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MINI-REVIEW

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# Amino acid and small GTPase regulation of mTORC1

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**ABSTRACT**

The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that belongs to the phosphatidylinositol 3-kinase-related kinase (PIKK) family. mTOR is the catalytic subunit of mTOR complex 1 (mTORC1), which integrates multiple environmental signals to control cell growth and metabolism. Nutrients, specifically amino acids, are the most potent stimuli for mTORC1 activation. Multiple studies have focused on how leucine and arginine activate mTORC1 through the Rag GTPases, with mechanistic details slowly emerging. Recently, a Rag GTPase-independent glutamine signaling pathway to mTORC1 has been identified, suggesting that mTORC1 is differentially regulated through distinct pathways by specific amino acids. In this review, we summarize our current understanding of how amino acids modulate mTORC1, and the role of other small GTPases in the regulation of mTORC1 activity.

## Introduction

Rapamycin is a natural product that was first isolated from the bacterium *Streptomyces hygroscopicus* found in soil samples collected from Easter Island (also known as Rapa Nui).[1] Rapamycin is a macrolide lactone that was first characterized as having antifungal, antibacterial, and immunosuppressive activity.[2,3] Suspecting a potential therapeutic role for rapamycin, Dr. Suren Sehgal sent a sample of rapamycin to the National Cancer Institute (NCI) Developmental Therapeutics Program for anti-tumor activity screening. Subsequently, rapamycin and its analogs (rapalogs) have been approved by the Food and Drug Administration (FDA) as cancer therapeutics and immunosuppressive agents.[4] The discovery of rapamycin, and the identification of the cellular target of rapamycin, has initiated the elucidation of an essential cellular signaling pathway that controls a wide range of biological processes. Genetic studies in the budding yeast *Saccharomyces cerevisiae* identified two genes, later named target of rapamycin 1 and 2 (TOR1 and TOR2), whose mutations conferred growth resistance in *Saccharomyces cerevisiae* treated with rapamycin.[5] Tor1 and Tor2 are structurally and functionally similar, but they are not identical.[6] Simultaneous disruption of both TOR genes, but not the disruption of a single TOR gene, produces a growth phenotype that resembles rapamycin treatment. Subsequent biochemical work in mammalian cell lines identified the mammalian target of rapamycin (mTOR), as the binding partner for rapamycin and the small immunophilin protein, FK506 binding protein 12 (FKBP12).[7-9] mTOR is found in two distinct complexes referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is composed of mTOR, mammalian lethal with SEC13 protein 8 (mLST8), DEP domain-

containing mTOR-interacting protein (DEPTOR), proline-rich AKT substrate of 40 kDa (PRAS40), and regulatory associated protein of mTOR (raptor).[10-17] Similarly, there are two separate TOR complexes in yeast, called TORC1 and TORC2. TORC1 consists of Tor1 or Tor2, controller of growth 1 (Kog1, raptor ortholog), lethal with SEC13 8 (Lst8, mLST8 ortholog), and Tor complex one 89 (Tco89, no obvious mammalian homolog).[18,19] Despite mTORC1 and mTORC2 both containing the catalytic subunit mTOR, each complex responds to distinct stimuli and regulates different downstream signaling pathways. Both complexes respond to growth factor stimulation, however, only mTORC1 responds to amino acids. Therefore, this review will only discuss mTORC1 and mTORC2 is reviewed elsewhere.[20] Nutrients utilized by eukaryotic cells include carbohydrates, lipids, and amino acids. Amino acids are particularly important, as they are the essential building blocks for protein synthesis as well as an energy and carbon source for many cellular metabolic processes.[21] By sensing amino acid availability, mTORC1 can regulate various cellular processes, including anabolic pathways such as protein, lipid, and nucleotide biosynthesis and catabolic pathways such as autophagy.[22,23] Similarly, in yeast, TORC1 controls protein synthesis, ribosome synthesis, and autophagy in response to environmental signals.[24] How mTORC1 senses the nutrient status of the cell is a long-standing question in the field. In this review, we will summarize our current understanding of the regulation of mTORC1 by amino acids, particularly how leucine, arginine, and glutamine activate mTORC1 in mammals and how methionine, leucine, and glutamine activate TORC1 in yeast. Furthermore, we will examine the role of small guanosine triphosphatases (GTPases) involved in this regulation.

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## Rag GTPase-dependent activation of mTORC1

The quest to decipher mTORC1 regulation by amino acids started to take off with the identification of the Ras-related guanosine triphosphate (GTP)-binding (Rag) proteins as mediators of amino acid signaling to mTORC1. The first Rag GTPase to be identified was Gtr1 in *Saccharomyces cerevisiae*, which was thought to play a role in phosphate uptake.[25] Later, RagA was shown to be the mammalian ortholog of Gtr1 and involved in the Ran/Gsp1-GTPase pathway.[26] The function of the Rag GTPases was largely unknown until two independent groups, one employing an RNA interference (RNAi) screen of GTPases in *Drosophila* S2 cells,[27] and the other utilizing co-immunoprecipitation-mass spectrometry (IP-MS) with overexpressed raptor in human embryonic kidney 293T (HEK293T) cells,[28] identified the Rag GTPases as important mediators of amino acid signaling to mTORC1 (Fig. 1). In mammals, the Rag GTPases consist of RagA, RagB, RagC, and RagD. RagA and RagB are functionally redundant and highly similar proteins that share ∼90% sequence identity. RagC and RagD are also functionally redundant and share ∼81% sequence identity. RagA or RagB bind to RagC or RagD to form a functional and stable heterodimeric complex.[29] The yeast ortholog of RagA/B is Gtr1 and the yeast ortholog of RagC/D is Gtr2. Gtr1 and Gtr2 also form a functional obligate heterodimer.[30] Similar to other GTP-binding proteins, the nucleotide loading state of the Rag GTPases regulates their function. Under amino acid-sufficient conditions, RagA/B is GTP-bound and RagC/D is GDP-bound; this constitutes an active Rag complex. When amino acids are scarce, RagA/B is GDP-bound and RagC/D is GTP-bound; this constitutes an inactive Rag complex. Similarly, when nutrients are plentiful Gtr1 and Gtr2 are GTP- and GDP-bound, respectively.[31] The nucleotide status of RagA/B (or Gtr1 in yeast) is sufficient for regulating the activation of mTORC1. Expression of constitutively GTP- or GDP-bound RagA/B (or Gtr1 in yeast) can activate or inactivate mTORC1 activity, respectively, regardless of amino acid availability.[27,28,31]

