critical component of the TORC1 signaling pathway in mammals and flies. p18 directly interacts with the Rag GTPases (Figure S2A) as well as with p14 and MP1 (Nada et al., 2009) and so may serve as a scaffold to bring the Rag GTPases and MP1-p14 next to each other. In vitro we have not detected a direct interaction between the Rag GTPases and either MP1 or p14, but both proteins are, like p18, necessary for localizing the Rag GTPases to the lysosomal surface. p14 is required to maintain normal p18 expression levels (Figure S2B), suggesting that within cells p14 and MP1 form a crucial part of the Ragulator structure. Given the nonspecific nature of the p14 and p18 names, in the future it may be best to rename these proteins, perhaps to names that reflect their essential roles in the mTORC1 pathway.

The location of the Rag GTPases, the Ragulator, and mTORC1 on the lysosomal surface implicates this organelle as the site of a yet to be discovered sensing system that signals amino acid availability to the Ragulator-Rag complex. The lysosomal location of the amino acid sensing branch of the mTORC1 pathway is consistent with increasing evidence that lysosomes, and their yeast counterparts, vacuoles, are at the nexus of amino acid metabolism within cells. Lysosomes are a major site of protein degradation and amino acid recycling, and vacuoles store amino acids at high concentrations (reviewed in Li and Kane, 2009). Thus, mTORC1 and its regulators may reside on the lysosomal surface so as to sense a currently unknown aspect of lysosomal function that reflects the intracellular pools of amino acids.

It is interesting to consider the differences and similarities between the still poorly understood amino acid signaling mechanisms employed by the mTORC1 and yeast TORC1 pathways. Consistent with previous work in mammalian cells (Sancak et al., 2008), the Gtr1p-Gtr2p heterodimer that is orthologous to RagA/B-RagC/D interacts with yeast TORC1 when Gtr1p is GTP loaded (Binda et al., 2009). TORC1 and the Gtr proteins are located on the surface of the vacuole (Berchtold and Walther, 2009; Binda et al., 2009), the yeast equivalent of lysosomes, but, unlike in mammals, yeast TORC1 does not leave the vacuolar surface upon amino acid deprivation although amino acids do control the interaction of TORC1 with Gtr1p-Gtr2p (Binda et al., 2009). This finding suggests that there must be a distinct mechanism for retaining TORC1 at the vacuolar surface and that in yeast the interaction between TORC1 and Gtr1p-Gtr2p serves other purposes besides controlling the intracellular location of TORC1. In contrast, our current work argues that in mammals, the main role of the Rag GTPase and the associated Ragulator complex is to control the association of mTORC1 with the cellular endomembrane system, in particular, lysosomes. Rheb, which is essential for the activation of mTORC1 by all upstream signals, does not appear to be part of the TORC1 pathway in yeast (reviewed in Berchtold and Walther, 2009). As we suggest that the Rag-dependent and amino acid-regulated translocation of mTORC1 to the lysosomal surface may ultimately be a mechanism for controlling the access of mTORC1 to Rheb, the absence of Rheb in the yeast TORC1 pathway may make regulation of TORC1 localization unnecessary. That known Rag- and Gtr-interacting proteins share no sequence homology also suggests that the mechanisms through which the Rag and Gtr GTPases regulate mTORC1 and yeast TORC1, respectively, have diverged. Although it is clear that

