Glutamine and asparagine activate mTORC1 independently of Rag GTPases

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Delong Meng[‡§¶], Qianmei Yang[‡§¶], Huanyu Wang[‡§¶], Chase H. Melick[‡§¶1], Rishika Navlani[‡§¶], Anderson R. Frank[‡§¶], and Jenna L. Jewell[‡§¶2]

From the [‡]Department of Molecular Biology, the [§]Harold C. Simmons Comprehensive Cancer Center, and the [¶]Hamon Center for Regenerative Science and Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75390

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Nutrient sensing by cells is crucial, and when this sensing mechanism is disturbed, human disease can occur. mTOR complex 1 (mTORC1) senses amino acids to control cell growth, metabolism, and autophagy. Leucine, arginine, and methionine signal to mTORC1 through the well-characterized Rag GTPase signaling pathway. In contrast, glutamine activates mTORC1 through a Rag GTPase–independent mechanism that requires ADP-ribosylation factor 1 (Arf1). Here, using several biochemical and genetic approaches, we show that eight amino acids filter through the Rag GTPase pathway. Like glutamine, asparagine signals to mTORC1 through Arf1 in the absence of the Rag GTPases. Both the Rag-dependent and Rag-independent pathways required the lysosome and lysosomal function for mTORC1 activation. Our results show that mTORC1 is differentially regulated by amino acids through two distinct pathways.

Nutrient sensing is important to sustain normal cell growth and proliferation (1–4). mTOR[3] complex 1 (mTORC1) senses nutrients to regulate cell growth, autophagy, and other mTORC1-mediated processes. Importantly, mTORC1 hyperactivation is observed in many human diseases, including cancer, obesity, type 2 diabetes, neurodegeneration, and metabolic disorders.

Growth factors, amino acids, energy status, and stress control mTORC1 (1–4). Amino acids are essential for mTORC1 activation. Growth factors alone cannot attain maximal mTORC1 activity without amino acid supplementation (5). Increased amino acid concentrations promote mTORC1 lysosomal localization and subsequent activation. Downstream of growth factors, the small G-protein Rheb binds to and activates mTORC1 at the lysosome. Rheb resides at the lysosomal surface via the last 15 amino acids, including a C-terminal CAAX box (6, 7). GTP-bound Rheb directly interacts with mTORC1, and structural analysis of this interaction reveals a conformational change in the mTOR active site and allosteric activation of the kinase (8). Tuberous sclerosis complex (TSC) dissociates away from the lysosome and Rheb, downstream of growth factors, resulting in mTORC1 activation (6). TSC is a GTPase-activating protein for Rheb and promotes inactive Rheb bound to GDP (9–11). Mutations in TSC elevate mTORC1 activity and cause tuberous sclerosis and lymphangioleiomyomatosis (12–14). Thus, amino acids and growth factors intersect at the lysosome to promote mTORC1 activation.

The Rag GTPases link amino acid signaling to mTORC1 activation at the lysosome (5, 15). RagA or RagB forms a heterodimer with RagC or RagD, and dimerization is essential for Rag GTPase protein stability and mTORC1 activation (16). In mammals, there are four Rag proteins: RagA and RagB are high in sequence similarity and functionally redundant; likewise, RagC and RagD are also highly related in sequence and redundant. The guanine nucleotide loading of the Rag GTPases is important for their physiological function, where GTP-bound RagA or RagB interacts with the mTORC1 component Raptor at the lysosome. RagC or RagD GDP-bound forms a heterodimer with the GTP-bound RagA or RagB. Other components have been reported to be involved in the Rag GTPase signaling cascade to mTORC1. For example, the Ragulator complex (7, 17), the vacuolar H^+-ATPase (v-ATPase) (18), GATOR complexes (referred to as GATOR1 and GATOR2) (19), FLCN-FNIP complex (20), KICSTOR complex (21), and SLC38A9 (also referred to as SNAT9) (22–25) were shown to regulate mTORC1 in a Rag-dependent manner.

Leu (5, 26, 27), Arg (26, 28, 29), Met (30), and Gln (16, 27, 31, 32) have been shown to regulate mTORC1. Leu and Arg sensors have been identified as Sestrin2 and CASTOR1, respectively (29, 33–36). Sestrin2 and CASTOR1 proteins are upstream of the Rag GTPases and are required for mTORC1 activation. Moreover, SAMTOR was shown to be an *S*-adenosylmethionine sensor that couples Met to mTORC1 in a Rag-dependent

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This article contains Figs. S1–S4.

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[2] To whom correspondence should be addressed: Dept. of Molecular Biology, University of Texas Southwestern Medical Center, 6000 Harry Hines Blvd., Rm. NA5.508A, Dallas, TX 75390-9148. Tel.: 214-648-1685; Fax: 214-648-1488; E-mail: Jenna.Jewell@UTSouthwestern.edu.

[3] The abbreviations used are: mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; DMEM, Dulbecco's modified Eagle's medium; MEM, minimum Eagle's medium; TSC, tuberous sclerosis complex; v-ATPase, vacuolar H^+-ATPase; MEF, mouse embryo fibroblast; HEK293A, human embryonic kidney 293A; KO, knockout; BFA, brefeldin A.

manner (30). Recently, we discovered a novel signaling pathway where Gln signals to mTORC1 independent of the Rag GTPases and requires ADP-ribosylation factor-1 (Arf1) (16). Importantly, this Gln-TORC1 pathway is conserved in yeast, where VPS34 and Pib2 are thought to be involved (37–40). However, the Gln sensor and other components involved in the Rag-independent pathway in mammals have yet to be discovered.

Results and discussion

Multiple individual amino acids activate mTORC1

Extensive research has demonstrated that amino acids potently activate mTORC1. However, the precise molecular mechanisms by which individual amino acids activate mTORC1 are only slowly beginning to be unraveled. Leu (5, 26, 27), Arg (26, 28, 29, 41), Met (30), and Gln (16, 27, 31, 32, 42) have previously been shown to modulate mTORC1 activity. Utilizing the 20 standard amino acids, we found that 10 of these (Ala, Arg, Asn, Gln, His, Leu, Met, Ser, Thr, and Val; hereafter referred to as AA^{mTORC1}) activate mTORC1 in mouse embryonic fibroblast (MEF) and human embryonic kidney 293A (HEK293A) cells as judged by immunoblotting for the phosphorylation of its well-characterized substrate S6K1 at threonine 389 (pS6K1) (Fig. 1*A*). Cys activated mTORC1 in MEF cells but not in HEK293A cells.