The Rag GTPases do not directly stimulate kinase activity of mTORC1; rather they help anchor mTORC1 at the lysosome in response to amino acids. In particular, the active Rag complex interacts with raptor, while the inactive Rag complex does not interact with raptor.[28] The lysosomal localization of mTORC1 in response to amino acids promotes mTORC1 activity by bringing the complex in contact with Rheb, a potent
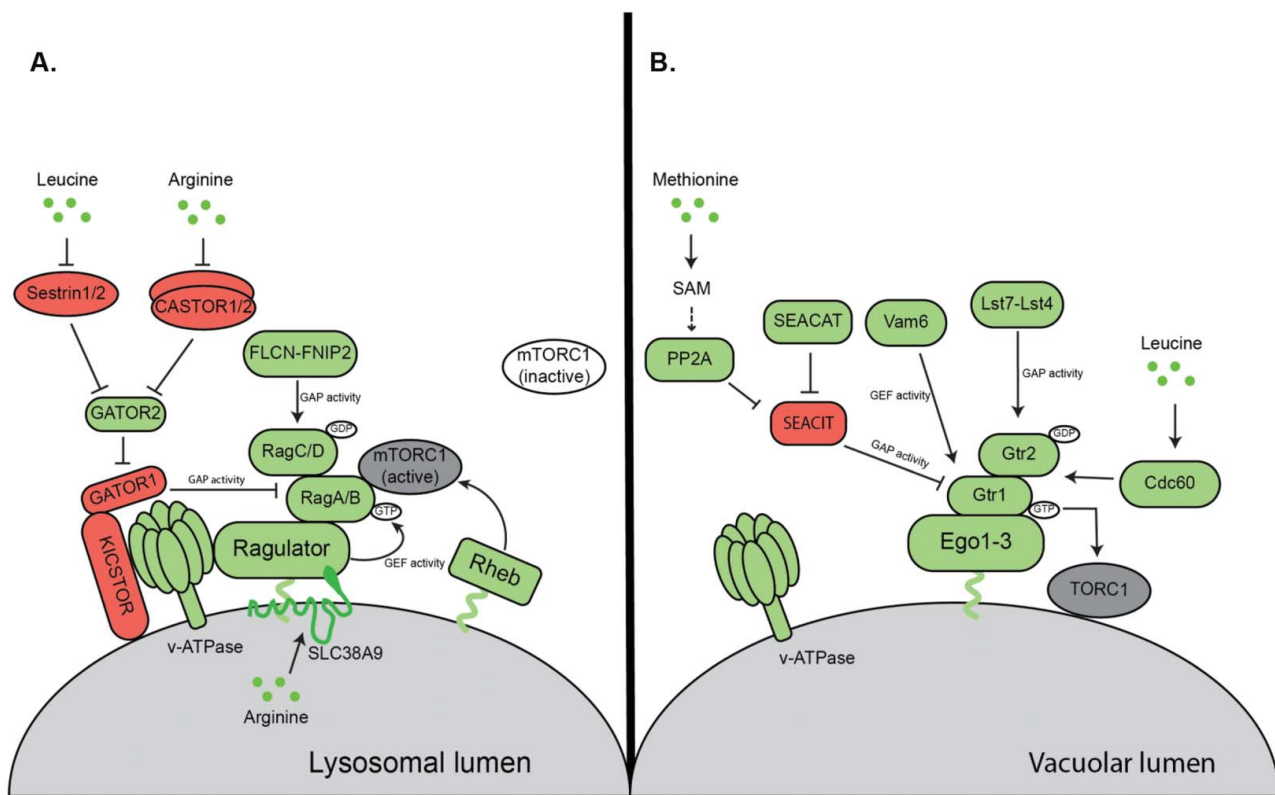


**Figure 1.** Rag GTPase-dependent amino acid signaling pathway upstream of mTORC1. (A) Current model of mTORC1 activation by amino acids in mammals. In the presence of amino acids, the heterodimeric Rag GTPase complex is comprised of GTP-bound RagA/B and GDP-bound RagC/D (active Rag complex). The active Rag complex anchors mTORC1 to the lysosome. During amino acid scarcity, mTORC1 is dispersed throughout the cell at an unknown location. Ragulator functions as a scaffold to anchor the Rag complex to the lysosome, and is a GEF for RagA/B. v-ATPase binds to Ragulator and is required for mTORC1 activity. GATOR1 functions upstream of the Rag complex as a GAP for RagA/B. KICKSTOR is thought to tether GATOR1 to the lysosome. GATOR2 functions upstream of GATOR1 and inhibits GATOR1 through an unknown mechanism. Sestrin1/2 is a cytosolic leucine sensor and CASTOR1/2 is a cytosolic arginine sensor. In the absence of amino acids, Sestrin1/2 and CASTOR1/2 bind to and inhibit GATOR2. The presence of leucine or arginine interrupts the interaction between Sestrin1/2 and GATOR2 or CASTOR1/2 and GATOR2. FLCN-FNIP2 is a GAP for RagC/D. Positive regulators of mTORC1 are indicated in green and negative regulators are indicated in red. (B) Current model of TORC1 activation by amino acids in yeast. In their active form, Gtr1 binds to GTP and Gtr2 binds to GDP. Ego1, Ego2, and Ego3 form the EGO complex that anchors the Gtr complex to the lysosome. Vam6 is a GEF for Gtr1. Lst7-Lst4 acts as a GAP for Gtr2. SEACAT inhibits SEACIT, which is a GAP for Gtr1. Methionine activates TORC1 activity by promoting SAM synthesis, which in turn activates PP2A and inhibits SEACIT. Leucine activates TORC1 through Cdc60. Positive regulators of TORC1 are indicated in green and negative regulators are indicated in red.