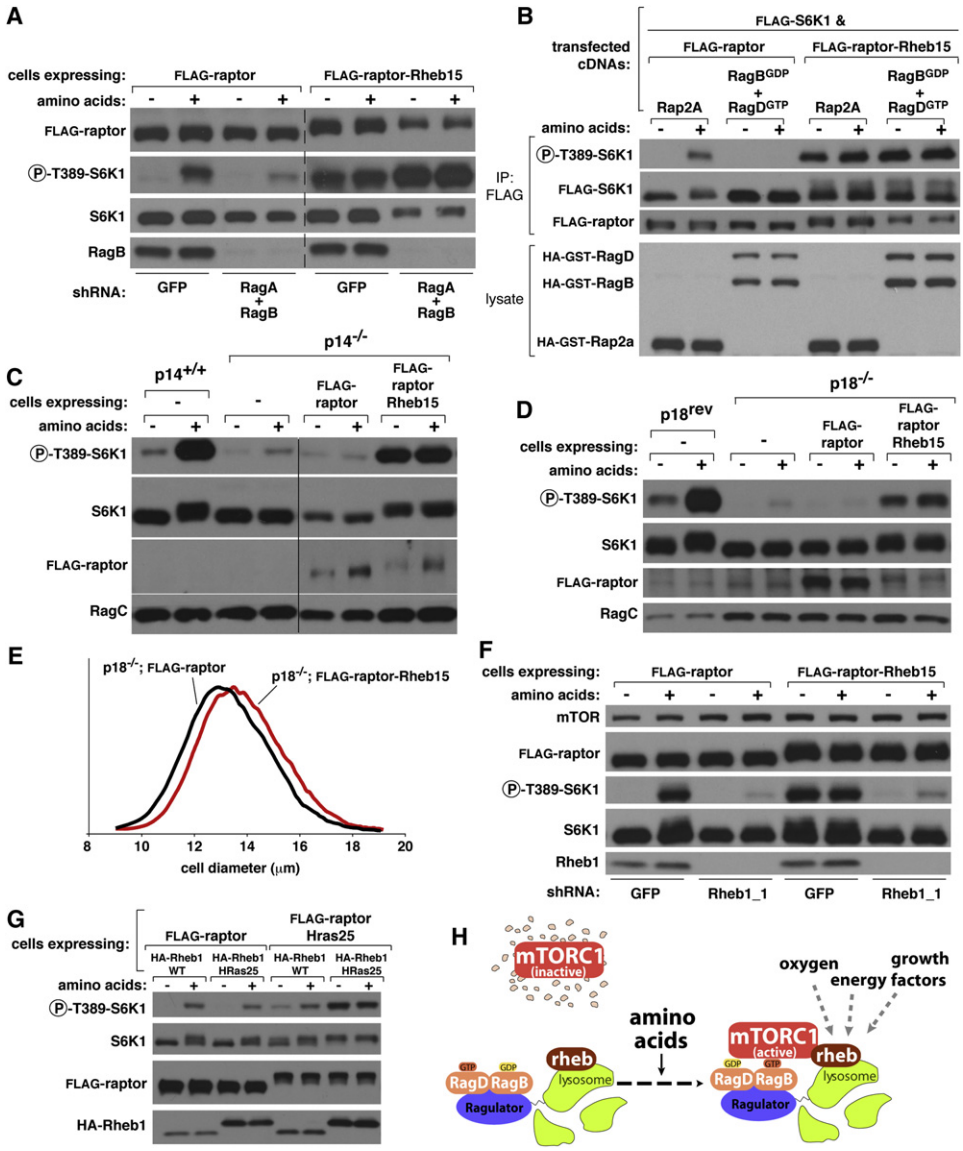

Figure 7. Targeting of mTORC1 to the Lysosomal Surface Makes the Activity of the mTORC1 Pathway Independent of Rag and Ragulator, but Not Rheb, Function

(A) In cells that express FLAG-raptor-Rheb15, mTORC1 pathway activity is independent of Rag GTPase function. Lysates of HEK293T cells expressing FLAG-raptor or FLAG-raptor-Rheb15 were analyzed by immunoblotting for the indicated proteins and phosphorylation states after disruption of Rag function by RNAi-mediated coknockdown of RagA and RagB. Cells were starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min before lysis.

(B) In cells that express FLAG-raptor-Rheb15, mTORC1 pathway activity is independent of Rag GTPase function. Lysates of HEK293T cells expressing FLAG-raptor or FLAG-raptor-Rheb15 were analyzed as in (A) after disruption of Rag function by expression of the dominant negative $RagB^{GDP}$-$RagD^{GTP}$ heterodimer. Cells were treated and processed as in (A).

(C) Stable expression of FLAG-raptor-Rheb15 but not FLAG-raptor in p14 null cells is sufficient to reactivate the mTORC1 pathway and make it insensitive to amino acid starvation. Cells stably expressing the indicated proteins were treated and analyzed as in (A).

(D) Stable expression of FLAG-raptor-Rheb15 but not FLAG-raptor in p18 null cells is sufficient to reactivate the mTORC1 pathway and make it insensitive to amino acid starvation. Cells stably expressing the indicated proteins were treated and analyzed as in (A).

(E) In p18 null cells expression of raptor-Rheb15, but not wild-type raptor, increases cell size. Cell size distributions of p18 null cells that stably express FLAG-raptor or FLAG-raptor-Rheb15 are shown.

(F) In cells that express FLAG-raptor-Rheb15, the activity of the mTORC1 pathway is still Rheb dependent. Lysates of HEK293T cells that stably express FLAG-raptor or FLAG-raptor-Rheb15 were analyzed by immunoblotting for the indicated proteins and phosphorylation states after disruption of Rheb function by an RNAi-mediated knockdown of Rheb1. Cells were treated as in (A).

(G) Coexpression of plasma membrane-targeted raptor and plasma membrane-targeted Rheb1 renders the mTORC1 pathway insensitive to amino acid starvation. HEK293T cells stably expressing the indicated proteins were treated and analyzed as in (A).

the Ragulator and EGO complexes both control the intracellular localization of the Rag (this paper) and Gtr (Gao and Kaiser, 2006) GTPases, respectively, whether these complexes have additional functions remains to be determined.

Previous studies suggest that MP1-p14-p18 complex plays an adaptor role in the MAP kinase (MAPK) pathway (reviewed in Dard and Peter, 2006), and our current findings do not contradict these results. However, considering the very strong inhibition of the mTORC1 pathway that occurs in cells lacking p14 or p18, it seems possible that some of the impairment in MAPK signaling observed in those cells reflects an altered feedback signaling from Akt to the MAPK pathway. For example, in Ragulator null cells, Akt is slightly activated, almost certainly because the well-known inhibitory signal from mTORC1 to PI3K is absent. As Akt suppresses MAPK signaling by phosphorylating and inhibiting Raf (Zimmermann and Moelling, 1999), it is conceivable that the activation of Akt that occurs in Ragulator null cells could account, at least in part, for the inhibition of MAPK signaling that has been observed in these cells.

Mice lacking either p14 or p18 die around embryonic day 7.5–8 and have obvious growth defects (Nada et al., 2009; Teis et al., 2006). We would not be surprised if, when generated, mice lacking the Rag proteins die at around the same age and present similar defects. On the other hand, mice lacking the core mTORC1 component raptor die earlier (before embryonic day 6.5) than p14 and p18 null mice (Guertin et al., 2006). This may be expected because although loss of p14 or p18 completely blocks mTORC1 activation by amino acids, cells lacking the Ragulator proteins are likely to retain a low residual level of mTORC1 activity that may be sufficient to support development further than in embryos completely lacking mTORC1 function. Lastly, our results suggest that the strong growth retardation observed in humans with a mutation that reduces p14 expression (Bohn et al., 2007) is a result of partial suppression of the mTORC1 pathway. If this turns out to be the case, it would represent the first human example of a loss-of-function mutation in a positive component of the mTORC1 pathway.