In human serum, amino acid concentrations range from 6 to 650 μM, depending on diet, age, and disease (43, 44). Therefore, to determine the kinetics of AA^{mTORC1}-induced activation of mTORC1, we starved MEF and HEK293A cells of all amino acids and performed a dose response, utilizing concentrations of each individual amino acid ranging from 100 nM to 1 mM and analyzed mTORC1 activity (Fig. 1*B* and Fig. S1 (*A* and *B*)). Concentrations in which individual amino acids activated mTORC1 ranged from 10 μM to 1 mM (except His in MEF), comparable with the concentrations of amino acids found in human plasma and cell culturing medium. AA^{mTORC1} could induce the activation of mTORC1 between 15 min and 1 h (Fig. 1*C*). To exclude the possibility that amino acids that take awhile to activate mTORC1 (e.g. Gln and Asn), indirectly signal to mTORC1 through the degradation of serum proteins, we performed amino acid starvation and stimulation experiments in the absence of serum. Gln and Asn can still signal to mTORC1 (Fig. S1*C*). Furthermore, because growth factors and amino acids merge at the lysosome to achieve optimal mTORC1 activation, the addition of the growth factor insulin further increased mTORC1 signaling (Fig. S1*C*).

Amino acid withdrawal experiments were performed, where each individual amino acid was removed from the Dulbecco's modified Eagle's medium (DMEM), and mTORC1 activity was analyzed (Fig. S1*D*). HEK293A cells were maintained in DMEM supplemented with Ala and Asn (amino acids absent in the DMEM) for several weeks prior to withdrawal experiments. Short-term withdrawal (4 h) of Ala, Met, and Arg decreased mTORC1 activity (Fig. S1*D*, *top*). Also, Asn and Gln withdrawal significantly inhibited mTORC1 after 4 h. Long-term withdrawal (48 h) of Ala, Leu, Asn, Gln, Arg, and Ser inhibited mTORC1 (Fig. S1*D*, *bottom*). Starvation of cells of all amino acids or Met for 48 h resulted in cell death. We did not observe a dramatic change of mTORC1 activity when starving cells of His, Thr, or Val for 4 or 48 h. Because there are multiple mTORC1 sensors (23, 24, 29, 30, 33), withdrawal of only one amino acid may not significantly alter mTORC1 activity. Consistent with the amino acid stimulation experiments (Fig. 1), the withdrawal of seven amino acids (Ala, Met, Arg, Leu, Asn, Gln, and Ser) appears to regulate mTORC1.

To exclude the possibility that phosphorylation of S6K1 at threonine 389 was due to a compensating kinase or inactivation of a phosphatase, we analyzed other mTORC1 substrates, such as the phosphorylation of ULK1 at serine 758 and the electrophoretic mobility shift of 4EBP1. Phosphorylation of S6K1 and 4EBP1 promotes protein synthesis, whereas phosphorylation of ULK1 inhibits autophagy (45, 46). Stimulation of cells with AA^{mTORC1} resulted in the phosphorylation of 4EBP1 (as measured by electrophoretic mobility shift) and ULK1 (Fig. S1*E*). Furthermore, cells pretreated with the mTORC1 inhibitor rapamycin prior to the addition of each individual amino acid blocked AA^{mTORC1} signaling to mTORC1 (Fig. 1*D*). Thus, 10 amino acids activate mTORC1 in both MEF and HEK293A cells. In agreement with previous work, Leu, Arg, Gln, and Met modulate mTORC1 activity (5, 26–32, 42).

Individual amino acids promote mTORC1 lysosomal localization and require lysosomal function

The lysosome and lysosome machinery are essential for mTORC1 regulation by amino acids (16, 18, 47, 48). Amino acid availability promotes mTORC1 lysosomal localization, where it is subsequently activated by Rheb downstream of growth factors (45, 46). Stimulation of cells with only Leu or Gln is sufficient to promote mTORC1 lysosomal localization (5, 16). Because our data show that 10 amino acids activate mTORC1 in MEF and HEK293A cells, we investigated whether mTORC1 lysosomal localization was required. Amino acid stimulation promoted the translocation of mTOR (*green*) to LAMP2-positive lysosomal membranes (*red*) in MEF cells (Fig. 2, *A* (*left column*, *first row*) and *B*) (6, 7, 16). In contrast, significantly less mTOR localized to LAMP2-positive lysosomal membranes in MEF cells starved of amino acids (Fig. 2, *A* (*right column*, *first row*) and *B*). Each of the 10 amino acids (AA^{mTORC1}) alone was able to induce the co-localization of mTOR and LAMP2-positive lysosomal membranes (Fig. 2, *A* (*left column*, *rows 2–7*; *right column*, *rows 2–5*) and *B*). However, amino acids such as Lys, Phe, and Trp, which do not activate mTORC1 in MEF and HEK293A cells (Fig. S1, *A* and *B*), were unable to induce the co-localization of mTOR and LAMP2-positive lysosomal membranes (Fig. 2, *A* (*right column*, *row 6*) and *B*). Corresponding immunoblots were performed in parallel to the imaging experiments as controls and showed that AA^{mTORC1} induced mTORC1 activation, whereas the other amino acids did not (Fig. 2*C*). Similar patterns of mTOR localization were also confirmed in HEK293A cells stimulated with Leu, Asn, and Ser (Fig. S2, *A* and *B*). Based on our results, the same 10 amino acids (AA^{mTORC1}) that regulate mTORC1 activity also promote its lysosomal localization (Figs. 1 and 2 and Figs. S1 and S2 (*A* and *B*)).