mTORC1 activator downstream of growth factor signaling. GTP-bound Rheb is thought to bind and activate mTORC1, through an unclear mechanism.[32] Because the Rag GTPases lack any obvious lipid anchoring motifs, a pentameric complex termed Ragulator facilitates their localization to the lysosomal surface. The Ragulator is comprised of p18, p14, MEK partner 1 (MP1), C7orf59, and hepatitis B virus X-interacting protein (HBXIP) (also referred to as LAMTOR1-5, respectively).[33,34] The N-terminal region of p18 is myristoylated and palmitoylated, and these lipid modifications anchor the Ragulator, Rags, and mTORC1 to the lysosomal surface.[33-35] Although sequence homology searches have failed to identify Ragulator orthologs in yeast, a genetic screen for mutants that are unable to recover from rapamycin treatment (*ego* mutants: <u>e</u>xit from rapamycin-induced <u>gro</u>wth arrest) has revealed that Gtr2 forms a complex with Ego1 and Ego3.[36] Subsequent independent studies have confirmed Gtr1, Gtr2, Ego1, and Ego3 are all components of the EGO complex (EGOC). More recently, Ego2 has been identified as an essential part of EGOC that is required for complex integrity and localization.[37] In addition, structural comparison of EGOC and Ragulator revealed several similar features, suggesting that the structure and scaffolding function of Ragulator is conserved in yeast.[38] Similar to p18, Ego1 anchors EGOC to the vacuolar membrane through its N-terminal myristoylated and palmitoylated residues. C7orf59 and HBXIP are also structurally similar to Ego2, which functions to stabilize EGOC. p14 and MP1 form a heterodimer with each other that is structurally similar to Ego3.[39] In addition, p14, MP1, HBXIP, and C7orf59 contain a roadblock domain, which is thought to serve as a platform for Rag protein binding. Consistently, Ego2 and Ego3 contain roadblock domain-like folds which serve as the interaction surface for the Gtr1-Gtr2 dimer, reinforcing the structural and functional similarities between Ragulator and EGOC.[40] Although there is significant functional and structural similarity between the Rag and Gtr complexes as well as the Ragulator and EGOC complexes, it is worthwhile to note that mTORC1 shuttles to and from the lysosome in response to amino acids while TORC1 remains on the vacuole regardless of amino acid status.[31] These localization differences suggest that there might be additional unknown regulatory mechanisms and differences in mammalian mTORC1 and yeast TORC1 under different nutrient conditions.

The activity of the Rag GTPases, like other small GTPases, is modulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs).[41,42] Besides being a scaffold for the Rag complex, Ragulator also functions as a GEF for RagA/B. Ragulator's role as a RagA/B GEF is supported by the fact that Ragulator has a stronger interaction with nucleotide-free or GDP-bound RagA/B than GTP-bound RagA/B, and pentameric Ragulator purified from cells accelerated GTP and GDP dissociation from RagA and RagB. However, pull-down of Ragulator subassemblies (p14-MP1, HBXIP-C7orf59, p14-MP1-p18, etc.) did not change the nucleotide dissociation rate, indicating that the entire Ragulator complex is necessary for GEF activity.[34] Previous work also demonstrated that Ragulator interacts with the vacuolar H$^+$-ATPase (v-ATPase), which consists of multicomponent V$_0$ and V$_1$ domains.[43] Current understanding of how the v-ATPase functions suggests that ATP hydrolysis by the V$_1$ domain provides torque to rotate the transmembrane domain, V$_0$. Rotation of the V$_0$ domain transfers protons into the lysosomal lumen and consequently acidifies the lysosome.[44] While the v-ATPase does not appear to affect Ragulator-Rag complex binding, it is believed to regulate Ragulator's GEF activity towards RagA/B. In yeast, however, the role of the v-ATPase in amino acid signaling to TORC1 is unclear. Unlike Ragulator, which serves both as an anchor and GEF for RagA/B, EGOC functions solely as a scaffold for Gtr1-Gtr2 binding, and work demonstrating that EGOC is dispensible when Gtr1-Gtr2 is tethered to the vacuolar membrane reinforces this role.[37] Although EGOC does not demonstrate GEF activity towards Gtr1, the vacuolar protein Vam6 has been proposed to be the GEF for Gtr1.[45] Interestingly, VPS39, the mammalian ortholog of Vam6, neither binds to RagA/B nor affects RagB's nucleotide exchange rate.[34] In addition to GEFs, which activate GTPases by promoting exchange of GDP for GTP, GTPase activity is also regulated by GAPs that stimulate GTP hydrolysis and inactivate GTPases. Using RagB as bait, IP-MS studies have also identifed the GAP for the RagA/B GTPases.[46] The RagA/B GAP is not a single protein, but instead is a multiprotein complex known as GAP activity toward Rags 1 (GATOR1), which is composed of DEP domain containing 5 (DEPDC5), nitrogen permease regulator 2-like protein (NPRL2), and nitrogen permease regulator 3-like protein (NPRL3).[46] GATOR1 inhibits mTORC1 activity by increasing the rate of RagA/B GTP hydrolysis. A second complex known as GATOR2 was also identified in these IP-MS studies. GATOR2 is composed of meiosis regulator for oocyte development (Mios), WD repeat-containing protein 24 (WDR24), WD repeat-containing protein 59 (WDR59), SEH1 like nucleoporin (SEH1L), and SEC13. GATOR2 indirectly activates mTORC1 by binding to and inhibiting GATOR1 through a currently unknown mechanism.[46] Folliculin (FLCN) and its interacting proteins, folliculin interacting protein 1 and 2 (FNIP1 and FNIP2), serve as a GAP for RagC/D in mammals.[47,48] As mentioned above, GDP-bound RagC/D is found in the active Rag complex, thus, FLCN-FNIP is a positive regulator of mTORC1 activity and provides an additional layer for Rag GTPase-dependent regulation of mTORC1.