EXPERIMENTAL PROCEDURES

Cell Lines and Tissue Culture

HEK293E cells, HEK293T cells, and TSC2$^{+/+}$, TSC2$^{-/-}$, p14$^{+/+}$, and p14$^{-/-}$ MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% inactivated fetal calf serum. p18^{rev}, p18^{mito}, and p18$^{-/-}$ cells were cultured in DMEM with 10% fetal bovine serum. HEK293E and HEK293T cells express E1a and SV40 large T antigen, respectively. In HEK293E, but not HEK293T, cells the mTORC1 pathway is strongly regulated by serum and insulin (Sancak et al., 2007). TSC2$^{-/-}$, p53$^{-/-}$, and TSC2$^{+/+}$, p53$^{-/-}$ MEFs were kindly provided by David Kwiatkowski (Harvard Medical School). The HEK293E cell line was kindly provided by John Blenis (Harvard Medical School). p14$^{-/-}$ and control MEFs were kindly provided by Lukas A. Huber (Innsbruck Medical University) and described are in Teis et al. (2006). p18$^{-/-}$ cells are epithelial in nature, and p18^{rev} cells are p18$^{-/-}$ cells in which wild-type p18 has been re-expressed (Nada et al., 2009). Patient-derived cells with a homozygous mutation in the *ROBLD3* (p14) gene 3′ untranslated region and control healthy donor-derived cells were kindly provided by Christoph Klein (Universität München) and have been described in Bohn et al. (2007)

Amino Acid and Serum Starvation and Stimulation of Cells

Serum and/or amino acid starvation of HEK293T cells, HEK293E cells, p14 null and control cells, p18 null and control cells, MEFs, and patient-derived and healthy donor-derived cells were performed essentially as described (Sancak et al., 2008). Serum was dialyzed against phosphate-buffered saline (PBS) in dialysis cassettes (Thermo Scientific) having a 3500 molecular weight cutoff.

Preparation of Cell Lysates and Immunoprecipitations

Cell lysate preparation, cell lysis, and immunoprecipitations were done as described in the Extended Experimental Procedures.

For cotransfection experiments, 2,000,000 HEK293T or HEK293E cells were plated in 10 cm culture dishes. Twenty-four hours later, cells were transfected with the indicated plasmids as follows: 50 ng or 1500 ng myc-mTOR in pRK5; 20 ng or 500 ng HA-, myc-, or FLAG-Raptor in pRK5 or pLJM1 with or without the targeting signals; 100 ng HA-GST-Rap2a in pRK5; 100 ng HA-GST-Rheb1 in pRK5; 100 ng HA-GST-RagB in pRK5; 100 ng HA-GST-RagD in pRK5; 1 ng FLAG-S6K1 in pRK7; 50 ng or 600 ng HA- or FLAG-p14 in pRK5; 75 ng or 600 ng HA-MP1 in pRK5; and 50 ng or 800 ng HA-p18 in pRK5. The total amount of plasmid DNA in each transfection was normalized to 2 μg with empty pRK5.

Cell Size Determinations

For measurement of cell size, 2,000,000 HEK293T cells or 200,000 of other cell types were plated into 10 cm culture dishes. Twenty-four hours later, the cells were harvested by trypsinization in a 4 ml volume and diluted 1:20 with counting solution (Isoton II Diluent, Beckman Coulter). Cell diameters were determined with a particle size counter (Coulter Z2, Beckman Coulter) running Coulter Z2 AccuComp software.

Mammalian Lentiviral shRNAs and cDNAs

Lentiviral short hairpin RNAs (shRNAs) targeting human Rheb1, RagB, and RagC have been described (Sancak et al., 2008). Lentiviral shRNAs targeting mouse Rheb1 and human p14 were obtained from Sigma-Aldrich. Lentiviral shRNAs targeting the messenger RNA for human MP1 and human p18 were cloned into pLKO.1 vector as described (Sarbassov et al., 2005). The target sequences are provided in the Extended Experimental Procedures. Virus generation and infection was done as previously described (Sancak et al., 2008).

Raptor was cloned into the AgeI and BamHI sites of a modified pLKO.1 vector (pLJM1) (Sancak et al., 2008) with or without the Rheb1, Rap1b, and HRas targeting signals or cloned into the pRK5 vector with or without the same localization signals. After sequence verification, pLJM1 based plasmids were used in transient cDNA transfections or to produce lentivirus needed to generate cell lines stably expressing these proteins. pRK5 based plasmids were also used for transient transfection experiments. The p18^{mito} expression plasmid was generated by cloning of a mutant p18 with amino acids 2–5 changed to alanines into a modified version of the pLKO.1 vector that added, to the C terminus of p18, the mitochondrial localization signal of OMP25 protein. This plasmid was used in transient cDNA transfections or to produce lentivirus needed to generate stable cell lines. HA-Rheb1 and HA-Rheb1-HRas25 were cloned into pLJM5, a derivative of pLJM1 carrying a hygromycin instead of puromycin resistance gene. The vectors were used as above for lentivirus production.

Immunofluorescence Assays

Fifty thousand HEK293T cells or 20,000 of other cell types were plated on fibronectin-coated glass coverslips in 12-well tissue culture plates. Twenty-four hours later, the slides were rinsed with PBS once and fixed for 15 min with 4% paraformaldehyde in PBS warmed to 37°C. The slides were rinsed

(H) Model for amino-acid induced mTORC1 activation. In the absence of amino acids, mTORC1 cannot associate with the endomembrane system and has no access to its activator Rheb. In the presence of amino acids, the Rag GTPases, which are tethered to the lysosomal surface by the Ragulator, serve as a docking site for mTORC1, allowing mTORC1 to associate with endomembranes and thus encounter and become activated by Rheb.
See also Figure S5.

twice with PBS and cells were permeabilized with 0.05% Triton X-100 in PBS for 30 s. After rinsing twice with PBS, the slides were incubated with primary antibody in 5% normal donkey serum for 2 hr at room temperature, rinsed four times with PBS, and incubated with secondary antibodies produced in donkey (diluted 1:1000 in 5% normal donkey serum) for 1 hr at room temperature in the dark, washed four times with PBS. Slides were mounted on glass coverslips using Vectashield (Vector Laboratories) and imaged. Transient transfections for immunofluorescence assays were performed as described in the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and Five Figures and can be found with this article online at doi:10.1016/j.cell.2010.02.024.