A

Amino Acid	MEF Cells mTORC1 Activation	HEK293A Cells mTORC1 Activation
Alanine (Ala)	+	+
Arginine (Arg)	+	+
Asparagine (Asn)	+	+
Aspartic Acid (Asp)	-	-
Cysteine (Cys)	+	-
Glutamic Acid (Glu)	-	-
Glutamine (Gln)	+	+
Glycine (Gly)	-	-
Histidine (His)	+	+
Isoleucine (Ile)	-	-
Leucine (Leu)	+	+
Lysine (Lys)	-	-
Methionine (Met)	+	+
Phenylalanine (Phe)	-	-
Proline (Pro)	-	-
Serine (Ser)	+	+
Threonine (Thr)	+	+
Tryptophan (Trp)	-	-
Tyrosine (Tyr)	-	-
Valine (Val)	+	+

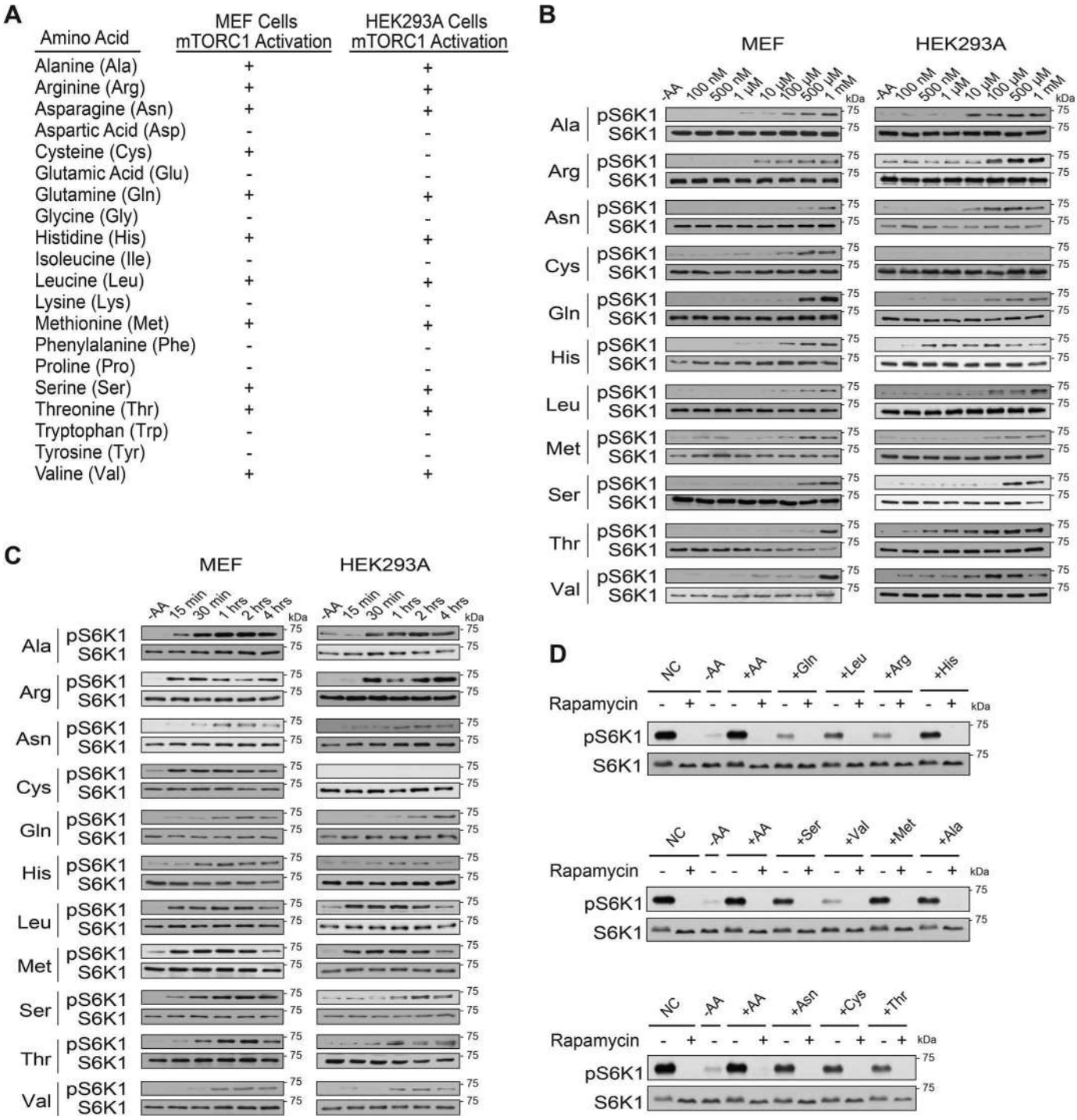
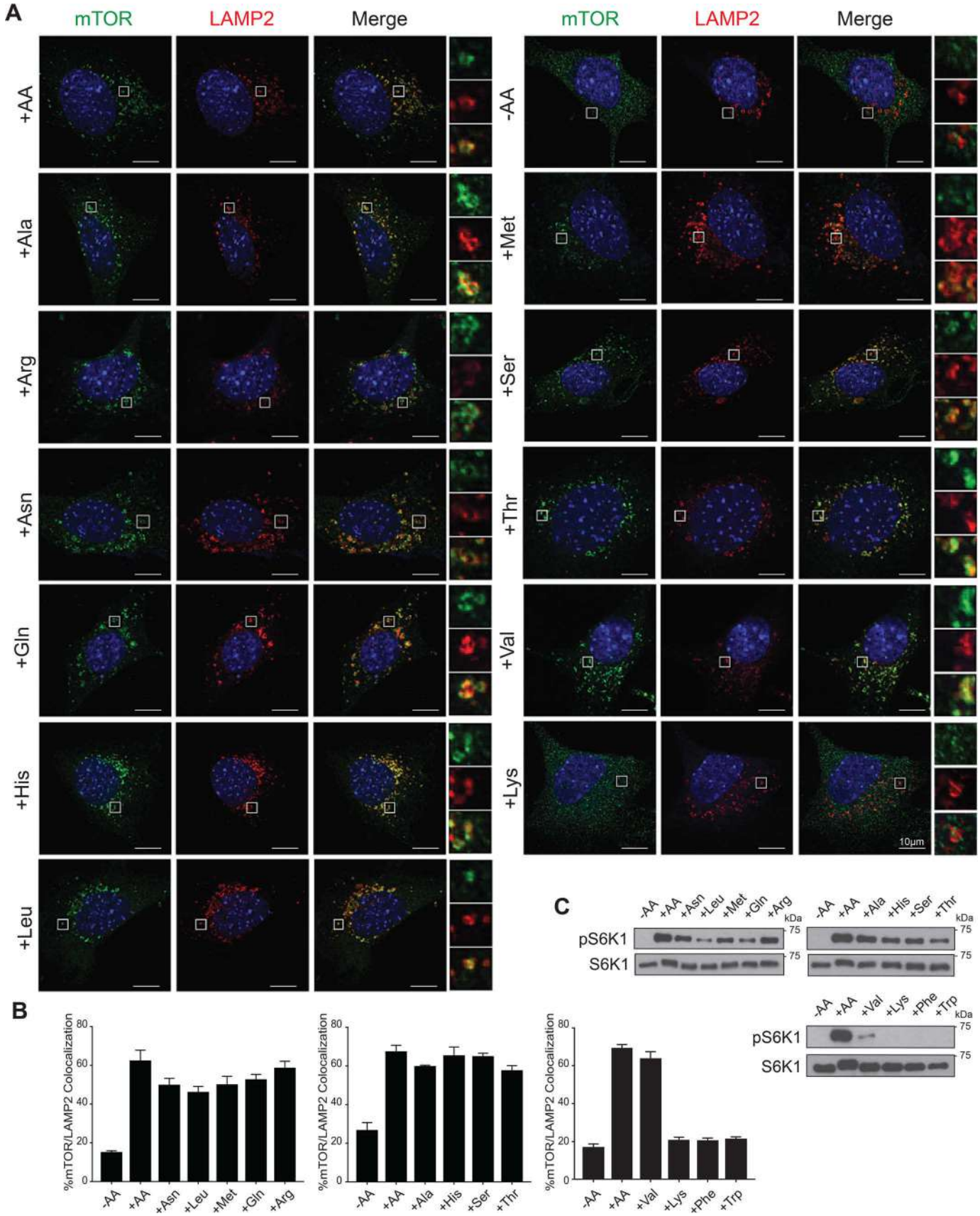


