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14. ABSTRACT The multiprotein mTORC1 protein kinase complex is the central component of a pathway that promotes growth in response to insulin, energy levels, and amino acids and is deregulated in common cancers. In particular, the mTOR pathway is hyperactive in Tuberous Sclerosis Complex (TSC), a mental retardation and cancer-prone syndrome affecting 1 in 6,000 people in the United States. With the long-term goal of developing anti-cancer therapeutics based on the mTORC1 regulatory mechanism, we recently found that the Rag proteins, a family of four related small GTPases, interact with mTORC1 in an amino acid-sensitive manner and are necessary for the activation of the mTORC1 pathway by amino acids. The Rag proteins do not directly stimulate the kinase activity of mTORC1, but, like amino acids, promote the intracellular localization of mTOR to a compartment that also contains its activator Rheb. In addition, our structural analysis of mTORC1 generated preliminary cryo-EM reconstructions of the mTORC1 dimer and Raptor at resolutions of 25Å and 30Å, respectively.					
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INTRODUCTION

It is well established that Tuberous Sclerosis Complex (TSC) is caused by inactivating mutations of the TSC1 and TSC2 tumor suppressor genes, but how TSC1/2 loss of function leads to tumor formation is not well understood. Recently, the mTORC1 (mammalian Target of Rapamycin Complex 1) protein complex has emerged as a key downstream regulator of TSC1/2, which negatively regulates the small guanosine triphosphatases (GTPase) Rheb, an activator of the mTORC1 pathway. Given that the mTOR pathway is an important target in TSC treatment, understanding how impairment of TSC1/2 function results in the activation of mTORC1 is critical. In particular, there is a lack of information about the nutrient and growth factor input to the mTORC1 complex and the inhibitory mechanism of rapamycin. With the long-term goal of developing anti-cancer therapeutics based on mTORC1 regulatory mechanisms, our structural and biochemical studies aim to find efficient means of regulating the mTOR signaling network. Therefore, we propose structural analysis of mTORC1 as well as mechanistic analysis of mTORC1 activation via Rheb.

BODY

Aim 1: Understand the role of Rheb-mediated phosphorylation of Raptor in the regulation of mTORC1

One of the major aims of this project is to understand the role of Raptor in mTORC1 signaling. While analyzing the Raptor phosphorylation, we found, by mass spectrometry, a new Raptor-interacting protein, Rag, which interacts with mTORC1 in an amino acid-sensitive manner and is necessary for the activation of the mTORC1 pathway by amino acids. The Rag proteins are a unique family of small GTPases with a canonical Ras-like GTPase domain at the N-termini and a unique Rag A conserved region at the C-termini. In mammals, there are four Rag genes, Rag A, B, C and D. Rag A and B are very similar with 98% amino acid identity in their overlapping sequences. Rag C and D are 81% homologous, and they differ at their N- and C-termini. The Rag proteins were shown to interact with each other in mammalian cells and in yeast.

Gain and loss of function studies of the Rag proteins pointed to their specific roles in amino acid signaling to mTORC1 rather than growth factor signaling. When over-expressed, a GTP-bound Rag B mutant can rescue mTORC1 inactivation induced by amino acid but not serum starvation. When the Rag proteins are knocked down by RNAi, mTORC1 becomes insensitive to amino acid stimulation. Although we do not completely understand how they activate mTORC1 in detail, a critical observation prompted us to hypothesize that they may regulate mTORC1 localization. We observed that amino acid stimulation induces an mTOR localization change. Supporting our hypothesis, when the Rag proteins and Raptor are knocked down, mTORC1 localization change in response to amino acid-availability is prevented. Similarly, in the presence of the GTP bound Rag B mutant, mTORC1 localization mimics amino acid-induced state in the absence of amino acids. Our study, for the first time, pointed to a role of Raptor in mTORC1 localization and regulation of mTORC1 by changing its localization. A detailed description of this particular aspect of the research can be found in an attached appendix (1).

Aim 2: Elucidate the structural features of mTORC1 and its interacting proteins via X-ray crystallography, cryo-EM and SAXS

In our attempt to crystallize the mTOR kinase domain, we utilized the mammalian expression system to obtain soluble recombinant proteins necessary for initial screening. Our previous experience in over-expressing mTOR and its interacting proteins confirmed that mammalian cells were best suited for generating properly folded and difficult-to-obtain target genes at adequate expression levels. For example, 2 billion HEK-293T cells typically produced 5mg of S6K1 following transient transfection. However, a similar approach failed to yield several mTOR kinase domain constructs in quantities necessary for X-ray crystallography, largely due to misfolding of the target proteins. In order to overcome this difficulty, we attempted to minimize the cellular toxicities associated with recombinant proteins by generating kinase-dead constructs, as well as, adding known mTORC1 inhibitors to suppress the kinase activity. In addition, selected constructs were inserted into retro- or lenti-viral backbones to create stable cell lines that utilize the CMV promoter for the optimal level of expression while adapting cells in synthesizing non-native proteins.

As a part of comprehensive structural studies, we aim to characterize the molecular envelope of mTORC1 via cryo-electron microscopy (cryo-EM). Over the past year, we made a significant progress in characterizing the molecular architecture of mTORC1. Due to a recent departure of Dr. Kazuyoshi Murata of the WI-MIT Bioimaging Center, we continued our work in collaboration with Dr. Tom Walz and Dr. Calvin Yip of the Harvard Medical School. Following extensive refinements in both

expression and purification protocols, we successfully obtained the structures of both mTORC1 and Raptor at resolutions of 25Å and 30Å, respectively (Fig. 2).

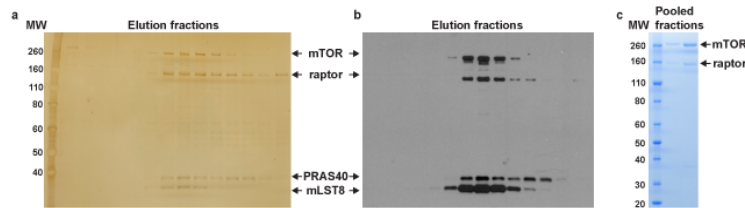


Figure 1. Purification of mTORC1

SDS gels of the fractions collected after gel filtration: silver staining (a), western blot (b) and Coomassie blue staining (c).

Utilization of the mammalian expression system was crucial in purifying mTORC1 in a highly monodisperse form. By transducing a retrovirus, we established a stable cell line that can selectively over-express a tagged recombinant protein. Due to strong affinities among known components of mTORC1, we were able to recruit endogenous proteins to form an intact complex. Following the affinity tag purification and gel

filtration, samples were carefully analyzed by various biochemical assays (Fig. 1). Contrary to our initial assessment, we discovered that mTORC1 only exists as a dimer, and this observation was further evidenced by the cryo-EM reconstruction of mTORC1, which contained the 2-fold symmetry (Figs. 2a-c). Additionally, by labeling mTOR, mLST8, Raptor and PRAS40 with monoclonal antibodies, we mapped individual subunits within mTORC1 (Fig. 2d), thereby providing an adequate framework in which a preliminary Raptor model generated by negatively stained EM can be meaningfully fitted (Figs. 2e and f).