ACKNOWLEDGMENTS

We thank all members of the Sabatini Lab for helpful suggestions and Eric Spooner for the mass spectrometric analysis of samples. We thank Lukas A. Huber for providing p14 null and control cells and Christoph Klein for providing patient-derived and healthy donor-derived cells. This work was supported by grants from the National Institutes of Health (CA103866 and AI47389) and Department of Defense (W81XWH-07-0448) to D.M.S., awards from the W.M. Keck Foundation and LAM Foundation to D.M.S, and fellowship support from the LAM Foundation and from the Jane Coffin Childs Memorial Fund for Medical Research to R.Z. D.M.S. is an investigator of the Howard Hughes Medical Institute.

Received: October 16, 2009
Revised: December 28, 2009
Accepted: February 5, 2010
Published online: April 8, 2010

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EXTENDED EXPERIMENTAL PROCEDURES

Materials

Reagents were obtained from the following sources: antibodies to phospho-T389 S6K1, S6K1, mTOR, raptor, RagA/B, RagC, p14, p18, MP1, the myc epitope, the HA epitope, the FLAG epitope (unconjugated and alexa fluor conjugated), TSC2, phospho-T398 dS6K, phospho-S473 Akt, Akt1, phospho-T70 4E-BP1, 4E-BP1, and Rheb from Cell Signaling Technology; antibodies to LAMP2 from Abcam (ab25631 and ab13524); antibody to raptor (for immunostaining) from Millipore; antibody to Cytochrome c from BD Biosciences; HRP-labeled anti-mouse, anti-goat, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; FLAG M2 affinity gel, FLAG M2 antibody, human recombinant insulin, from Sigma Aldrich; protein G-sepharose and dialysis cassettes from Thermo Scientific; DMEM from SAFC Biosciences; FuGENE 6 and Complete Protease Cocktail from Roche; alexa fluor conjugated secondary antibodies from Invitrogen; 16% paraformaldehyde solution from Electron Microscopy Sciences; fibronectin from Jackson Immunoresearch Laboratories; 35 mm glass bottom dishes from Mattek Corporation; glass coverslips from Ted Pella, Inc; amino acid and glucose-free RPMI from United States Biological; Schneider's medium, *Drosophila*-SFM, and Inactivated Fetal Calf Serum (IFS) from Invitrogen. The dS6K antibody was a generous gift from Mary Stewart (North Dakota State University).

Preparation of Cell Lysates and Immunoprecipitations

Cells were rinsed once with ice-cold PBS and lysed in ice-cold lysis buffer (40 mM HEPES [pH 7.4], 2 mM EDTA or 5mM $MgCl_2$, 10 mM pyrophosphate, 10 mM glycerophosphate, 0.3% CHAPS, or 1% Trition X-100 and one tablet of EDTA-free protease inhibitors (Roche) per 25 ml). The soluble fractions of cell lysates were isolated by centrifugation at 13,000 rpm for 10 min by centrifugation. For immunoprecipitations, primary antibodies were added to the lysates and incubated with rotation for 1.5 hr at 4°C. 60 μl of a 50% slurry of protein G-sepharose was then added and the incubation continued for an additional 1 hr. Immunoprecipitates were washed three times with lysis buffer containing 150mM NaCl. Immunoprecipitated proteins were denatured by the addition of 20 μl of sample buffer and boiling for 5 min, resolved by 8%–16% SDS-PAGE, and analyzed by immunoblotting. For Flag purifications, Flag M2 affinity gel was washed with lysis buffer 3 times. 20 ul of beads in 50% slurry was then added to pre-cleared cell lysates and incubated with rotation for 2 hr at 4°C. Finally, The beads were washed 3 times with lysis buffer containing 150 mM NaCl. Immunoprecipitated proteins were denatured by the addition of 50 μl of sample buffer and boiling for 5 min.

Mammalian Lentiviral shRNAs and cDNAs

The sequences of shRNAs targeting human MP1 and p18 are as follows:

MP1_1: GAGATGGAGTACCTGTTATTA
MP1_2: ATATCAATCCAGCAATCTTTA
p18: AGACAGCCAGCAACATCATTG

Identification of Ragulator Components as Rag-Associated Proteins

Ragulator components (MP1, p14, and p18) were detected in anti-FLAG immunoprecipitates prepared from HEK293T cells stably expressing FLAG-RagB or FLAG-RagD as well as in immunoprecipitates of endogenous RagC prepared from HEK293T cells. Immunoprecipitates were prepared as described (Sancak et al., 2008). Proteins were eluted with the FLAG peptide from the anti-FLAG affinity matrix or recovered from the protein G-sepharose by boiling with sample buffer, resolved by SDS-PAGE, and stained with simply blue stain (Invitrogen). Each gel lane was sliced into 10-12 pieces and the proteins in each gel slice digested overnight with trypsin. The resulting digests were analyzed by mass spectrometry as described (Sancak et al., 2008). 2-3 peptides corresponding to each Ragulator component were identified in the FLAG-RagB and endogenous RagC immunoprecipitates, while no peptides corresponding to any of the proteins were ever found in the FLAG-Rap2a, p53, or α-tubulin immunoprecipitates that served as controls.

Amino Acid Starvation and Stimulation and dsRNA-Mediated Knockdowns in *Drosophila* Cells

Amino acid starvation and stimulation of *Drosophila* S2 cells was performed as described (Sancak et al., 2008). The design and synthesis of dsRNAs has also been described (Sancak et al., 2008).