The yeast orthologs of the GATOR components were identified earlier in many independent studies, but their involvement in TORC1 regulation was not fully described until recently.[49] The Seh1-associated complex (SEAC) is made up of eight proteins that can be divided into two subcomplexes – one that inhibits TORC1 and one that activates TORC1. The inhibitory subcomplex, which is equivalent to GATOR1, is referred to as SEAC inhibiting TORC1 (SEACIT) and is composed of increased minichromosome loss (Iml1), nitrogen permease regulator 2 (Npr2), and nitrogen permease regulator 3 (Npr3).[50,51] Iml1 increases the rate of Gtr1, but not Gtr2, GTP hydrolysis *in vitro* and provides the GAP activity for the SEACIT complex. The activating subcomplex, which is similar to GATOR2, is known as SEAC activating TORC1 (SEACAT), and is composed of SEC13 homolog 1 (Seh1), Sec13, and SEH1 associated 2, 3, and 4 (Sea2, Sea3, and Sea4). SEACAT opposes SEACIT activity in order to activate TORC1.[51] Similar to FLCN and FNIP1/2, the orthologous yeast complex, Lst4-Lst7, exhibits GAP activity toward Gtr2.[52] The Rag GTPases are tightly regulated by GEFs and GAPs, and the fact that this regulation is

well-conserved in both yeast and mammals implies evolutionary importance of this process.

Recently, a complex referred to as KICSTOR, which is composed of kaptin, actin binding protein (KPTN), integrin alpha FG-GAP repeat containing 2 (ITFG2), C12orf66, and seizure threshold 2 (SZT2), was shown to tether GATOR1 to the lysosomal surface through interaction between SZT2 and GATOR1. KICSTOR appears to be required for mTORC1 sensitivity to amino acid deprivation as knockout (KO) of any KICSTOR component results in the inability to shut down mTORC1 activity in response to amino acid starvation.[53,54] Together, KICSTOR anchors GATOR1 to the lysosomal surface, forming an extended complex that negatively regulates mTORC1 activity in response to amino acid withdrawal.

While it is well-known that amino acids are potent stimuli for mTORC1 activity, how many and which amino acids are capable of activating mTORC1 remains unclear. In mammals, it is well-established that mTORC1 responds to leucine, arginine, and glutamine; whereas in yeast, leucine, methionine, and glutamine have been shown to activate TORC1 activity.[55,56] How amino acids are sensed by the cell and how these signals are transmitted to mTORC1 has been unkown for some time, but recent work has provided potential sensors and mechanisms for signaling of specific amino acids. The intracellular arginine sensors for mTORC1 (CASTOR1 and 2) and solute carrier family 38 member 9 (SLC38A9) were recently identified as potential arginine sensors.[57-61] SLC38A9 is a previously uncharacterized lysosomal amino acid transporter that directly interacts with Ragulator and the Rag GTPases. Knockdown, KO, and overexpression analysis of SLC38A9 suggests it plays a role in mTORC1 activation by amino acids, especially ariginine. However, the direction of arginine transport across the lysosomal membrane is unclear, and more data is needed to support SLC38A9 as a direct arginine sensor. CASTOR1 and CASTOR2 are highly related proteins that can form homo- or heterodimers and interact directly with GATOR2 to inhibit mTORC1 activity. While the CASTOR2-GATOR2 complex is insentive to amino acid depletion, CASTOR1 interacts more strongly with GATOR2 during amino acid withdrawal. Arginine disrupts only the CASTOR1-GATOR2 complex by binding at the interface of two aspartate kinase, chorismate mutase, TyrA (ACT) domains of the CASTOR1 homodimer, promoting mTORC1 activation. However, despite having 63% sequence similarity to CASTOR1, CASTOR2 is incapable of binding arginine, which explains its constitutive interaction with GATOR2 regardless of amino acid presence. Residues only found in CASTOR1 (His108-Val110) appear to be critical for arginine binding *in vitro*, and replacing these residues with the one's found in CASTOR2 (Gln108-Ile110), disrupts CASTOR1 binding to arginine.[60,61]

The Sestrins are a family of 3 related, highly conserved proteins (Sestrin1/2/3) which are thought to be negative regulators of mTORC1 activation.[62] The Sestrins were first proposed to interact with and function as guanine nucleotide dissociation inhibitors (GDI) for the Rag GTPases.[63] Structural studies of Sestrin2, however, disproved the Sestrins as GDIs for Rag GTPases because important residues for GDI function are buried and Sestrin2 is structurally different from other GDIs.[64] IP-MS and tandem affinity purification studies later identifed a direct interaction between Sestrin2 and GATOR2.[65,66] While leucine disrupts the Sestrin1/2-GATOR2 interaction, Sestrin3 binds to GATOR2 regardless of the presence of leucine. Moreover, Sestrin1 and 2, but not Sestrin 3, bind leucine *in vitro*.[67] Similar to arginine's disruption of the CASTOR1-GATOR2 interaction, leucine binds to Sestrin2, and this binding might cause conformational changes which disrupt the association of Sestrin2 and GATOR2.[64] Why Sestrin3 does not bind to leucine is currently unclear, due to the lack of structural studies. Hence, Sestrin1 and 2, but especially Sestrin2, have been proposed as potential leucine sensors. However, there are debates for whether or not Sestrin2 is indeed a leucine sensor as Sestrins are still capable of inhibiting mTORC1 even in leucine-replete conditions.[68] As there is no Sestrin2 ortholog in yeast, TORC1 responds to leucine stimulation via a different mechanism. Leucyl-tRNA synthetase (LeuRS), or cell division cycle 60 (Cdc60), was proposed to be a cytosolic leucine sensor that controls TORC1 activation through EGOC.[69] Leucine-bound LeuRS interacts with Gtr1, and this interaction is necessary and sufficient to activate TORC1. Methionine is another amino acid that potently regulates TORC1. Methionine induces the synthesis of S-adenosylmethionine (SAM), which in turn serves as a methyl donor for type 2A protein phosphatase (PP2A) methylation. Methylated PP2A promotes dephosphorylation of Npr2, a component of the SEACIT complex (yeast ortholog of GATOR1), which inhibits SEACIT GAP activity and activates TORC1.[56] Continued work on amino acid activation of mTORC1 will likely identify additional amino acid sensors and provide better understanding of how cells sense the nutrients present in their environment.