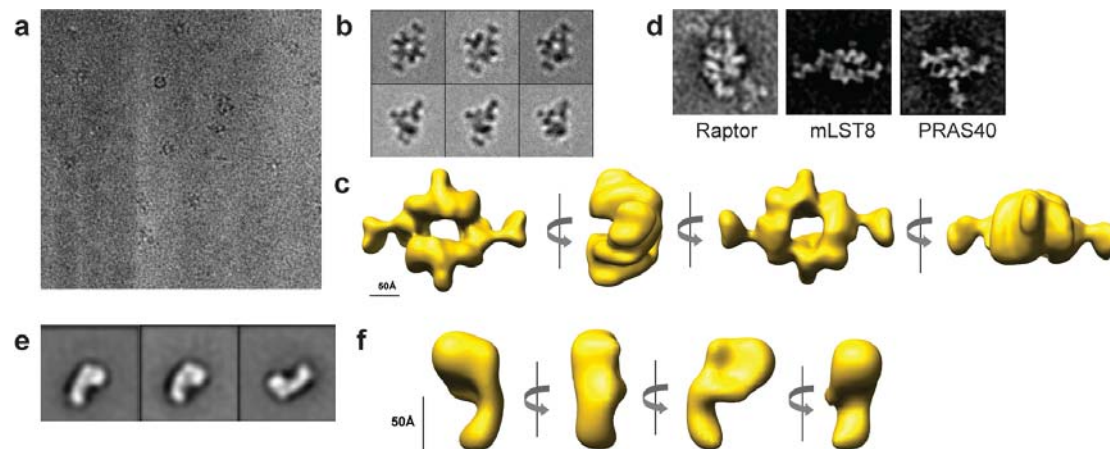


Figure 2. 3D reconstructions of mTORC1 and raptor.

(a and b) Cryo-EM field of mTORC1 and projections. (c) Cryo-EM structure of mTORC1 at 25Å resolution. (d) Antibody-labeling of mTORC1. (e and f) Projections and structure of raptor from negatively stained EM

KEY RESEARCH ACCOMPLISHMENTS

- Identification of Rag as a key Raptor-interacting protein
- Demonstration of mTOR localization based on amino acid availability
- Showing the regulation of mTOR localization by Rag and Raptor in response to nutrients
- Purification of mTORC1 containing mTOR, Raptor, mLST8 and PRAS40
- Cryo-EM reconstruction of mTORC1
- Antibody labeling of mTORC1
- EM structure of Raptor via negative staining
- High-throughput small-molecule screening for mTOR inhibitors using purified mTORC1

REPORTABLE OUTCOMES

- Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., and Sabatini, D.M. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* **2008**, 302 (5882), 1496-1501.

- Sancak, Y. and Sabatini, D.M. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Gordon Conference: Phosphorylation and G-Protein Mediated Signaling Networks*, University of New England, Biddeford, ME, June 15-20, 2008.

CONCLUSION

Although the regulation of mTORC1 by amino acids is very robust, signaling events that lead to mTORC1 activation in response to amino acid availability are largely unknown. We found that binding of the Rag GTPase to Raptor is necessary and sufficient to mediate amino acid signaling to mTORC1, and also mediates the amino acid-induced relocalization of mTOR within the endomembrane system of the cell. Given the prevalence of cancer-linked mutations in the pathways that control mTORC1, it is possible that Rag function is also deregulated in human tumors. Motivated by the clinical significance of the mTORC1 pathway, we are currently assessing the details of amino-acid induced mTORC1 activation while trying to identify other Rag interacting proteins.

As for our structural analysis, we demonstrated that mTORC1 exists only as a dimer, and this structural characteristic could be essential in mediating its catalytic activity. In continuation of our cryo-EM study, we aim to locate the rapamycin binding site and to describe how rapamycin ultimately inhibits mTORC1 based on our preliminary reconstructions of mTORC1 and Raptor. It will also answer an important question regarding the location of the mTOR catalytic domain within the complex. Consequently, these information will uncover key structural features that differentiate the specificity of mTOR into mTORC1 and mTORC2. Additionally, our imaging techniques will allow us to map regions within mTORC1 that can facilitate the protein-protein interactions with known upstream/downstream substrates, such as Rheb, Rag, and S6K1.

REFERENCES

1. Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., and Sabatini, D.M. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* **2008**, 302 (5882), 1496-1501.

in others, such as adult liver, it does not substantially affect protein secretory function but rather controls select transcriptional programs such as lipogenesis. Preservation of the normal hepatic lipid profile suggests that compounds that inhibit XBP1 activation in the liver may reduce serum lipids without causing hepatic steatosis in patients with dyslipidemias.

Given XBP1's known function as a key mediator of the UPR, it was surprising that its function in regulating lipogenesis was unrelated to the ER stress response. Indeed, apoB-100 folding and secretion, as well as the overall hepatocyte protein secretory function, were minimally compromised by loss of XBP1, likely because XBP1 independent basal chaperone gene expression is sufficient to accommodate moderate secretory loads. Interestingly, IRE1 α , the upstream activator of XBP1, was constitutively active in the *Xbp1* Δ liver, suggesting the presence of a negative feedback loop that precisely maintains XBP1s protein levels even in the absence of ER stress. The nature of this signal, and its relationship to the ER stress response and to the activation of XBP1 in the

liver by carbohydrate feeding, require further investigation.

References and Notes

1. H. N. Ginsberg, Y. L. Zhang, A. Hernandez-Ono, *Obesity* **14** (suppl. 1), 415 (2006).
2. F. Foufelle, P. Ferre, *Biochem. J.* **366**, 377 (2002).
3. D. Ron, P. Walter, *Nat. Rev. Mol. Cell Biol.* **8**, 519 (2007).
4. A. L. Shaffer *et al.*, *Immunity* **21**, 81 (2004).
5. A. H. Lee, N. N. Iwakoshi, L. H. Glimcher, *Mol. Cell Biol.* **23**, 7448 (2003).
6. D. Acosta-Alvear *et al.*, *Mol. Cell* **27**, 53 (2007).
7. A. M. Reimold *et al.*, *Nature* **412**, 300 (2001).
8. A. H. Lee, G. C. Chu, N. N. Iwakoshi, L. H. Glimcher, *EMBO J.* **24**, 4368 (2005).
9. A. M. Reimold *et al.*, *Genes Dev.* **14**, 152 (2000).
10. R. Sriburi, S. Jackowski, K. Mori, J. W. Brewer, *J. Cell Biol.* **167**, 35 (2004).
11. N. O. Davidson, G. S. Shelleness, *Annu. Rev. Nutr.* **20**, 169 (2000).
12. M. M. Hussain, J. Iqbal, K. Anwar, P. Rava, K. Dai, *Front. Biosci.* **8**, s500 (2003).
13. M. C. Schotz, A. Scannu, I. H. Page, *Am. J. Physiol.* **188**, 399 (1957).
14. S. J. Stone *et al.*, *J. Biol. Chem.* **279**, 11767 (2004).
15. X. X. Yu *et al.*, *Hepatology* **42**, 362 (2005).
16. J. M. Ntambi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 11482 (2002).
17. P. Cohen *et al.*, *Science* **297**, 240 (2002).