Figure 1. Multiple amino acids regulate mTORC1. *A*, *table* summarizing the amino acids that activate mTORC1. *B*, MEF or HEK293A cells were starved of amino acids (−*AA*) for 1–2 h, followed by the addition of each amino acid at the indicated concentration for 30 min. mTORC1 activity was analyzed by immunoblotting for the phosphorylation status of S6K1 (*pS6K1*) at threonine 389. S6K1 was used as loading control. *C*, MEF or HEK293A cells were starved of amino acids for 1–2 h, and each amino acid (500 μM to 1 mM) was added for the indicated time. *D*, MEF cells were starved of amino acids for 1–2 h and pretreated with or without 100 nM rapamycin for 30 min, and then amino acids (+*AA*) or individual amino acids (500 μM to 1 mM) were added for 30 min. mTORC1 activity was analyzed as in *B*. *NC*, normal conditions.

Both Rag-dependent and Rag-independent activation of mTORC1 require the v-ATPase, responsible for acidifying the lysosome and maintaining lysosomal function (16, 18, 49). For example, Leu was suggested to accumulate inside the lysosome and activate mTORC1 through an "inside-out" model, whereas v-ATPase serves as a critical component (18). However, it is unclear whether v-ATPase and lysosomal function is a general requirement for all amino acids to activate mTORC1. To test whether v-ATPase function is involved in AA^{mTORC1} signaling to mTORC1, we treated cells with the v-ATPase inhibitor bafilomycin A (50). Pretreatment of cells with bafilomycin A inhibited mTORC1 activation, even after the addition of individual


A
mTOR
LAMP2
Merge
+AA
+Ala
+Arg
+Asn
+Gln
+His
+Leu
-AA
+Met
+Ser
+Thr
+Val
+Lys
10μm
B
%mTOR/LAMP2 Colocalization
-AA
+AA
+Asn
+Leu
+Met
+Gln
+Arg
+Ala
+His
+Ser
+Thr
+Val
+Lys
+Phe
+Trp
C
pS6K1
S6K1
kDa
75

amino acids (AA^{mTORC1}) or amino acids combined (Fig. S2*C*). Choloroquine, a v-ATPase–independent inhibitor of the lysosomal pH gradient, also inhibited AA^{mTORC1} signaling to mTORC1 (Fig. S2*D*). Collectively, these results show that the v-ATPase and lysosomal function are required for the 10 amino acids (AA^{mTORC1}) to activate mTORC1.

Glutamine and asparagine activate mTORC1 independently of the Rag GTPase signaling pathway

We previously discovered that mTORC1 is activated in response to amino acid stimulation in the absence of the Rag GTPases (16). As described previously, there are four Rag proteins in mammals: RagA, RagB, RagC, and RagD (5, 15). Rag A/B knockout (KO) cells have depleted levels of RagC and RagD, because RagC and RagD protein levels are stabilized by RagA/B-RagC/D heterodimerization. Thus, RagA/B KO cells do not have intact Rag GTPase complexes. Moreover, RagA/B directly interacts with and anchors mTORC1 to the lysosome, whereas RagC/D does not (51). By systematically testing the 20 standard amino acids in Rag GTPase (RagA/B) KO cells, using amino acid concentrations found in DMEM and MEM nonessential amino acid solution, we found that Gln could still activate mTORC1 (16). DMEM and MEM nonessential amino acid solution contain amino acid concentrations ranging from ~80–800 μM, with a high concentration of Gln (4 mM). Because the concentrations of each amino acid in DMEM and MEM nonessential amino acid solution can vary significantly, we tested the 10 amino acids (AA^{mTORC1}) at similar concentrations (500 μM or 1 mM) (Fig. 1*C*). We starved MEF or HEK293A cells of amino acids and replenished cells with either amino acids or individual amino acids (Fig. 3 (*A* (*left*) and *B* (*left*)) and Fig. S3 (*A* (*left*) and *B* (*left*)). Interestingly, Asn like Gln activates mTORC1 in the absence of the Rag GTPases (Fig. 3*A* (*middle*) and Fig. S3*A* (*right*)). Gln and Asn activation of mTORC1 in the Rag GTPase KO cells is comparable with that in WT cells (Fig. 3*A*, *right*). Likewise, Asn and Gln signaled to mTORC1 without other crucial components in the Rag GTPase signaling pathway, such as GATOR2 (Mios) (Fig. 3*B* (*right*) and Fig. S3*B*). Ser, Thr, and Ala slightly activate mTORC1 in RagA/B and Mios KO cells (Fig. 3, *A* and *B*). These amino acids can be metabolized into pyruvate, and it has previously been revealed that pyruvate can regulate mTORC1 through the TTT-RUVBL1/2 complex (42). As mentioned previously, the activity of the Rag GTPases is determined by their guanine nucleotide status, and the overexpression of a constitutively inactive Rag GTPase complex (RagA/B GDP-bound and RagC/D GTP-bound) inhibits amino acid signaling through the Rag GTPases (5, 15). Gln and Asn can still activate mTORC1 in WT and RagA/B KO cells expressing $RagA/B^{GDP}$-$RagC/D^{GTP}$ (Fig. S3*C*). As mentioned above, amino acids promote lysosomal localization and activation of mTORC1 (Figs. 1 and 2 and Fig. S1 and S2 (*A* and *B*)). Even in the absence of Rag GTPases, Gln still induces lysosomal localization of mTORC1 (16). In addition, like Gln, Asn also promotes mTOR localizing to lysosomes (Fig. S3, *D* and *E*). Collectively, these data suggest that Asn and Gln can activate mTORC1, independently of the Rag GTPase signaling pathway (Fig. 3 (*A*–*C*) and Fig. S3).