Primer sequences used to amplify DNA templates for dsRNA synthesis for dp14, dp18, and dMP1, including underlined 5′ and 3′ T7 promoter sequences, are as follows:

dp14 (CG5189) dsRNA forward primer: GAATTAATACGACTCACTATAGGGAGACTCTATTGGCCTACTCCGGTTAT
dp14 (CG5189) dsRNA reverse primer: GAATTAATACGACTCACTATAGGGAGATATGAGGCCGAGATCTGCTTA
dp18 (CG14184) dsRNA forward primer: GAATTAATACGACTCACTATAGGGAGAGCAGAATACTGCGATAAACATGATA
dp18 (CG14184) dsRNA reverse primer: GAATTAATACGACTCACTATAGGGAGATGGATAGGTTGGCTTAGACAGATAG
dMP1 (CG5110) dsRNA forward primer: GAATTAATACGACTCACTATAGGGAGAGTCGGACGACATCAAGAAGTATTTA
dMP1 (CG5110) dsRNA reverse primer: GAATTAATACGACTCACTATAGGGAGAAGTACATGGAGATGATGGTCTTGTT

In Vitro Binding Assay

2 million HEK293T cells were transfected with 2 μg FLAG-p18 (lipidation mutant G2A), 2 μg HA-GST-Rap2a, or 2 μg HA-GST-RagB together with 2 μg of HA-GST-RagC. 2 days after transfection, the cells were lysed in lysis buffer containing 1% Triton X-100 as described (Sancak et al., 2007) and cleared lysates were incubated with glutathione- or FLAG-beads for 3 hr at 4°C with rotation. The beads were washed 3 times with lysis buffer and two times with lysis buffer containing 0.3% CHAPS. FLAG-p18 was eluted from FLAG beads with the FLAG peptide and 1/8 of the eluate was incubated with 1/4 of the Rag-containing glutathione beads in lysis buffer with 0.3% CHAPS for 45 min at 4°C. The glutathione beads were washed three times with lysis buffer containing 0.3% CHAPS and 150 mM NaCl. Proteins were denatured by the addition of 20 μl of sample buffer and boiling for 5 min and analyzed by SDS-PAGE and immunoblotting.

Transient Transfections for Immunofluorescence Assays

For myc-mTOR and HA-raptor co-transfection experiments, HEK293T cells were seeded in 60 mm culture plates. 24 hr later, cells were transfected with 500 ng myc-mTOR and 50 ng HA-Raptor. 24 hr after transfections, cells were split and plated on fibronectin coated glass coverslip in 12-well culture plates and processed as above.

For GFP-RagB, GFP-RagD, p18-GFP, GFP-Mito, RFP-RagB, and LAMP1-mRFP co-transfection experiments, HEK293T cells (250,000 cells/dish) or $p18^{-/-}$, $p18^{rev}$ or $p18^{mito}$ cells (50,000 cells/dish) were plated on 35 mm, glass-bottom Mattek dishes. The next day, each dish was transfected with 100 ng of GFP-RagB or GFP-RagD, p18-GFP, GFP-mito, RFP-RagB or LAMP1-mRFP using fugene. At 18-24 hr post transfections, cells were fixed and imaged. GFP-Mito has been described (Nemoto and De Camilli, 1999).

For GFP-LC3 localization experiments, 2 million cells were transfected by electroporation with 1 μg of GFP-LC3 plasmid, and plated on 35 mm glass-bottom Mattek dishes. The next day the cells were starved for 3 hr in serum- and amino acid-free RPMI to induce autophagy and processed for imaging as above.

All images were acquired with a spinning disk confocal microscope (Perkin Elmer) equipped with a Hamamatsu 1k X 1k EM-CCD camera. For each image, 8-10 optical slices were acquired and displayed as maximum projections.

Quantification of Number of Autophagosomes per Cell

After acquisition, the images were opened with Image J, made binary and the number of autophagosomes per cell was obtained using the "Analyze Particle" function.

SUPPLEMENTAL REFERENCES

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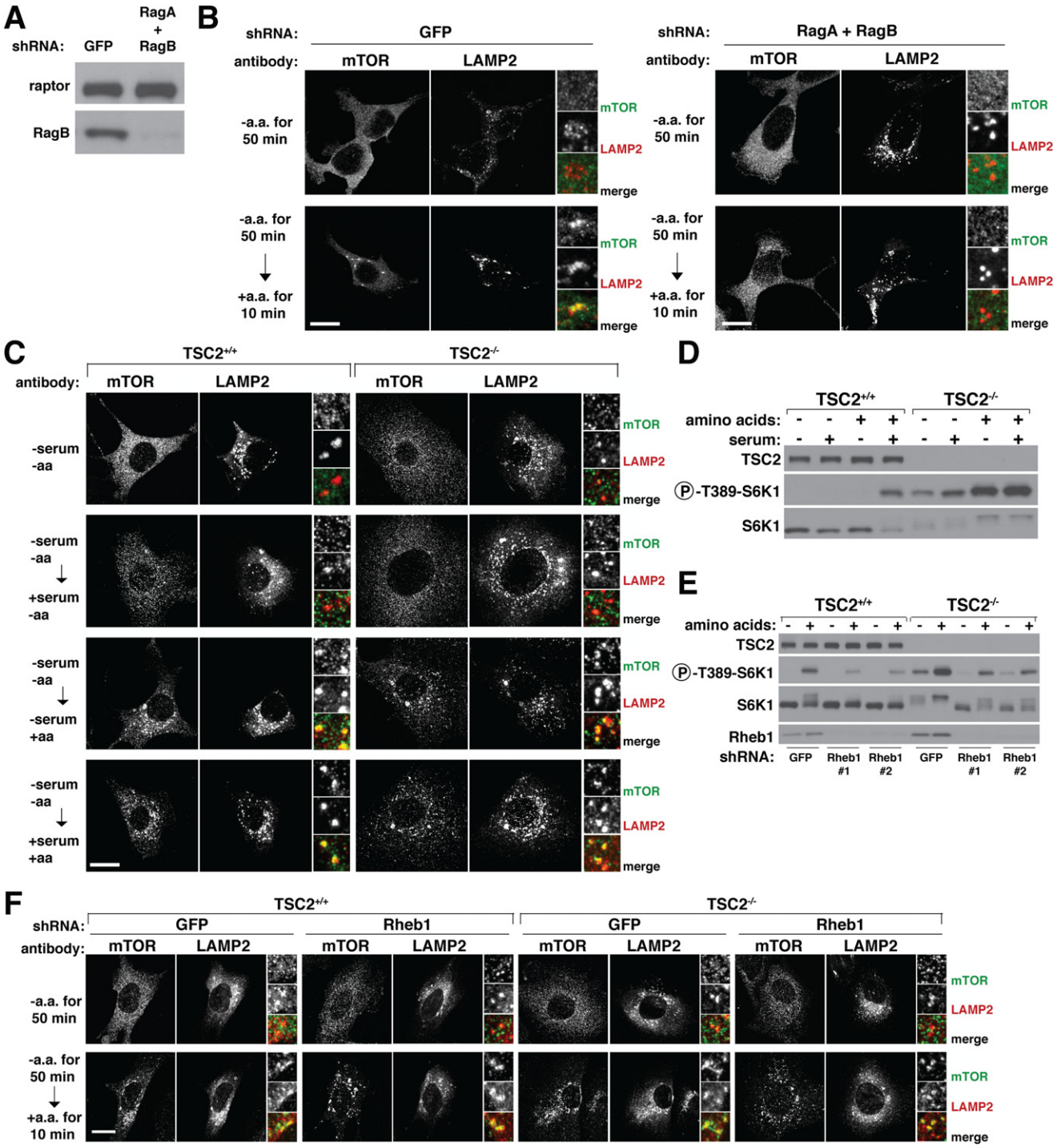