18. L. Abu-Elheiga, W. Oh, P. Kordari, S. J. Wakil, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 10207 (2003).
19. J. D. Horton, J. L. Goldstein, M. S. Brown, *J. Clin. Invest.* **109**, 1125 (2002).
20. H. C. Towle, E. N. Kaytor, H. M. Shih, *Annu. Rev. Nutr.* **17**, 405 (1997).
21. M. Miyazaki *et al.*, *J. Biol. Chem.* **279**, 25164 (2004).
22. H. Basciano, L. Federico, K. Adeli, *Nutr. Metab.* **2**, 5 (2005).
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Supporting Online Material

www.sciencemag.org/cgi/content/full/320/5882/1492/DC1
Materials and Methods
Figs. S1 to S5
Tables S1 to S4
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The Rag GTPases Bind Raptor and Mediate Amino Acid Signaling to mTORC1

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The multiprotein mTORC1 protein kinase complex is the central component of a pathway that promotes growth in response to insulin, energy levels, and amino acids and is deregulated in common cancers. We find that the Rag proteins—a family of four related small guanosine triphosphatases (GTPases)—interact with mTORC1 in an amino acid-sensitive manner and are necessary for the activation of the mTORC1 pathway by amino acids. A Rag mutant that is constitutively bound to guanosine triphosphate interacted strongly with mTORC1, and its expression within cells made the mTORC1 pathway resistant to amino acid deprivation. Conversely, expression of a guanosine diphosphate-bound Rag mutant prevented stimulation of mTORC1 by amino acids. The Rag proteins do not directly stimulate the kinase activity of mTORC1, but, like amino acids, promote the intracellular localization of mTOR to a compartment that also contains its activator Rheb.

The mTOR complex 1 (mTORC1) branch of the mammalian target of rapamycin (mTOR) pathway is a major driver of cell growth in mammals and is deregulated in many common tumors (1). It is also the target of the drug rapamycin, which has generated considerable interest as an anticancer therapy.

Diverse signals regulate the mTORC1 pathway, including insulin, hypoxia, mitochondrial function, and glucose and amino acid availability. Many of these are integrated upstream of mTORC1 by the tuberous sclerosis complex (TSC1-TSC2) tumor suppressor, which acts as an important negative regulator of mTORC1 through its role as a guanosine triphosphatase (GTPase)-activating protein (GAP) for Rheb, a small guanosine triphosphate (GTP)-binding protein that potentially activates the protein kinase activity of mTORC1 (2). Loss of either TSC protein causes hyperactivation of mTORC1 signaling, even in the absence of many of the upstream signals that are normally required to

maintain pathway activity. A notable exception is the amino acid supply, as the mTORC1 pathway remains sensitive to amino acid starvation in cells lacking either TSC1 or TSC2 (3–5).

The mechanisms through which amino acids signal to mTORC1 remain mysterious. It is a reasonable expectation that proteins that signal the availability of amino acids to mTORC1 are also likely to interact with it, but, so far, no good candidates have been identified. Because most mTORC1 purifications rely on antibodies to isolate mTORC1, we wondered if in previous work antibody heavy chains obscured, during SDS-polyacrylamide electrophoresis (SDS-PAGE) analysis of purified material, mTORC1-interacting proteins of 45 to 55 kD. Indeed, using a purification strategy that avoids this complication (6), we identified the 44-kD RagC protein as copurifying with overexpressed raptor, the defining component of mTORC1 (7–10).

RagC is a Ras-related small GTP-binding protein and one of four Rag proteins in mammals (RagA, RagB, RagC, and RagD). RagA and RagB are very similar to each other and are orthologs of budding yeast Gtr1p, whereas RagC and RagD are similar and are orthologs of yeast Gtr2p (11–13). In yeast and in human cells, the Rag and Gtr proteins function as heterodimers consisting of one Gtr1p-like (RagA or RagB) and one Gtr2p-like (RagC or RagD) component (14, 15). The finding that RagC copurifies with raptor was intriguing to us because, in yeast, Gtr1p and Gtr2p regulate the intracellular sorting of the Gap1p amino acid permease (16) and microautophagy (17), processes modulated by amino acid levels and

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the TOR pathway (18–20). The Gtr proteins have been proposed to act downstream or in parallel to TORC1 in yeast because their overexpression induces microautophagy even in the presence of rapamycin, which normally suppresses it (17).

To verify our identification of RagC as an mTORC1-interacting protein, we expressed raptor with different pairs of Rag proteins in human embryonic kidney (HEK)-293T cells. Consistent with the Rags functioning as heterodimers, raptor copurified with RagA-C or RagB-C, but not with RagA-B or the Rap2A control protein (Fig. 1A). Because the nucleotide loading state of most GTP-binding proteins regulates their functions, we generated RagB, RagC, and RagD mutants predicted (14, 16, 17) to be restricted to the GTP- or guanosine diphosphate (GDP)-bound conformations (for simplicity, we call these mutants RagB^{GTP}, RagB^{GDP}, etc.) (6). When expressed with mTORC1 components, Rag heterodimers containing RagB^{GTP} immunoprecipitated with

more raptor and mTOR than did complexes containing wild-type RagB or RagB^{GDP} (Fig. 1B). The GDP-bound form of RagC increased the amount of copurifying mTORC1, so that RagB^{GTP}-C^{GDP} recovered the highest amount of endogenous mTORC1 of any heterodimer tested (Fig. 1C). Giving an indication of the strength of the mTORC1-RagB^{GTP}-C^{GDP} association, in this same assay, we could not detect coimmunoprecipitation of mTORC1 with Rheb1 (Fig. 1C), an established interactor and activator of mTORC1 (1). When expressed alone, raptor, but not mTOR, associated with RagB^{GTP}-D^{GDP}, which suggests that raptor is the key mediator of the Rag-mTORC1 interaction (Fig. 1D). Consistent with this, rictor, an mTOR-interacting protein that is only part of mTORC2 (1), did not copurify with any Rag heterodimer (Fig. 1C and fig. S1). Last, highly purified raptor interacted in vitro with RagB-D and, to a larger extent, with RagB^{GTP}-D^{GDP}, which indicates that the Rag-raptor interaction is most likely direct (Fig. 1E).

We tested whether various Rag heterodimers affected the regulation of the mTORC1 pathway within human cells. In HEK-293T cells, expression of the RagB^{GTP}-D^{GDP} heterodimer, which interacted strongly with mTORC1, not only activated the pathway, but also made it insensitive to deprivation for leucine or total amino acids, as judged by the phosphorylation state of the mTORC1 substrate T389 of S6K1 (Fig. 2, A and B). The wild-type RagB-C heterodimer had milder effects than RagB^{GTP}-C^{GDP}, making the mTORC1 pathway insensitive to leucine deprivation, but not to the stronger inhibition caused by total amino acid starvation (Fig. 2, A and B). Expression of RagB^{GDP}-D^{GTP}, a heterodimer that did not interact with mTORC1 (Fig. 1, C and D), had dominant-negative effects, as it eliminated S6K1 phosphorylation in the presence, as well as absence, of leucine or amino acids (Fig. 2, A and B). Expression of RagB^{GDP} alone also suppressed S6K1 phosphorylation (fig. S2). These results suggest that the activity of the mTORC1 pathway