Stimulation of HEK293A cells with saturating concentrations of either Gln or Asn activated mTORC1. However, the simultaneous addition of saturating concentrations of both amino acids further increased mTORC1 activity, suggesting synergy between the amino acids (Fig. 3*D*). These results are consistent with different sensors or modes of action used by Gln and Asn to activate mTORC1. As expected, the combination of Leu and Arg also synergistically enhanced mTORC1 activity in HEK293A cells. Leu and Arg activate mTORC1 by binding to Sestrin2 and CASTOR1, respectively (29, 33–35). Taken together, eight amino acids (Ala, Arg, His, Leu, Met, Ser, Thr, and Val) primarily utilize the Rag GTPase-dependent pathway to activate mTORC1, whereas Gln and Asn signal to mTORC1 in the absence of the Rag GTPases.

VPS34 and Pib2 have been shown to play a role in the Rag GTPase–independent TORC1 signaling pathway in yeast (38–40). To test whether VPS34, a class III phosphatidylinositol 3-kinase, plays a role in Gln and Asn signaling to mTORC1, we inhibited VPS34 (VPS34-IN1) in RagA/B KO HEK293A cells. mTORC1 activity was decreased with treatment of VPS34-IN1 under normal culturing conditions. However, Gln- or Asn-induced mTORC1 activation was not significantly changed when cells were treated with VPS34-IN1 (Fig. S4*A*). Pib2 is a vacuolar membrane–associated phosphatidylinositol 3-phosphate–binding protein with no orthologs in mammals. The Pib2 FYVE domain shares high sequence similarity with LARPF/phafin1 (gene PLEKHF1), whereas the domain on Pib2 (motif E) that interacts with TORC1 is similar to a region in R3H and coiled-coil domain–containing protein 1 (gene R3HCC1). Knockdown of PLEKHF1 or R3HCC1 failed to alter Gln- or Asn-induced mTORC1 activation (Fig. S4, *B* and *C*).

Gln (31, 42, 52, 53) and Asn withdrawal decreases mTORC1 activity (Fig. S1*D*). Gln and Asn are both nonessential amino acids that can be made by the cell via Gln and Asn synthetases (GLUL and ASNS, respectively) (54). Glutamine synthesis does not appear to compensate for acute withdrawal of exogenous glutamine, and we did not observe further decrease of mTORC1 when depleting glutamine from GLUL stable knockdown cells (Fig. 3*E*). In addition, we performed asparagine deprivation through acute ASNS knockdown for cells growing in regular Asn-free DMEM and observed impaired mTORC1 activity (Fig. 3*F*), similar to a previous report (52). These data

Figure 2. Multiple amino acids promote mTORC1 lysosomal localization. *A*, immunofluorescence analysis of mTOR (*green*) and the lysosomal marker LAMP2 (*red*) in MEF cells. Cells were starved of amino acids (−*AA*) for 4 h and then stimulated with amino acids (+*AA*) or the individual amino acids (500 μM to 1 mM) as indicated for 2 h. *Higher-magnification images* of the depicted area are shown on the *right*. *B*, quantification of the percentage of mTOR/LAMP2 colocalization. *p* values were as follows: −AA *versus* +AA ($p < 0.0001$); −AA *versus* +Asn ($p < 0.0001$); −AA *versus* +Leu ($p < 0.0001$); −AA *versus* +Met ($p < 0.0001$); −AA *versus* +Gln ($p < 0.0001$); −AA *versus* +Arg ($p < 0.0001$); −AA *versus* +Ala ($p < 0.0001$); −AA *versus* +His ($p < 0.0001$); −AA *versus* +Ser ($p < 0.0001$); −AA *versus* +Thr ($p < 0.0001$); −AA *versus* +Val ($p < 0.0001$); −AA *versus* +Lys (not significant); −AA *versus* +Phe (not significant); −AA *versus* +Trp (not significant). *C*, corresponding Western blots were performed in parallel to the imaging (*A*) and quantification (*B*). mTORC1 activity was analyzed by immunoblotting for the phosphorylation status of S6K1 (*pS6K1*) at threonine 389. S6K1 was used as loading control. *Error bars*, S.E.