Figure S1. Movement of mTORC1 to Lysosomal Membranes in Response to Amino Acids Depends on the Rag GTPases and Is Independent of TSC1/2, Rheb, and Growth Factors, Related to Figure 1

(A) Immunoblot analysis of RagB and raptor protein levels in HEK293T cells with an RNAi-mediated knockdown of a control protein or RagA and RagB.

(B) Images of cells with knockdowns of RagA and RagB and co-immunostained for mTOR (green) and LAMP2 (red) after starvation and restimulation with amino acids for the indicated times. HEK293T cells expressing the indicated shRNAs were starved and restimulated with amino acids as indicated and processed in the immunofluorescence assay. In all images, insets show selected fields that were magnified five times and their overlay. Scale bar is 10 μm.

(C) mTOR co-localizes with LAMP2 only in the presence of amino acids and independently of serum stimulation. Images show co-immunostaining of mTOR (green) and LAMP2 (red) in $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs after indicated treatments. Cells were starved for serum and amino acids, and stimulated with dialyzed

serum, amino acids, or both before processing in the immunofluorescence assay.
(D) Lysates from $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs starved and stimulated as in (A) were analyzed by immunobloting for the activity of the mTORC1 pathway.
(E) Loss of Rheb expression inhibits mTORC1 signaling in $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs. Cells expressing the indicated shRNAs were starved for amino acids or starved and restimulated with amino acids and lysates analyzed by immunobloting for mTORC1 pathway activity and Rheb1 levels.
(F) mTOR co-localizes with LAMP2 only in the presence of amino acids and independently of Rheb or TSC2. Images show co-immunostaining of mTOR (green) and LAMP2 (red) in $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs treated as in (C).
In all images, insets show selected fields that were magnified five times and their overlays. Scale bar is 10 μm.

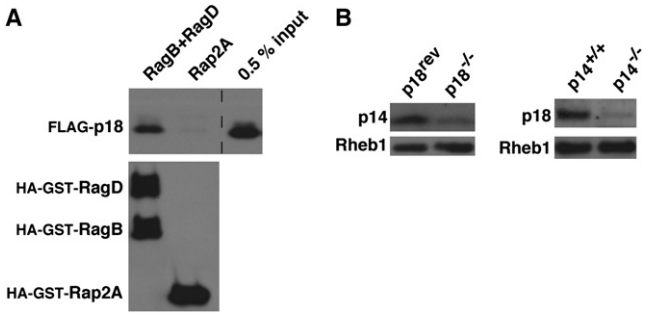

Figure S2. The Expression of Ragulator Proteins Is Coregulated and Purified FLAG-p18 Interacts with Purified HA-GST-RagB/HA-GST-RagD Dimer In Vitro, Related to Figure 2

(A) In vitro binding assay using purified soluble FLAG-p18 and HA-GST-RagB/HA-GST-RagD heterodimer bound to glutathione beads was performed as described in the Experimental Procedures.

(B) p14 protein levels are lower in p18 null cells than in p18 null cells expressing FLAG-p18 ($p18^{rev}$). Similarly, in cells that lack p14 ($p14^{-/-}$), p18 expression is reduced compared to control cells ($p14^{+/+}$). Cells were grown to confluency, lysates were prepared, and the levels of the indicated proteins analyzed by immunoblotting.