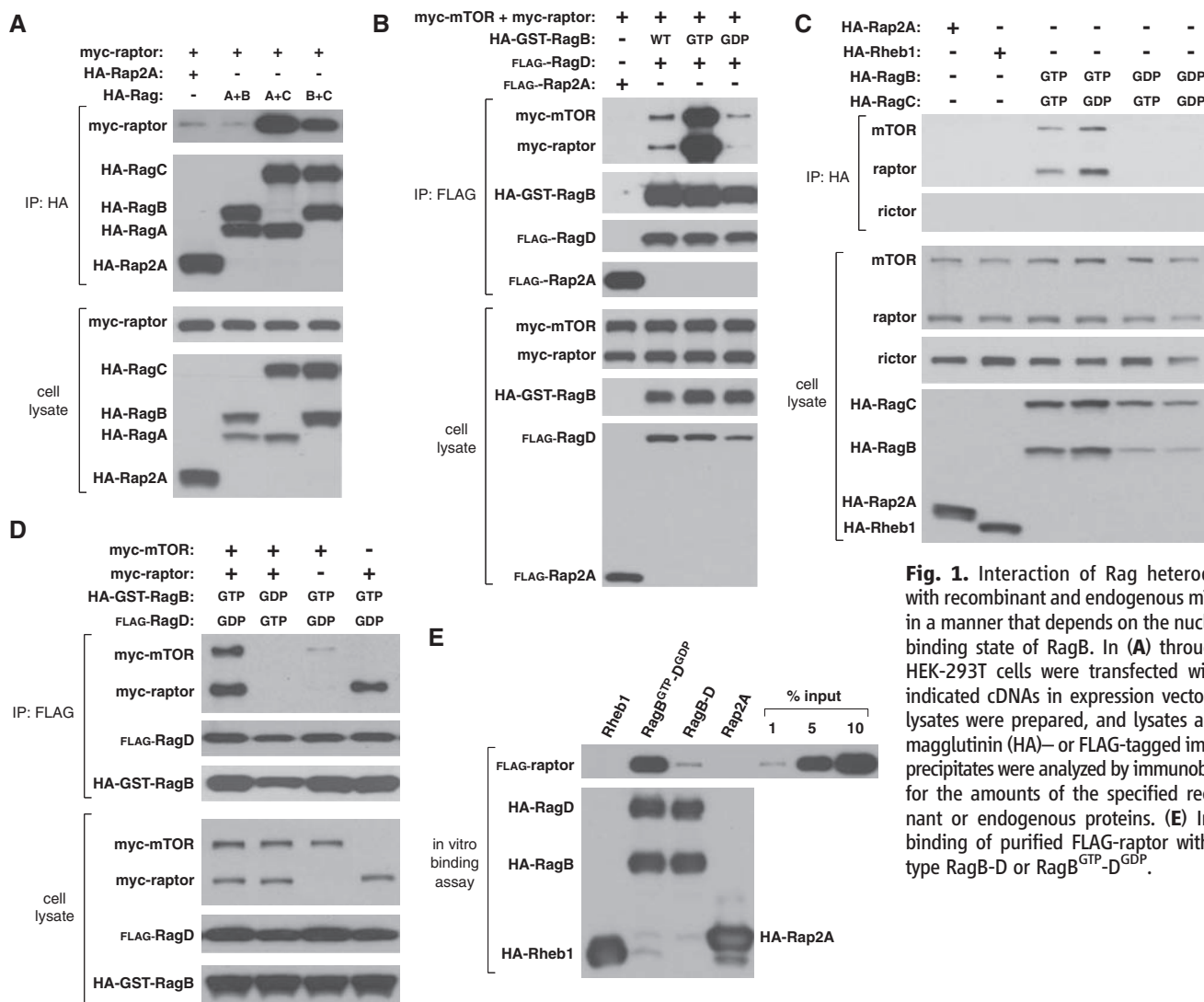


Fig. 1. Interaction of Rag heterodimers with recombinant and endogenous mTORC1 in a manner that depends on the nucleotide binding state of RagB. In (A) through (D) HEK-293T cells were transfected with the indicated cDNAs in expression vectors, cell lysates were prepared, and lysates and heparagglutinin (HA)- or FLAG-tagged immunoprecipitates were analyzed by immunoblotting for the amounts of the specified recombinant or endogenous proteins. (E) In vitro binding of purified FLAG-raptor with wild-type RagB-D or RagB^{GTP}-D^{GDP}.

under normal growth conditions depends on endogenous Rag function.

To verify the actions of the Rags in a more physiological setting than that achieved by transient cDNA transfection, we generated HEK-293T cell lines stably expressing Rheb1, RagB, or RagB^{GTP} (attempts to generate lines stably expressing RagB^{GDP} failed). Under normal growth conditions, these cells were larger than control cells and had higher levels of mTORC1 pathway activity (Fig. 3A). Unlike transient Rheb1 overexpression (Fig. 2, A and B), stable expression did not make the mTORC1 pathway insensitive to leucine or amino acid starvation (Fig. 3, B and C), consistent with evidence that transiently overexpressed Rheb may have non-physiological consequences on amino acid signaling to mTORC1 (4, 5). Stable expression of a Rheb1^{GTP} mutant was also unable to make the mTORC1 pathway resistant to amino acid deprivation (fig. S3). In contrast, stable expression of RagB^{GTP} eliminated the sensitivity of the mTORC1 pathway to leucine or total amino acid withdrawal, whereas that of wild-type RagB overcame sensitivity to leucine but not to amino

acid starvation (Fig. 3, B and C). Thus, transient or stable expression of the appropriate Rag mutants is sufficient to put the mTORC1 pathway into states that mimic the presence or absence of amino acids.

To determine if the Rag mutants affect signaling to mTORC1 from inputs besides amino acids, we tested whether in RagB^{GTP}-expressing cells the mTORC1 pathway was resistant to other perturbations known to inhibit it. This was not the case, as oxidative stress, mitochondrial inhibition, or energy deprivation still reduced S6K1 phosphorylation in these cells (fig. S4). Moreover, in HEK-293E cells, expression of RagB^{GTP}-D^{GDP} did not maintain mTORC1 pathway activity in the absence of insulin (Fig. 2C). Expression of the dominant-negative RagB^{GDP}-D^{GTP} heterodimer did, however, block insulin-stimulated phosphorylation of S6K1 (Fig. 2C), as did amino acid starvation (Fig. 2D). Thus, although RagB^{GTP} expression mimics amino acid sufficiency, it cannot substitute for other inputs that mTORC1 normally monitors.

This evidence for a primary role of the Rag proteins in amino acid signaling to mTORC1

raised the question of where, within the pathway that links amino acids to mTORC1, the Rag proteins might function. The existence of the Rag-mTORC1 interaction (Fig. 1), the effects on amino acid signaling of the Rag mutants (Figs. 2 and 3), and the sensitivity to rapamycin of the S6K1 phosphorylation induced by RagB^{GTP} (fig. S4), strongly suggested that the Rag proteins function downstream of amino acids and upstream of mTORC1. To verify this, we took advantage of the established finding that cycloheximide reactivates mTORC1 signaling in cells starved for amino acids by blocking protein synthesis and thus boosting the levels of the intracellular amino acids sensed by mTORC1 (21–23). Thus, if the Rag proteins act upstream of amino acids, cycloheximide should overcome the inhibitory effects of the RagB^{GDP}-C^{GTP} heterodimer on mTORC1 signaling, but if they are downstream, cycloheximide should not reactivate the pathway. The results were clear: cycloheximide treatment of cells reversed the inhibition of mTORC1 signaling caused by leucine deprivation, but not that caused by expression of RagB^{GDP}-C^{GTP} (fig. S5). Given the place-