Although our knowledge of Rag GTPase-dependent amino acid signaling to mTORC1 has rapidly expanded, several outstanding questions related to how amino acids are sensed by the cell and how amino acid sufficiency is transmitted to mTORC1 remain. For example, can other amino acids activate mTORC1? If so, which amino acids activate mTORC1 and how do these amino acids activate mTORC1? Earlier work has sought to address these questions, and demonstrated that individual amino acids affect ribosomal protein S6 kinase beta-1 (S6K1; a substrate of mTORC1) activity to varying degrees depending on different experimental conditions.[70] In addition, do some amino acids have more than 1 sensor? What is the physiological role of mTORC1 in sensing leucine and arginine? Continued work on the regulation of Rag GTPase-dependent amino acid signaling will hopefully provide answers to these questions in the near future.

## Rag GTPase-independent activation of mTORC1

Recent work has demonstrated that glutamine can activate mTORC1 independently of the Rag GTPases.[71] Importantly, these findings are consistent with prior studies in mice where cardiac-specific deletion of RagA did not dramatically reduce mTORC1 activity in heart tissue.[72] Glutamine stimulation of Rag GTPase KO cells also induces mTOR translocation to the lysosome, and requires the action of the v-ATPase in order to activate mTORC1, which is similar to previous findings.[33,43,71] Rag GTPase-independent amino acid signaling to mTORC1 is
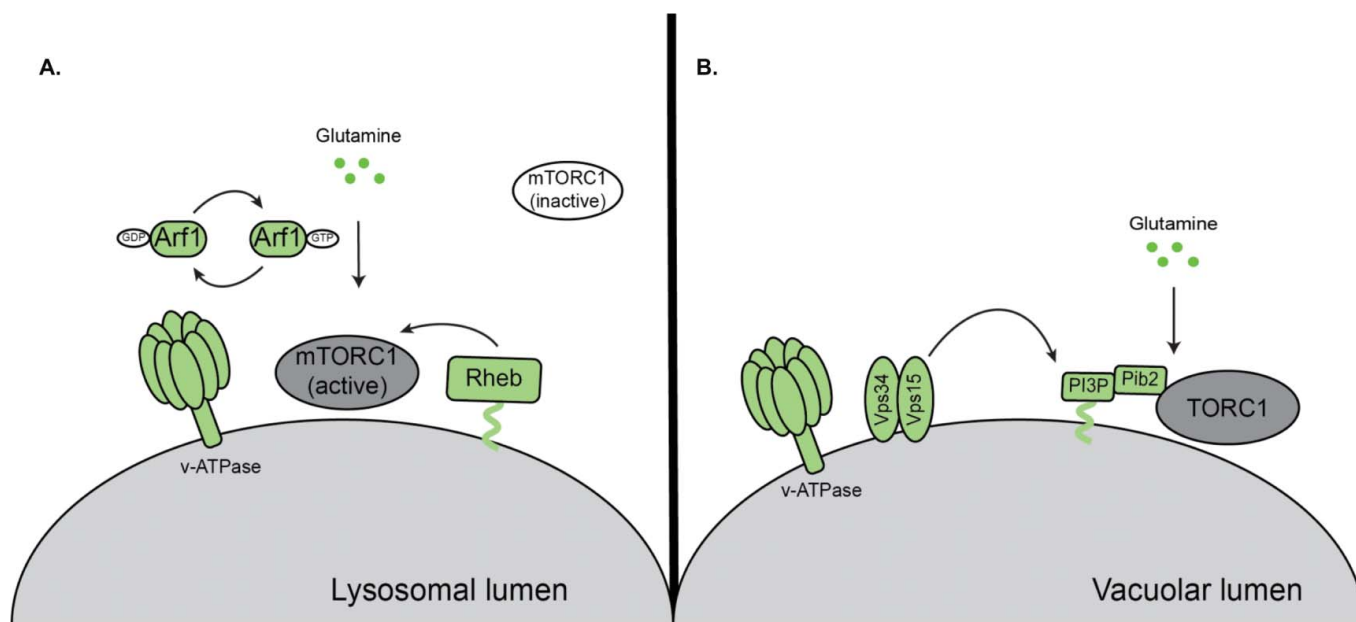
**Figure 2.** Rag GTPase-independent amino acid signaling pathway upstream of mTORC1. (A) Current model of mTORC1 activation by glutamine independently of Rag GTPases. v-ATPase is required for mTORC1 activity. The cycling of GTP-bound and GDP-bound Arf1 facilitates mTORC1 lysosomal localization and activation by glutamine. Positive regulators of mTORC1 are indicated in green. (B) Current model of Gtr1-independent TORC1 activation by glutamine. Pib2, Vsp34, and Vsp15 are required for TORC1 activation. Positive regulators of mTORC1 and TORC1 are indicated in green.