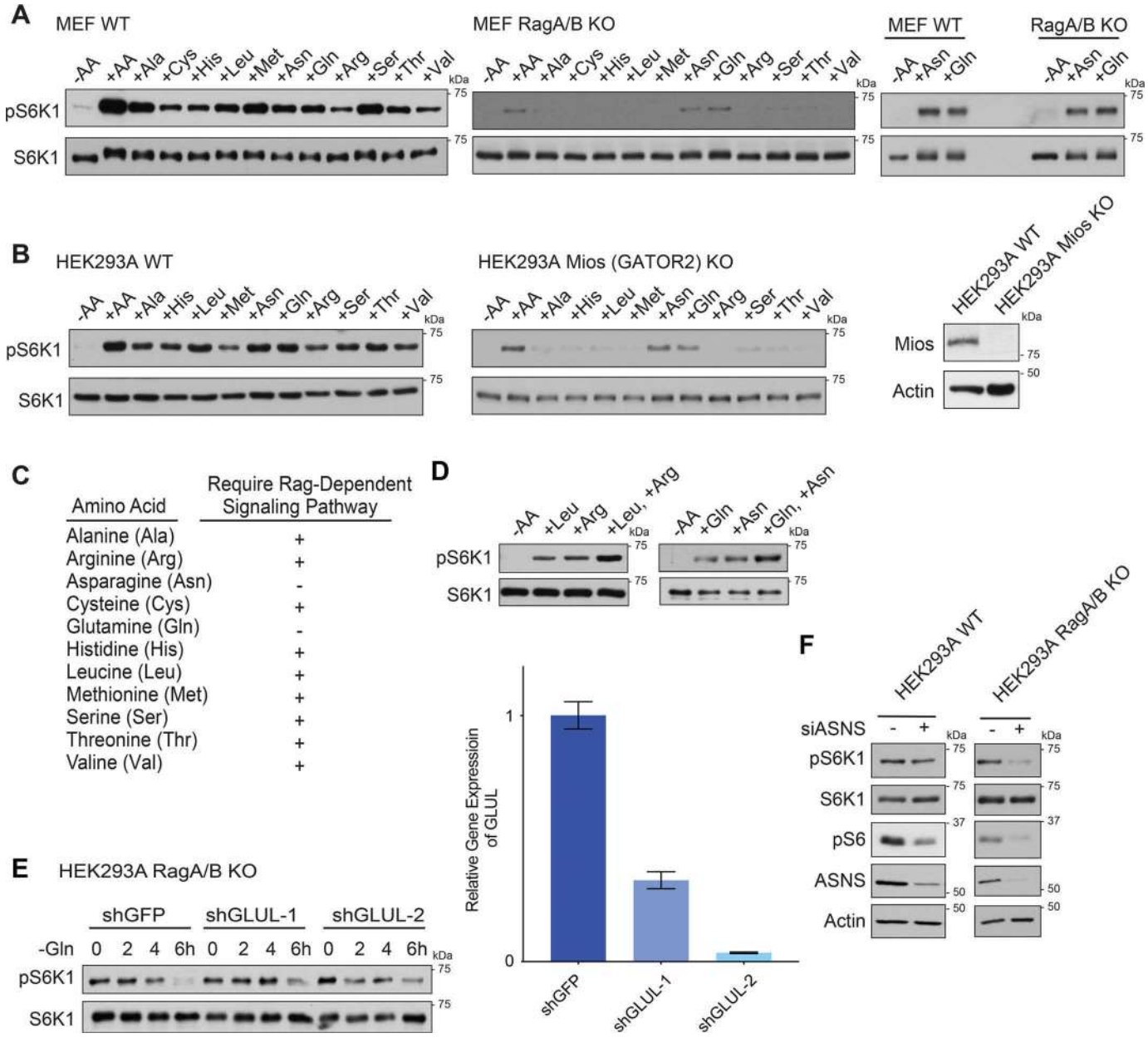
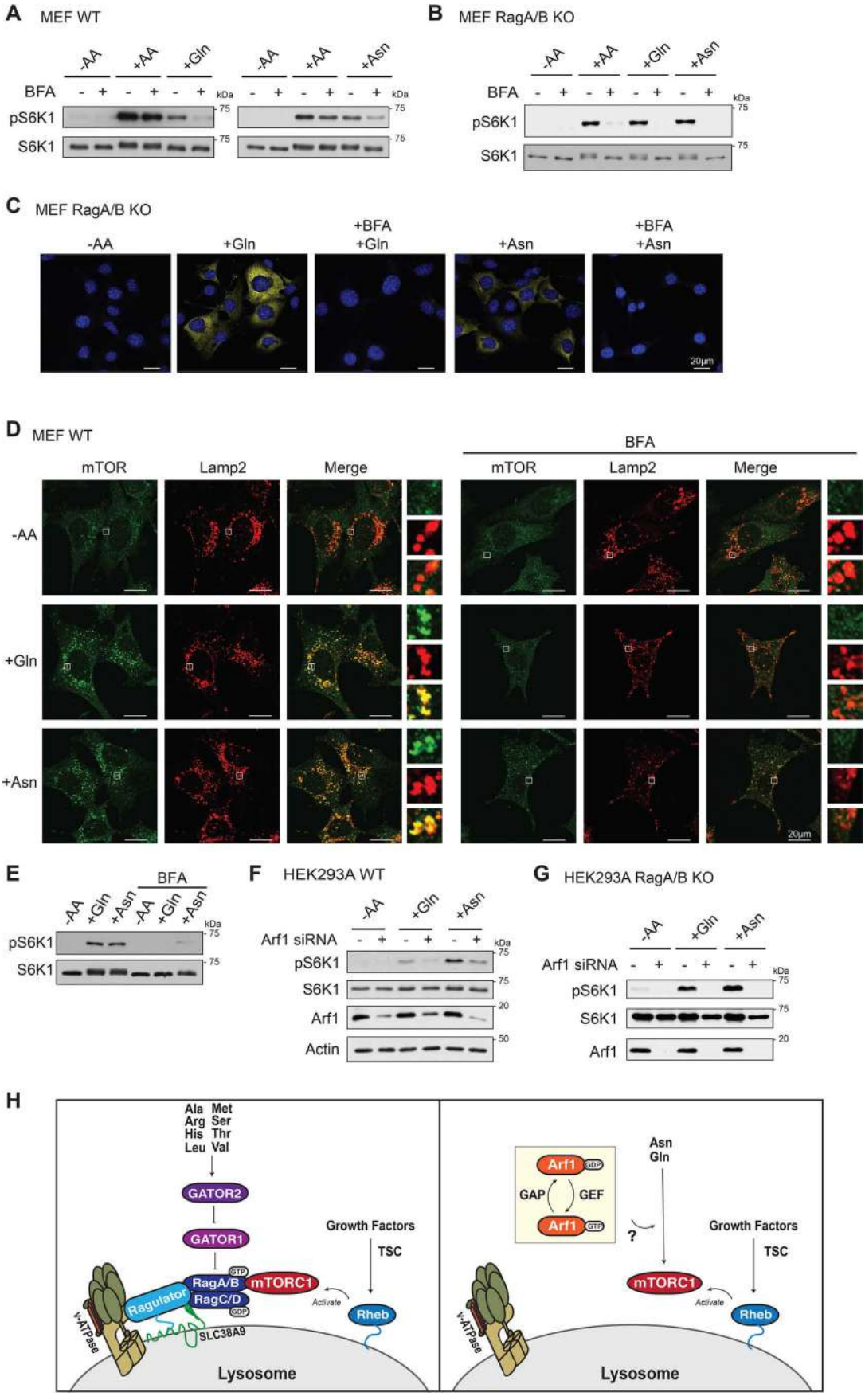