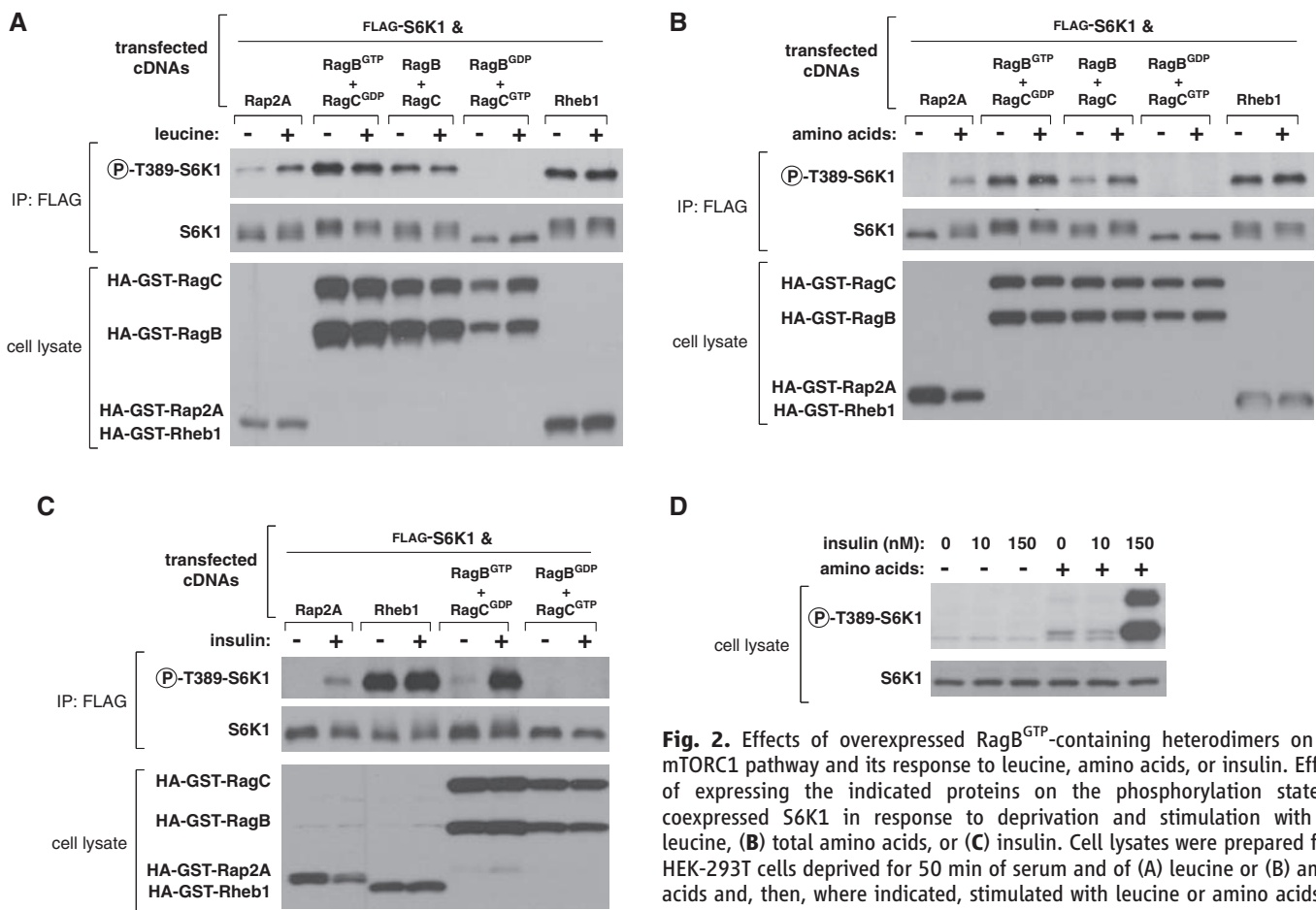


Fig. 2. Effects of overexpressed RagB^{GTP}-containing heterodimers on the mTORC1 pathway and its response to leucine, amino acids, or insulin. Effects of expressing the indicated proteins on the phosphorylation state of coexpressed S6K1 in response to deprivation and stimulation with (A) leucine, (B) total amino acids, or (C) insulin. Cell lysates were prepared from HEK-293T cells deprived for 50 min of serum and of (A) leucine or (B) amino acids and, then, where indicated, stimulated with leucine or amino acids for 10 min. HEK-293E cells (C) were deprived of serum for 50 min and, where indicated, stimulated with 150 nM insulin for 10 min. Lysates and FLAG-immunoprecipitates were analyzed for the levels of the specified proteins and the phosphorylation state of S6K1. (D) Effects of amino acid deprivation on insulin-mediated activation of mTORC1. HEK-293E cells were starved for serum and amino acids or just serum for 50 min, and where specified, stimulated with 10 or 150 nM insulin. Cell lysates were analyzed for the level and phosphorylation state of S6K1.

ment of the Rag proteins downstream of amino acids and upstream of mTORC1, we determined whether amino acids regulate the Rag-mTORC1 interaction within cells. Initial tests using transiently coexpressed Rag proteins and mTORC1 components did not reveal any regulation of the interaction. Because we reasoned that pronounced overexpression might overcome the normal regulatory mechanisms that operate within the cell, we developed an assay (6), based on a reversible chemical cross-linker, that allows us to detect the interaction of stably expressed FLAG-tagged Rag proteins with endogenous mTORC1. With this approach, we readily found that amino acids, but not insulin, promote the Rag-mTORC1 interaction when we used either FLAG-tagged RagB or

RagD to isolate mTORC1 from cells (Fig. 3D and fig. S6A). As the GTP-loading state of the Rag proteins also regulates the Rag-mTORC1 interaction (Fig. 1), we determined whether amino acids modulate the amount of GTP bound to RagB. Indeed, amino acid stimulation of cells increased the GTP loading of RagB (Fig. 3E). Consistent with this, amino acids did not further augment the already high level of interaction between mTORC1 and the RagB^{GTP} mutant (Fig. 3D).

To determine whether the Rag proteins are necessary for amino acids to activate the mTORC1 pathway, we used combinations of lentivirally delivered short hairpin RNAs (shRNAs) to suppress RagA and RagB or RagC and RagD at the same time. Loss of RagA and RagB also led

to the loss of RagC and RagD and vice versa, which suggests that, within cells, the Rag proteins are unstable when not in heterodimers (Fig. 3F). In cells with a reduction in the expression of all the Rag proteins, leucine-stimulated phosphorylation of S6K1 was strongly reduced (Fig. 3G). The role of the Rag proteins appears to be conserved in *Drosophila* cells as double-stranded RNA-mediated suppression of the *Drosophila* orthologs of RagB or RagC eliminated amino acid-induced phosphorylation of dS6K (Fig. 3H). Consistent with amino acids being necessary for activation of mTORC1 by insulin, a reduction in Rag expression also suppressed insulin-stimulated phosphorylation of S6K1 (fig. S6B). Thus, the Rag proteins appear to be both necessary and