controlled by another small GTPase, adenosine ribosylation factor 1 (Arf1) (Fig. 2). Knockdown of multiple small GTPases in *Drosophila* S2 and mammalian cells identified Arf1 as an essential mediator of glutamine signaling to mTORC1 in the absence of Rags.[71] Although it is currently unclear how glutamine is sensed and integrated into this Arf1-dependent pathway, it is worth noting that glutamine-induced activation of mTORC1 requires Arf1 for mTORC1 lysosomal localization and requires the ability of Arf1 to cycle between the GTP- and GDP-bound states. Overexpression of constitutively GTP-bound Arf1 inhibits mTORC1 activity, even in the presence of amino acids,[73] and overexpression of GTP-bound Arf1 does not rescue mTORC1 activity during amino acid starvation.[71] In addition, treatment with brefeldin A (BFA), an inhibitor for some Arf1 GEFs, blocks glutamine-induced mTORC1 lysosomal localization and activation. Also of note, leucine activation of mTORC1 through the Rags and glutamine stimulation of mTORC1 through Arf1 occur on different timescales. Leucine quickly activates mTORC1, typically within five minutes, while glutamine activates mTORC1 more slowly, on the order of one hour.[71] The role of Arf1 seems to be specific to the Rag GTPase-independent pathway, as treatment with BFA and knockdown of Arf1 does not affect leucine activation of mTORC1.[71] Whether or not Arf1 plays a role in arginine signaling has yet to be tested. Consistently, glutamine is able to activate TORC1 independently of Vam6 and Gtr1 in *Saccharomyces cerevisiae*, and yeast with deletion of VAM6, GTR1, and GTR2 are able to grow on glutamine-containing media.[55] Subsequent genetic and *in vitro* work has implicated the class III phosphatidylinositol 3-phosphate (PI3P) kinase complex (PIK3C), Vps34-Vps15, as well as the PIP3-binding FYVE domain-containing vacuolar protein, Pib2, as important mediators of Gtr1- and EGOC-independent activation of TORC1.[74,75] Vps34, a lipid kinase which produces PI3P, has been

extensively characterized for its role in yeast protein sorting, membrane trafficking, and autophagy.[76-78] Vps34 is conserved across eukaryotes and the mammalian ortholog, PIK3C3, has been linked to mTORC1 activation in response to amino acids.[79-83] Recent work has also shown that mTORC1 inhibits PIK3C3 activity via the autophagy-promoting ULK1 kinase complex.[84,85] Previous reports indicate that PIK3C3 and the PI3P-binding protein phospholipase D1 (PLD1) signal amino acid sufficiency to mTORC1 in a Rag GTPase parallel mechanism, so the PIK3C3/PLD1 system represents a potential Rag GTPase-independent amino acid signaling complex.[79,80,86] Please see Table 1 for a summary of components involved in amino acid signaling to mTORC1. While the majority of amino acid signaling to mTORC1 has focused on Rag/Gtr-dependent mechanisms, the identification of a conserved, Rag/Gtr-independent pathway represents an exciting opportunity to develop a more complete picture of amino acid signaling to mTORC1. Given the importance of glutamine in a number of cellular processes including protein synthesis, nucleotide synthesis, and the tricarboxylic acid cycle, as well as the prevalence of "glutamine addiction" in cancer,[87] the study of this Rag/Gtr-independent pathway will likely improve our understanding of cellular metabolism as a whole.

## Other small GTPases and mTORC1

In addition to the Rag GTPases, Arf1, and Rheb (which was briefly mentioned above), several other small GTPases have been linked to the regulation of mTORC1 activity. The role of small GTPases as molecular switches that readily activate cellular signaling pathways makes them ideal regulators of mTORC1 in response to stimuli such as nutrients and growth factors. Recent work has identified members of the Ral, Rab,

**Table 1.** Comparison of Amino Acid signaling components in mammals and yeast.

| Mammalian Complex | Function | Yeast Complex | Function | Reference |
|---|---|---|---|---|
| **Rag GTPase** (RagA, RagB, RagC, RagD) | Bind and anchor mTORC1 to the lysosome. | **Gtr** (Gtr1, Gtr2) | Bind TORC1 at the vacuole. | 27, 28, 31 |
| **Ragulator** (p18, p14, MP1, C7orf59, HBXIP) | Anchors Rag complex to the lysosome. p18 has lipid modification for membrane localization and anchoring. | **EGOC** (Gtr1, Gtr2, Ego1, Ego2, Ego3) | Anchors Gtr complex to vacuole. Ego1 has lipid modification for membrane localization and anchoring. | 33, 34, 35, 36, 37, 38, 39, 40 |
| **Ragulator** (p18, p14, MP1, C7orf59, HBXIP) | GEF for RagA/B. | **Vam6** | GEF for Gtr1. | 34, 45 |
| **GATOR1** (DEPDC5, NPRL2, NPRL3) | GAP for RagA/B. | **SEACIT** (Iml1, Npr2, Npr3) | GAP for Gtr1. | 46, 49, 50, 51 |
| **GATOR2** (Mios, WDR24, WDR59, SEH1L, Sec13) | Inhibits GATOR1 activity. | **SEACAT** (Seh1, Sec13, Sea2, Sea3, Sea4) | Inhibits SEACIT activity. | 46, 51 |
| **Folliculin** (Folliculin, FNIP1, FNIP2) | GAP for RagC/D. | **Lst4-Lst7** (Lstp, Lst7) | GAP for Gtr2. | 47, 48, 52 |
| **KICKSTOR** (KPTN, ITFG2, C12orf66, SZT2) | Anchors GATOR1 to the lysosome. | — | — | 53, 54 |
| **CASTOR** (CASTOR1) | Cytosolic sensor for arginine. | — | — | 60, 61 |
| **SLC38A9** | Potential lysosome arginine sensor. | — | — | 57, 58, 59 |
| **Sestrin** (Sestrin1, Sestrin2) | Potential cytosolic sensor for leucine. | — | — | 64, 65, 66, 67 |
| — | — | **Cdc60** | Cytosolic sensor for leucine. | 69 |
| **v-ATPase** | Acidification of the lysosome, activity is necessary for mTORC1 activation. | — | — | 43, 71 |
| **Arf1** | Essential for RagA/B-independent lysosome localization and activation of mTORC1. | — | — | 71 |
| **PIK3C-p150** | Implicated in amino acid activation of mTORC1 | — | — | 79, 80, 81, 82 |
| — | — | **Vps34-Vps15** | Important for Gtr-independent glutamine-induced TORC1 activation. | 74 |
| — | — | **Pib2** | Possibly interacts with TORC1 in a Gtr-independent fashion at the vacuole when glutamine is present. | 74 |

and Rho GTPase families as potential regulators of mTORC1 activity.