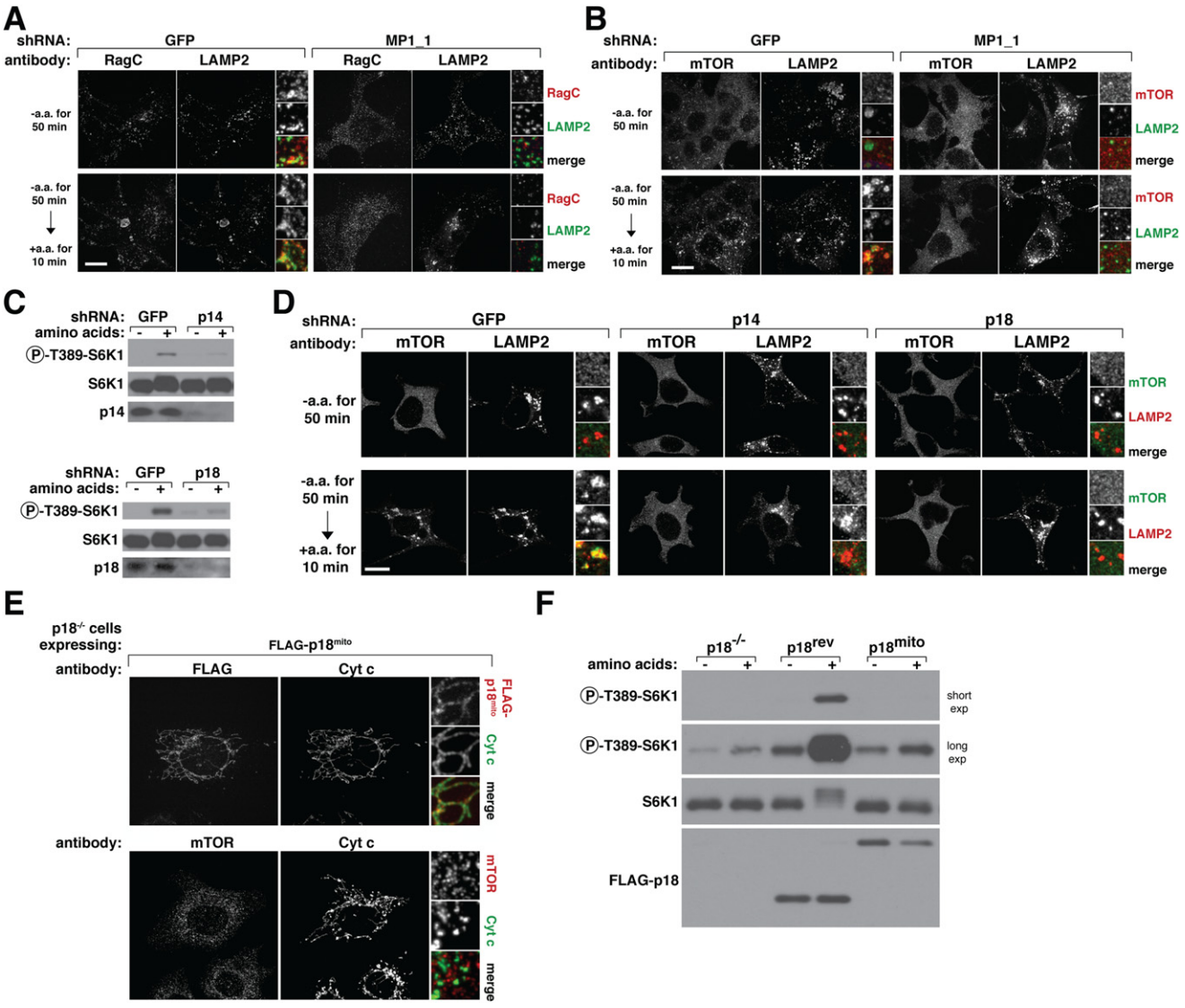

Figure S3. The Ragulator Is Required for RagC Localization to Lysosomal Membranes and Amino Acid-Induced mTOR Lysosomal Localization, Related to Figure 3

(A) An MP1 knockdown displaces RagC from the lysosomal surface. Images of cells with shRNA-mediated knockdowns of a control protein or MP1 and co-immunostained for RagC (red) and LAMP2 (green). HEK293T cells expressing the indicated shRNAs were starved of and restimulated with amino acids for the stated times and then processed in the immunofluorescence assay.

(B) An MP1 knockdown impairs the recruitment of mTOR to the lysosomal surface in response to amino acid stimulation. Images of cells with shRNA-mediated knockdowns of a control protein or MP1 and co-immunostained for mTOR (red) and LAMP2 (green). HEK293T cells expressing the indicated shRNAs were starved of and restimulated with amino acids for the stated times and then processed in the immunofluorescence assay.

(C) Knockdown of p18 or p14 in HEK293T cells impair amino acid-induced mTORC1 activation. HEK293T cells with RNAi-mediated knockdown of p14 or p18, or control cells, were starved for amino acids for 50 min or starved and restimulated with amino acid for 10 min. Cell lysates were prepared and analyzed by immunoblotting for the phosphorylation states and levels of indicated proteins.

(D) Knockdown of p18 or p14 in HEK293T cells impairs amino acid-induced lysosomal recruitment of mTOR. Control cells and cells with p14 or p18 knockdown were treated as in (C) and immunostained for mTOR (green) and LAMP2 (red). In all images, insets show selected fields that were magnified five times and their overlays. Scale bar is 10 μm.

(E) Images of $p18^{-/-}$ cells stably expressing FLAG-$p18^{mito}$ and co-immunostained for FLAG-$p18^{mito}$ or mTOR (red) and Cytochrome c (Cyt c) (green).

(F) The mTORC1 pathway can be activated by amino acids in p18 null cells expressing wild-type p18 ($p18^{rev}$), but not mitochondrially-targeted p18 ($p18^{mito}$). Cells were starved for amino acids in the presence of dialyzed serum for 50 min, or starved and restimulated with amino acids for 10 min. Lysates were prepared and phosphorylation states and levels of indicated proteins were analyzed by immunoblotting.