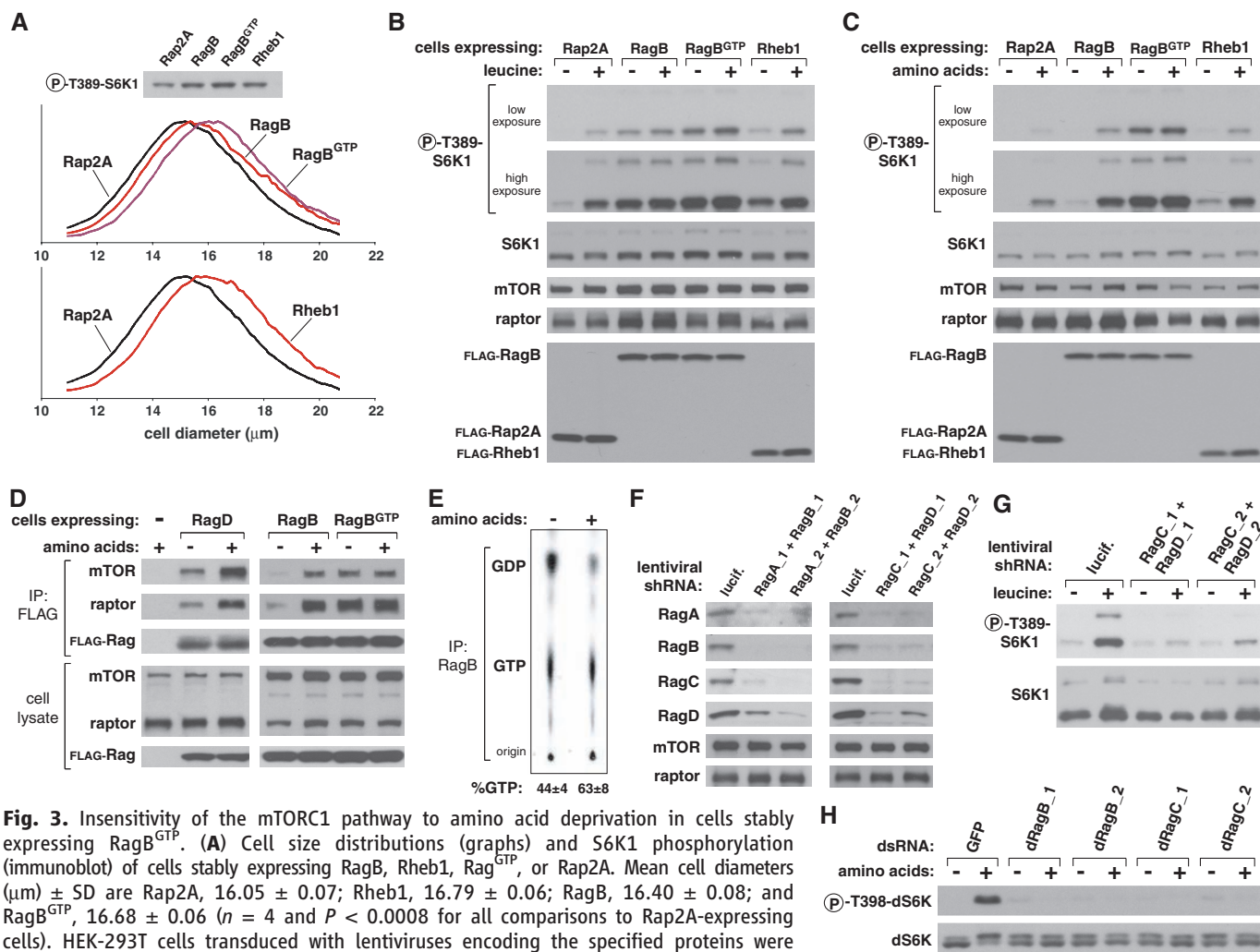


Fig. 3. Insensitivity of the mTORC1 pathway to amino acid deprivation in cells stably expressing RagB^{GTP}. (A) Cell size distributions (graphs) and S6K1 phosphorylation (immunoblot) of cells stably expressing RagB, Rheb1, Rag^{GTP}, or Rap2A. Mean cell diameters (μm) ± SD are Rap2A, 16.05 ± 0.07; Rheb1, 16.79 ± 0.06; RagB, 16.40 ± 0.08; and RagB^{GTP}, 16.68 ± 0.06 (*n* = 4 and *P* < 0.0008 for all comparisons to Rap2A-expressing cells). HEK-293T cells transduced with lentiviruses encoding the specified proteins were deprived for 50 min for serum and (B) leucine or (C) total amino acids, and, where indicated, restimulated with leucine or amino acids for 10 min. Cell lysates were analyzed for the levels of the specified proteins and the phosphorylation state of S6K1. (D) Amino acid-stimulated interaction of the Rag proteins with mTORC1. HEK-293T cells stably expressing FLAG-tagged RagB, RagD, or RagB^{GTP} were starved for amino acids and serum for 50 min and, where indicated, restimulated with amino acids for 10 min. Cells were then processed with a chemical cross-linking assay, and cell lysates and FLAG immunoprecipitates were analyzed for the amounts of the indicated proteins. (E) Effects of amino acid stimulation on GTP loading of RagB. Values are means ± SD for *n* = 3 (*P* < 0.02 for increase in GTP loading caused by amino acid stimulation). (F) Abundance of RagA, RagB, RagC, and RagD in HeLa cells expressing the indicated shRNAs. (G) S6K1 phosphorylation in HeLa cells expressing shRNAs targeting RagC and RagD. Cells were deprived of serum and leucine for 50 min, and, where indicated, were restimulated with leucine for 10 min. (H) Effects of double-stranded RNA (dsRNA)-mediated knockdowns of *Drosophila* orthologs of RagB or RagC on amino acid-induced phosphorylation of dS6K.