## Rheb GTPase

Ras homolog enriched in brain (Rheb) is a small GTPase that localizes to different intracellular organelles including the lysosome, mitochondria, and Golgi apparatus and is an important activator of mTORC1.[32,33,88] Rheb is a member of the Ras superfamily of small GTPases, and was originally identified in mice as a messenger RNA (mRNA) transcript whose expression increased following N-methyl-D-aspartic acid (NMDA)-stimulated synaptic activity and growth factor signaling.[89] Early work in the fission yeast Schizosaccharomyces pombe demonstrated that deletion of rhb1 which encodes for Rhb1, the fission yeast homolog of mammalian Rheb, caused a growth arrest phenotype similar to nitrogen starvation.[90] The first evidence linking Rheb to TORC1 signaling was originally provided in Drosophila melanogaster using chemical and genetic screens,[91,92] and subsequent work confirmed a role for Rheb in mTORC1 activity.[93] GTP-bound Rheb is believed to stimulate mTORC1 activity by binding to the mTOR kinase domain, but the exact mechanistic details of this regulation remain unknown.[14,32] Given that Rheb is able to bind the kinase domain of other phosphatidylinositol-3 kinase-like kinase (PIKK) family members such as ataxia telangiecstasia mutated (ATM) and ataxia telangiecstasia and Rad3-related (ATR),[32]

structural studies examining the physical interaction between Rheb and mTOR could provide helpful insight into how Rheb activates mTOR. Unlike other Ras superfamily members, Rheb possesses low intrinsic GTPase activity, which is likely mediated by unique structural characteristics of Rheb.[94,95] Specifically, the tyrosine at residue 35 blocks access of the catalytic aspartic acid at residue 65, thus preventing GTP hydrolysis.[95] Interestingly, even though Rheb is a well-conserved protein across eukaryotes,[96] the functional role of Rheb in TORC1 activation is not conserved. Deletion of Rhb1 in Saccharomyces cerevisiae causes increased uptake of arginine; however, no overt growth defects have been observed in these deletion strains.[97]

Similar to other GTPases, Rheb GTPase activity is stimulated by a GAP. The heterotrimeric tuberous sclerosis complex (TSC),[93,98,99] which is composed of TSC1, TSC2, and TBC1D7, serves as the GAP for Rheb, with TSC2 containing the catalytic GAP domain.[93,98-100] Mutations in the genes encoding TSC can lead to the human diseases tuberous sclerosis or lymphangioleiomyomatosis (LAM), which are characterized by the growth of benign tumors and constitutively active mTORC1.[101,102] TSC serves as a hub for multiple signals which can activate and inhibit mTORC1. Growth factor-stimulated activation of multiple upstream kinases including AKT, extracellular signal-regulated kinase (ERK), ribosomal S6 kinase 1 (RSK1), and IκB kinase β (IKKβ), leads to the phosphorylation and inhibition of TSC, which ultimately increases mTORC1 activity.[103-107]

Conversely, cellular stresses such as nutrient starvation, hypoxia, osmotic stress, and DNA damage can inhibit mTORC1 via TSC.[108-112] The activity of 5′ adenosine monophosphate-activated protein kinase (AMPK), a serine/threonine protein kinase that is activated during nutrient starvation, can inhibit mTORC1 by phosphorylating TSC2.[108] AMPK phosphorylation of TSC2 enhances TSC2 GAP activity and primes TSC2 for phosphorylation by glycogen synthase kinase-3$\beta$ (GSK3-$\beta$), which also enhances TSC2 GAP activity.[113] In addition to nutrient starvation, hypoxia and DNA damage inhibit mTORC1 by increasing TSC activity via regulated in development and DNA damage response 1 (REDD1)[109] and tumor protein 53 (TP53),[112] respectively. Recent work has also shown that TSC is recruited to the lysosome in order to inactivate Rheb, however, there is currently debate as to whether or not this is due to amino acid or growth factor withdrawal.[114,115] The different outcomes between these studies could be due to differences in cell type or experimental conditions, i.e. different amino acid starvation time courses. Additional work has shown that TSC is recruited to the lysosome following arginine withdrawal,[116] but TSC recruitment was enhanced by growth factor withdrawal, suggesting that both amino acids and growth factors regulate the localization of TSC. Notably, the withdrawal of other amino acids does not affect lysosomal recruitment of TSC, and the authors suggest that arginine specifically disrupts the interaction between Rheb and TSC.[116] Continued work is necessary to understand why arginine plays an important role in regulating lysosomal recruitment of TSC. While the GAP for Rheb is well characterized, a GEF for Rheb has not been identified in mammals. Studies in *D. melanogaster* demonstrated that translationally controlled tumor protein (dTctp) physically interacts with dRheb and stimulates GDP exchange *in vitro*,[117] but subsequent biochemical and genetic studies in mammalian cells found that TCTP had no effect on Rheb GTP loading or mTORC1 activity.[118,119] Future work might reveal a GEF for Rheb, however, it is possible that Rheb does not require a GEF due to its intrinsically low GTPase activity.

## Ral GTPases

Shortly after the demonstration that the Rag GTPases are important for mediating amino acid sufficiency to mTORC1,[27,28] a role for the small GTPase, Ras-related protein Ral-A (RalA), was proposed for signaling nutrient sufficiency to mTORC1.[120] RalA is primarily known for its involvement in endocytosis, exocytosis, and cytoskeletal regulation.[121-123] While small interfering RNA (siRNA)-mediated knockdown of RalA blocked amino acid- and glucose-induced activation of mTORC1,[120] these effects could also be explained by RalA's importance in regulating the exocyst complex, a protein complex which mediates the fusion of secretory vesicles with the plasma membrane.[124] Given the importance of the exocyst in targeting nutrient receptors such as the glucose receptor, GLUT4, to the cell surface,[125] and the role of cell surface nutrient transporters in mTORC1 activity,[126] it's possible that RalA's role in mTORC1 activity is linked to receptor localization at the plasma membrane. Subsequent work, however, has corroborated the findings that RalA is important for glucose and amino acid activation of mTORC1 through a mechanism that involves regulation of PLD1 activity.[86] Additional

studies are needed to clarify the role of RalA in nutrient signaling to mTORC1.