Figure 3. Glutamine and asparagine activate mTORC1 independently of the Rag GTPase signaling pathway. *A*, WT (*left*) or RagA/B knockout (KO) MEF cells (*middle*) were starved of amino acids (−*AA*) for 2 h, followed by the addition of amino acids (+*AA*) or each individual amino acids (500 μM to 1 mM) for 1 h. Gln and Asn stimulation in WT (*left*) or RagA/B KO (*middle*) MEF cells were compared on the same Western blot (*right*). mTORC1 activity was analyzed by immunoblotting for the phosphorylation status of S6K1 (*pS6K1*) at threonine 389. S6K1 was used as loading control. *B*, WT (*left*) or MIOS (component of GATOR2) KO HEK293A cells (*right*) were starved of amino acids for 1 h, followed by the addition of amino acids or each individual amino acid (500 μM to 1 mM) for 1 h. mTORC1 activity was analyzed as in *A*. WT and MIOS KO HEK293A cells were immunoblotted for expression of MIOS protein. Actin was loading control. *C*, *table* summarizing which amino acids require the Rag GTPases to activate mTORC1. *D*, WT HEK293A cells were starved of amino acids for 2 h. Leu, Arg, Gln, and Asn were added either individually or in combination for 2 h. Cells were stimulated with individual amino acids (4 mM) or a combination of two amino acids (2 mM each, total concentration of 4 mM). mTORC1 activity was analyzed as in *A*. *E* (*left*), control (*shGFP*) or Gln synthetase (*GLUL*) shRNA knockdown RagA/B KO HEK293A cells were starved of Gln for the indicated time, and mTORC1 activity was analyzed as in *A*. *Right*, GLUL mRNA levels were confirmed by real-time quantitative PCR. Glyceraldehyde-3-phosphate dehydrogenase was used as internal control. *F*, ASNS was knocked down (*siASNS*) for 48 h in WT or RagA/B KO HEK293A cells, and mTORC1 activity was analyzed as in *A*. Phosphorylation of S6 at serine 235/236 also measures mTORC1 activity. ASNS protein level was determined by immunoblotting. *Error bars*, S.E.

support a critical role of Gln and Asn signaling to mTORC1 in the absence of the Rag GTPases.

Glutamine and asparagine signaling to mTORC1 requires Arf1

Gln signaling to mTORC1 in the absence of the Rag GTPases requires Arf1 (16). We previously demonstrated that Arf1 guanine nucleotide cycling is crucial for Gln, but not Leu or the Rag GTPase pathway, to induce mTORC1 activation and lysosomal localization. Because Asn, like Gln, can activate mTORC1 independently of the Rag GTPases (Fig. 3, *A*–*C*), we tested whether Asn signaling to mTORC1 also requires Arf1. Consistent with our previous findings, treatment of WT MEF cells with brefeldin A (BFA), an Arf1 guanine exchange factor inhibitor (55), modestly reduced mTORC1 activation in response to amino


A
MEF WT
-AA
+AA
+Gln
+Asn
BFA
kDa
pS6K1
S6K1
75
B
MEF RagA/B KO
C
MEF RagA/B KO
-AA
+Gln
+BFA
+Gln
+Asn
+BFA
+Asn
20μm
D
MEF WT
BFA
mTOR
Lamp2
Merge
-AA
+Gln
+Asn
20μm
E
F
HEK293A WT
Arf1 siRNA
Arf1
Actin
20
50
G
HEK293A RagA/B KO
H
Ala Met
Arg Ser
His Thr
Leu Val
GATOR2
GATOR1
Growth Factors
TSC
RagA/B
RagC/D
GTP
GDP
mTORC1
Ragulator
Activate
Rheb
v-ATPase
SLC38A9
Lysosome
Arf1
GAP
GEF
Asn
Gln
?

acids (Fig. 4*A*). BFA inhibited both Gln- and Asn-induced mTORC1 activation in both WT and Rag A/B KO MEFs (Fig. 4, *A–C*). Moreover, BFA treatment in WT MEF cells dramatically impaired lysosomal localization of mTORC1 induced by Gln and Asn (Fig. 4, *D* and *E*).

In addition to BFA treatment, we also utilized siRNA to knock down Arf1 in WT or RagA/B KO HEK293A cells and analyzed the ability of Gln and Asn to regulate mTORC1. Gln and Asn stimulated mTORC1 activation in WT and Rag A/B KO cells treated with a control siRNA; however, Gln and Asn failed to signal to mTORC1 in cells that were depleted of Arf1 (Fig. 4, *F* and *G*). Thus, Arf1 is involved in Gln and Asn signaling to mTORC1, independent of the Rag GTPase pathway.

In summary, we show that eight amino acids filter through the well-studied Rag GTPase pathway (Fig. 4*H*, *left*). Whereas the sensors of Leu, Arg, and Met have been identified (29, 30, 33–36), the mechanisms by which Ala, His, Ser, Thr, and Val signal to mTORC1 are still unclear. Importantly, in addition to Gln (16), we discovered that Asn also activates mTORC1 in a Rag GTPase–independent manner and requires Arf1 (Fig. 4*H*, *right*). Our results show that mTORC1 is differentially regulated by amino acids through two distinct pathways.