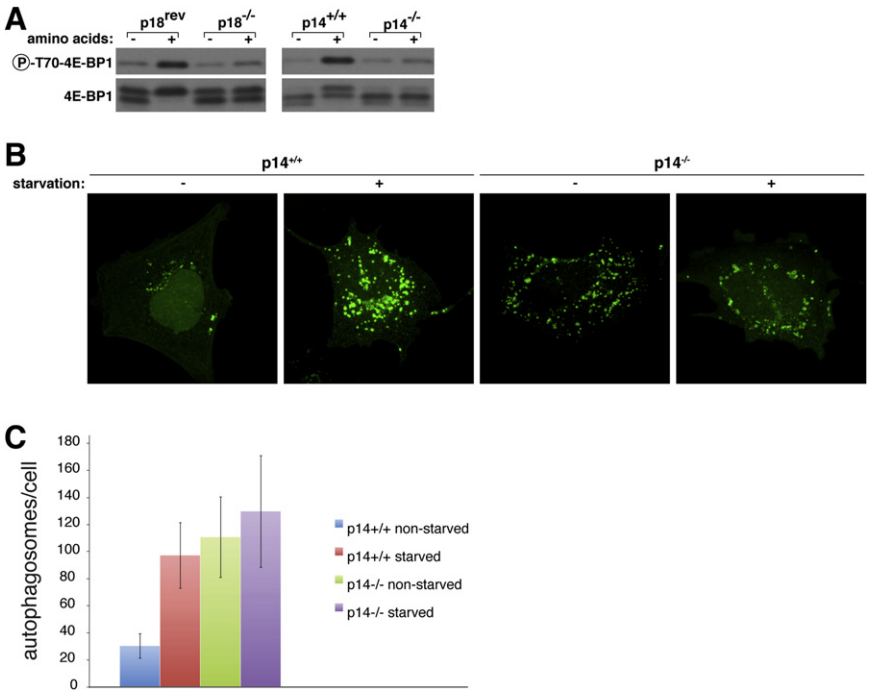

Figure S4. 4E-BP1 Phosphorylation Is Inhibited and Autophagy Is Induced in Cells Lacking Ragulator Components, Related to Figure 4

(A) Amino acids fail to stimulate 4E-BP1 phosphoryation in cells lacking p14 or p18. Cells were starved for amino acids in the presence of dialyzed serum for 50 min, or starved and restimulated with amino acids for 10 min. Lysates were prepared and 4E-BP1 phosphorylation and levels analyzed by immunoblotting.

(B) Autophagy is induced in p14 null cells. Images of cells transiently expressing GFP-LC3 and starved for amino acids and serum for 3 hr or growing in complete media. Accumulation of GFP-LC3 in large puncta in starved control cells and in the non-starved p14 null cells indicates increased levels of autophagy in these cells.

(C) Quantification of autophagosomes in wild-type or p14 null cells expressing GFP-LC3. Cells were treated as in (B), images were taken and the number of autophagosomes per cell was quantified using Image J. At least six cells were analyzed per sample. The data are represented as mean −/+ standard deviation. Starved wild-type cells, or p14 null cells, irrespective of being starved or not, show statistically significant increases in the number autophagosomes per cell compared to wild-type non-starved cells ($p < 0.000002$). There is no statistically significant difference between starved and non-starved p14 null cells ($p = 0.38$).

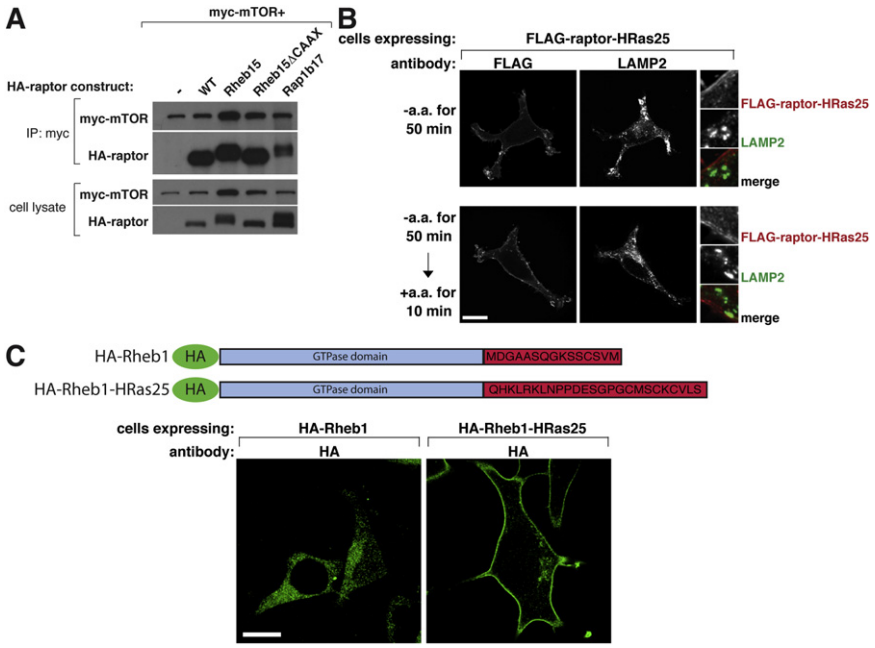

Figure S5. Addition of Rheb1 and Rap1b Targeting Signals to Raptor Does Not Interfere with Its Binding to mTOR and Raptor-HRas25 and Rheb-HRas25 Localize to the Plasma Membrane, Related to Figure 7

(A) HEK293T cells were co-transfected with plasmids encoding myc-mTOR and the indicated HA-raptor variants. Anti-myc immunoprecipitates as well as lysates were analyzed by immunobloting for the indicated proteins.

(B) Raptor fused at its C terminus with the localization signal of HRas localizes to the plasma membrane. Images of cells expressing FLAG-raptor-HRas25 and starved of and restimulated with amino acid for the indicated times and co-immunostained with antibodies to the FLAG epitope (red) and endogenous LAMP2 (green).

(C) Rheb1 localizes to the plasma membrane when its localization signal is swapped for that of HRas. Schematic shows composition of the HA-Rheb1-HRas25 variant. Images of cells expressing HA-Rheb1 or HA-Rheb1-HRas25 (green).