## Rab GTPases

Multiple studies have also examined the influence of the Ras-related protein Rab (Rab) GTPases, which are normally involved in intracellular trafficking,[127] on mTORC1 activity. Double-stranded RNA (dsRNA) knockdown of multiple Rab GTPases, including Rab1, Rab5, and Rab11, in *Drosophila* S2 cells impaired TORC1 activity, suggesting that the Rab GTPases may be important activators of TORC1.[73] In addition, yeast Vps21 (mammalian Rab5) and Ypt1 (mammalian Rab1A) are important for amino acid activation of TORC1.[88,128] The role of Rab GTPases in mammalian cells, however, appears to be inhibitory of mTORC1.[73] Overexpression of multiple constitutively active Rab GTPases, including Rab5, Rab7, Rab10, and Rab11, completely blocked mTORC1 activity in HEK293A cells. Overexpression of constitutively active (GTP-bound) and dominant negative (GDP-bound) Rab5, for example, blocked mTORC1 activity and interfered with the localization of mTOR to lysosomes.[73,128] Thus, the observed inhibitory role of Rab GTPases is potentially due to disruption of cellular trafficking and the subcellular localization of mTORC1. Recent work has proposed that another Rab GTPase, Rab1A, is important in mediating amino acid signaling to mTORC1.[88] siRNA knockdown of Rab1A suggests that this is due to Rab1A-dependent trafficking of mTORC1 to Rheb found on the Golgi apparatus.[88] Notably, the yeast homolog of Rab1A, Ytp1, is also necessary for amino acid activation of TORC1, and multiple cancer cell lines display elevated expression of Rab1A. mTORC1 activity in these cell lines mirrors Rab1A overexpression. Similar to the Rag GTPases, constitutively GTP-bound Rab1A remains associated with mTORC1 and is able to rescue cells from amino acid starvation.[88] Additional work is needed to understand whether or not the activity of mammalian transport protein particle complexes (mTRAPPCs), which can function as GEFs for Rab1,[129] are important for activating mTORC1. In addition, it is currently unknown whether or not TBC1D20, a Rab1A GAP, is involved in inactivating mTORC1 during amino acid starvation. Given the established role of Rab GTPases in intracellular trafficking, it would be interesting to see if additional Rabs are involved in mTORC1 trafficking, and whether or not these Rabs define unique subpopulations of mTORC1.

## Rho GTPases

The Ras homolog gene family (Rho) GTPases constitute an additional family within the Ras superfamily that are responsible for cytoskeletal regulation, cell migration, and cell proliferation.[130] Rho GTPases are further divided into the Rho, Ras-related C3 botulinum toxin substrate (Rac), and cell division control protein 42 homolog (Cdc42) subfamilies. The Rho subfamily regulates actin stress fiber and focal adhesion formation, the Rac subfamily regulates membrane ruffling, and the Cdc42 subfamily regulates filopodium formation.[130] Work in mammalian cells and yeast has identified potential regulation of mTORC1 activity by members of the Rho and Rac GTPase subfamilies. Deletion of *Rac1* in cultured mouse embryonic fibroblasts (MEFs) causes a marked reduction in cell size and

drastic reduction in mTORC1 activity. Immunofluorescence studies and binding experiments demonstrate that Rac1 and mTOR directly interact and that this interaction facilitates mTORC1 localization to the cytosolic region surrounding the nucleus, as well as the plasma membrane.[131] How Rac1 regulates mTORC1 localization is currently unknown. Given that Rac1-dependent mTORC1 localization is independent of actin polymerization and Rac1's effects on mTORC1 activity are independent of Rac1 binding to GTP, it will be interesting to see how Rac1 regulates mTORC1 localization and what other regulatory elements are involved. Recent work in yeast has identified Rho1 as a negative regulator of TORC1 activity that binds to Kog1 (ortholog of raptor) in response to cellular stresses such as heat, cell wall stress, and rapamycin treatment.[132] Rho1 binding to the raptor N-terminal conserved domain (RNC) of Kog1 blocks the interaction of TORC1 with some of its substrates.[132] Although RhoA, the mammalian homolog of Rho1, is able to inhibit mTORC1 activity, it appears as though RhoA and raptor do not interact *in vivo*.[133] In addition, inhibition of mTORC1 by RhoA in these studies is based on overexpression data, and whether or not endogenous RhoA suppresses mTORC1 activity in response to cellular stresses such as nutrient starvation is currently unknown. Hopefully future work will be able to provide a better understanding of the role Rho GTPases have in the regulation of mTORC1 activity.

## Conclusion

Multiple cellular processes are governed by the master regulatory complex mTORC1 in response to extracellular cues such as growth factors and nutrients. Given the diverse set of signals that ultimately filter through mTORC1, it is not surprising that multiple small GTPases – molecular switches that are regulators of cell signaling cascades – are involved in the regulation of mTORC1. In addition to Rheb, which has a well-established role as an mTORC1 activator downstream of growth factor signaling,[91-93] two small GTPases have emerged as regulators of amino acid signaling to mTORC1. The Rag GTPases are an evolutionarily conserved mechanism for amino acid signaling to mTORC1, recruiting mTORC1 to the lysosome and activating it in response to amino acids such as leucine and arginine.[27,28,31] In addition, the recently discovered Rag GTPase-independent pathway, which relies on Arf1, constitutes a conserved pathway for glutamine signaling to mTORC1.[55,71,74] Although complete mechanistic understanding of either pathway is far from complete, several broad questions remain: Why do multiple amino acid signaling mechanisms exist? What is the significance in the different activation times for each pathway?[71] Are different mTORC1-dependent processes activated by the two pathways? Do additional amino acid signaling mechanisms exist? Continued work on amino acid signaling to mTORC1 will likely reveal several other small GTPases that are important in mTORC1 regulation. In addition to identifying small GTPases that regulate mTORC1 activity, it will be important to understand how these small G-proteins regulate mTORC1 activity. Due to increasing numbers of small GTPases that are also involved in trafficking being linked to mTORC1 activity, it will be interesting to see how these proteins regulate mTORC1 subcellular localization,

and how these different G-protein families are responding to and integrating nutrient signals.

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The authors have no competing interests to disclose.

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