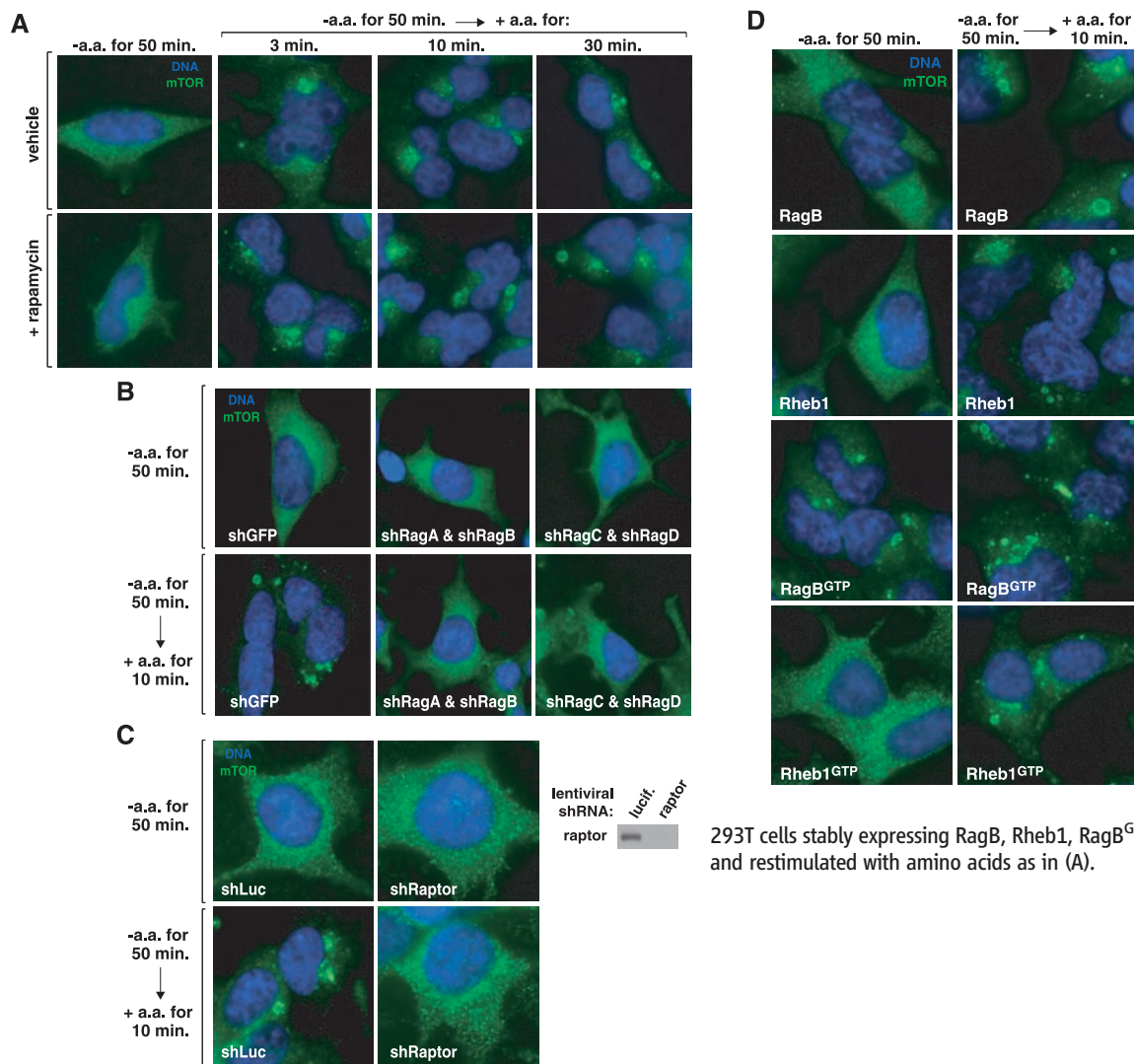


Fig. 4. Rag-dependent regulation by amino acids of the intracellular localization of mTOR. **(A)** HEK-293T cells were starved for serum and amino acids for 50 min or starved and then restimulated with amino acids for the indicated times in the presence or absence of rapamycin. Cells were then processed in an immunofluorescence assay to detect mTOR (green), costained with 4',6'-diamidino-2-phenylindole (DAPI) for DNA content (blue), and imaged. Of these cells, 80 to 90% exhibited the mTOR localization pattern shown. **(B)** and **(C)** mTOR localization in HEK-293T cells expressing the indicated shRNAs and deprived and restimulated with amino acids as in **(A)**. Immunoblot of raptor expression levels. **(D)** mTOR localization in HEK-293T cells stably expressing RagB, Rheb1, RagB^{GTP}, or Rheb1^{GTP} and deprived and restimulated with amino acids as in **(A)**.

sufficient for mediating amino acid signaling to mTORC1.

Unlike Rheb (24, 25), the Rag heterodimers did not directly stimulate the kinase activity of mTORC1 *in vitro* (fig. S7), so we considered the possibility that the Rag proteins regulate the intracellular localization of mTOR. mTOR is found on the endomembrane system of the cell, including the endoplasmic reticulum, Golgi apparatus, and endosomes (26, 27). The intracellular localization of endogenous mTOR, as revealed with an antibody that we validated recognizes mTOR in immunofluorescence assays (fig. S8), was strikingly different in cells deprived of amino acids than in cells starved and briefly restimulated with amino acids (Fig. 4A and fig. S11) or growing in fresh complete media (fig. S9). In starved cells, mTOR was in tiny puncta throughout the cytoplasm, whereas in cells stimulated with amino acids for as little as 3 min, mTOR localized to the perinuclear region of the cell, to large vesicular structures, or to both (Fig. 4A). Rapamycin did not block the change in mTOR

localization induced by amino acids (Fig. 4A), which indicated that it is not a consequence of mTORC1 activity but rather may be one of the mechanisms that underlies mTORC1 activation. The amino acid-induced change in mTOR localization required expression of the Rag proteins and of raptor (Fig. 4, B and C), and amino acids also regulated the localization of raptor (fig. S10).

In cells overexpressing RagB, Rheb1, or Rheb1^{GTP}, mTOR behaved as in control cells, its localization changing upon amino acid stimulation from small puncta to the perinuclear region and vesicular structures (Fig. 4D). In contrast, in cells overexpressing the RagB^{GTP} mutant that eliminates the amino acid sensitivity of the mTORC1 pathway, mTOR was already present on the perinuclear and vesicular structures in the absence of amino acids, and became even more localized to them upon the addition of amino acids (Fig. 4D). Thus, there is a correlation, under amino acid-starvation conditions, between the activity of the mTORC1 pathway and the subcellular localization of mTOR,

which implies a role for Rag-mediated mTOR translocation in the activation of mTORC1 in response to amino acids.

We failed to find an established marker of the endomembrane system that colocalized with mTOR in amino acid-starved cells. However, in cells stimulated with amino acids, mTOR in the perinuclear region and on the large vesicular structures overlapped with Rab7 (Fig. 5A), which indicated that a substantial fraction of mTOR translocated to the late endosomal and lysosomal compartments in amino acid-replete cells. In cells expressing RagB^{GTP}, mTOR was present on the Rab7-positive structures even in the absence of amino acids (Fig. 5B).

The perinuclear region and vesicular structures on which mTOR appears after amino acid stimulation are similar to the Rab7-positive structures where green fluorescent protein (GFP)-tagged Rheb localizes in human cells (28, 29). Unlike mTOR, however, amino acids did not appreciably affect the localization of Rheb, as GFP-Rheb1 colocalized with *Discosoma* red fluorescent protein (DsRed)-labeled Rab7 (DsRed-