Experimental procedures

Cell lines and tissue culture

HEK293A cells (described in Ref. 16) and MEFs (described in Ref. 16) were cultured in high-glucose DMEM (#D5796 from Sigma) supplemented with 10% FBS (#F2442 from Sigma) and penicillin/streptomycin (#P0781 from Sigma; 100 units of penicillin and 100 μg of streptomycin/ml) and maintained at 37 °C with 5% CO_2. RagA/B KO MEF and HEK293A cells were generated previously (16). Mios (GATOR2) KO HEK293A cells were generated by CRISPR/Cas9 genome editing (56).

Amino acid starvation and stimulation of cells

Amino acid–free medium was made following the Sigma (#D5796) high-glucose DMEM recipe with the exception that all amino acids were omitted. All experiments with amino acid starvation and stimulation contained 10% dialyzed FBS (#F0392 from Sigma) instead of regular FBS (#F2442 from Sigma) unless otherwise indicated. Amino acid starvation was performed by replacing regular medium with amino acid-free medium for ~1–2 h prior to amino acid stimulation unless otherwise indicated. For the confocal experiments, cells were starved of amino acids for 4 h before the addition of amino acids. Glutamine-free DMEM (#D5671 from Sigma) containing 10% dialyzed fetal bovine serum (#F0392 from Sigma) were used in glutamine starvation experiments. For all amino acid stimulation experiments, amino acids were used with the indicated concentration and time points.

Antibodies

The following antibodies were purchased from Cell Signaling Technology and used at the indicated dilution for Western blot analysis: phospho-S6K1 Thr-389 (#9234, 1:1000), S6K1 (#9202, 1:1000), phospho-S6 Ser-235/236 (#4803, 1:1000), 4EBP1 (#9452, 1:1500), phospho-ULK1 Ser-758 (#6888, 1:1000), ULK1 (#8054, 1:1000), Mios (#13557, 1:1000), and Actin (#3700, 1:100,000). Arf1 (#sc-53168, 1:200) and HA (#sc-7392 or #sc-805, 1:500) were obtained from Santa Cruz Biotechnology, Inc. ASNS (14681-1-AP) antibody was from Proteintech. Horseradish peroxidase–linked secondary antibodies (#NXA931V anti-mouse or #NA934V anti-rabbit, 1:4000) were from GE Healthcare. Antibody used for the immunofluorescent microscopy experiments: mTOR (#2983, 1:200) was purchased from Cell Signaling Technology; LAMP2 (#13524, 1:200) was obtained from Abcam; Phospho-S6 ribosomal protein (Ser-235/236) Alexa Fluor 555 conjugate antibody (#3985) was obtained from Cell Signaling Technology; Alexa Fluor 488, 555, 594, and 647 secondary antibodies (1:200) were obtained from Invitrogen.

Chemicals

Rapamycin was from Calbiochem (#53123-88-9). Bafilomycin A1 was from LC Laboratories (#B-1080). Brefeldin A (#B6542), insulin (#I1507), and chloroquine (#C6628) were from Sigma. VPS34-IN1 (#17392) was from Cayman Chemical. All amino acids were obtained from Sigma. For rapamycin, bafilomycin A1, chloroquine, brefeldin A, or VPS34-IN1 treatment experiments, cells were starved of amino acids for 1–2 h, with or without 100 nM rapamycin for 30 min, 10 μM Baf A for 1 h, 100 μM chloroquine for 2 h, 10 μM BFA for 1 h, or 1 μM VPS34-IN1 for 30 min, followed by amino acid stimulation.

Detailed procedures of amino acid stimulation of cells, plasmids, cDNA transfection, RNAi, RNA extraction, reverse transcription, real-time PCR, Western blotting, immunofluorescence microscopy, generation of stable cell lines, generation of Mios knockout cells using CRISPR/Cas9 genome editing, mTOR localization analysis, and statistical analysis are available in the supporting information.

Figure 4. Arf1 is required for glutamine- and asparagine- induced mTORC1 activation. *A*, WT MEF cells were starved of amino acids (−*AA*) for 2 h and pretreated with or without 10 μM BFA for 1 h, and then amino acids (+*AA*), Gln, or Asn was added for 2 h at 4 mM. mTORC1 activity was analyzed by immunoblotting for the phosphorylation status of S6K1 (pS6K1) at threonine 389. S6K1 was used as loading control. *B*, RagA/B KO MEF cells were starved of amino acids for 2 h and pretreated with or without 10 μM BFA for 1 h, and then amino acids, Gln, or Asn was added for 2 h at 4 mM. mTORC1 activity was analyzed as in *A*. *C*, immunofluorescence (*IF*) analysis depicting mTORC1 activity by staining for phospho-S6 (*yellow*). RagA/B KO MEF cells were starved of amino acids for 2 h and pretreated with or without 10 μM BFA for 1 h, and then Gln or Asn was added for 2 h at 4 mM. 4′,6-Diamidino-2-phenylindole is shown in *blue*. *D*, immunofluorescence analysis of mTOR (*green*) and the lysosomal marker LAMP2 (*red*) in WT MEF cells. Cells were amino acid–starved, BFA-pretreated, and Gln- or Asn-stimulated as described in *B*. Higher-magnification images of the depicted area are shown on the *right*. *E*, corresponding Western blots were performed in parallel to the imaging (*D*). mTORC1 activity was analyzed as in *A*. *F*, WT HEK293A cells were transfected with control siRNA (−) or siRNA against Arf1 (+) for 48 h and starved of amino acids for 2 h, and then Gln or Asn was added for 2 h at 4 mM. mTORC1 activity was analyzed as in *A*. *G*, RagA/B KO HEK293A cells were transfected with control siRNA (−) or siRNA against Arf1 (+) for 48 h and starved of amino acids for 2 h, and then Gln or Asn was added for 2 h at 4 mM. mTORC1 activity was analyzed as in *A*. *H*, model of Rag GTPase– dependent and –independent mTORC1 pathways. *Left*, eight amino acids (Ala, Arg, His, Leu, Met, Ser, Thr, and Val) activate mTORC1 through the Rag GTPase pathway. *Right*, two amino acids (Gln and Asn) activate mTORC1 independently of the Rag GTPases, whereas the activity of Arf1 is required through an unknown mechanism. Both pathways require the v-ATPase, the lysosome, and growth factor signaling (through TSC-Rheb) to achieve optical mTORC1 activation.

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