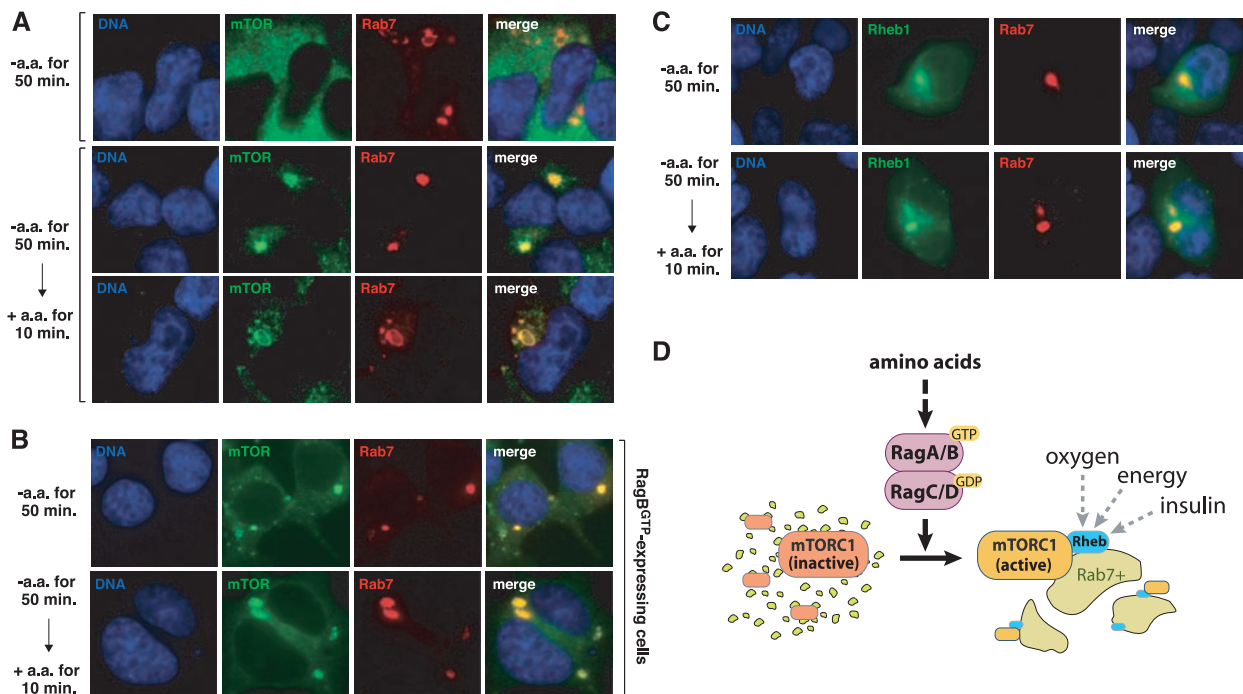


Fig. 5. Amino acids promote the localization of mTOR to a Rab7-positive compartment that also contains Rheb. **(A)** mTOR and Rab7 localization in cells deprived or stimulated with amino acids. HEK-293T cells transiently transfected with a cDNA for DsRed-Rab7 were starved for serum and amino acids for 50 min and, where indicated, stimulated with amino acids for 10 min. Cells were then processed to detect mTOR (green), Rab7 (red), and DNA content (blue), and imaged. Two examples are shown of mTOR localization in the

presence of amino acids. **(B)** HEK-293T cells stably expressing RagB^{GTP} and transiently transfected with a cDNA for DsRed-Rab7 were treated and processed as in **(A)**. **(C)** Rheb1 and Rab7 localization in cells deprived or stimulated with amino acids. HEK-293T cells transiently transfected with 1 to 2 ng of cDNAs for GFP-Rheb1 and DsRed-Rab7 were treated as in **(A)**, processed to detect Rheb1 (green), Rab7 (red), and DNA content (blue), and imaged. **(D)** Model for role of Rag GTPases in signaling amino acid availability to mTORC1.

Rab7) in the presence or absence of amino acids (Fig. 5C). Unfortunately, it is currently not possible to compare, in the same cells, the localization of endogenous mTOR with that of Rheb, because the signal for GFP-Rheb or endogenous Rheb is lost after fixed cells are permeabilized to allow access to intracellular antigens (28, 29). Nevertheless, given that both mTOR and Rheb are present in Rab7-positive structures after amino acid stimulation, we propose that amino acids might control the activity of the mTORC1 pathway by regulating, through the Rag proteins, the movement of mTORC1 to the same intracellular compartment that contains its activator Rheb (see model in Fig. 5D). This would explain why activators of Rheb, like insulin, do not stimulate the mTORC1 pathway when cells are deprived of amino acids and why Rheb is necessary for amino acid-dependent mTORC1 activation (4) (fig. S12). When Rheb is highly overexpressed, some might become mislocalized and inappropriately encounter and activate mTORC1, which could explain why Rheb overexpression, but not loss of TSC1 or TSC2, makes the mTORC1 pathway insensitive to amino acids (4, 5).

In conclusion, the Rag GTPases bind rapTOR, are necessary and sufficient to mediate amino acid signaling to mTORC1, and mediate the amino acid-induced relocalization of mTOR within the endomembrane system of

the cell. Given the prevalence of cancer-linked mutations in the pathways that control mTORC1 (1), it is possible that Rag function is also down-regulated in human tumors.

References and Notes

1. D. A. Guertin, D. M. Sabatini, *Cancer Cell* **12**, 9 (2007).
2. D. D. Sarbassov, S. M. Ali, D. M. Sabatini, *Curr. Opin. Cell Biol.* **17**, 596 (2005).
3. M. P. Byfield, J. T. Murray, J. M. Backer, *J. Biol. Chem.* **280**, 33076 (2005).
4. T. Nobukuni et al., *Proc. Natl. Acad. Sci. U.S.A.* **102**, 14238 (2005).
5. E. M. Smith, S. G. Finn, A. R. Tee, G. J. Browne, C. G. Proud, *J. Biol. Chem.* **280**, 18717 (2005).
6. Materials and methods are available as supporting material on Science Online.
7. D.-H. Kim et al., *Cell* **110**, 163 (2002).
8. R. Loewith et al., *Mol. Cell* **10**, 457 (2002).
9. K. P. Wedaman et al., *Mol. Biol. Cell* **14**, 1204 (2003).
10. K. Hara et al., *Cell* **110**, 177 (2002).
11. M. Bun-Ya, S. Harashima, Y. Oshima, *Mol. Cell. Biol.* **12**, 2958 (1992).
12. A. Schurmann, A. Brauers, S. Massmann, W. Becker, H. G. Joost, *J. Biol. Chem.* **270**, 28982 (1995).
13. E. Hirose, N. Nakashima, T. Sekiguchi, T. Nishimoto, *J. Cell Sci.* **111**, 11 (1998).
14. N. Nakashima, E. Noguchi, T. Nishimoto, *Genetics* **152**, 853 (1999).
15. T. Sekiguchi, E. Hirose, N. Nakashima, M. Ii, T. Nishimoto, *J. Biol. Chem.* **276**, 7246 (2001).
16. M. Gao, C. A. Kaiser, *Nat. Cell Biol.* **8**, 657 (2006).
17. F. Dubouloz, O. Deloche, V. Wanke, E. Camerini, C. De Virgilio, *Mol. Cell* **19**, 15 (2005).
18. J. B. Kunz, H. Schwarz, A. Mayer, *J. Biol. Chem.* **279**, 9987 (2004).
19. K. J. Roberg, N. Rowley, C. A. Kaiser, *J. Cell Biol.* **137**, 1469 (1997).
20. E. J. Chen, C. A. Kaiser, *J. Cell Biol.* **161**, 333 (2003).
21. A. Beugnet, A. R. Tee, P. M. Taylor, C. G. Proud, *Biochem. J.* **372**, 555 (2003).
22. G. R. Christie, E. Hajdich, H. S. Hundal, C. G. Proud, P. M. Taylor, *J. Biol. Chem.* **277**, 9952 (2002).
23. D. J. Price, R. A. Nemenoff, J. Avruch, *J. Biol. Chem.* **264**, 13825 (1989).
24. Y. Sancak et al., *Mol. Cell* **25**, 903 (2007).
25. X. Long, Y. Lin, S. Ortiz-Vega, K. Yonezawa, J. Avruch, *Curr. Biol.* **15**, 702 (2005).
26. R. M. Drenan, X. Liu, P. G. Bertram, X. F. Zheng, *J. Biol. Chem.* **279**, 772 (2004).
27. M. Mavrikis, J. Lippincott-Schwartz, C. A. Stratakis, I. Bossis, *Autophagy* **3**, 151 (2007).
28. K. Saito, Y. Araki, K. Kontani, H. Nishina, T. Katada, *J. Biochem.* **137**, 423 (2005).
29. C. Burger, B. DeVries, V. Stambolic, *Biochem. Biophys. Res. Commun.* **344**, 869 (2006).
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Supporting Online Material

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Materials and Methods
Figs. S1 to S12
References

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