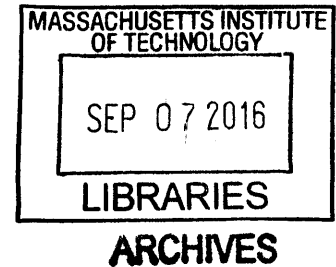Studies of amino acid sensing by the mTORC1 pathway

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Studies of amino acid sensing by the mTORC1 pathway

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Abstract

The mTOR complex 1 (mTORC1) protein kinase is a master growth regulator that responds to multiple cues from the local and systemic environment surrounding the cell. Nutrients and growth factors are both required to activate mTORC1 and to promote growth. While the mechanisms of growth factor signaling have been reasonably well established, we have only begun to unravel in recent years how amino acids signal to mTORC1, thanks in large part to the identification of the Rag GTPases, which recruit mTORC1 to the lysosomal surface for catalytic activation, and of the regulators of Rag function. An ultimate goal is to uncover the biochemical basis of the sensing event that triggers this signaling cascade in the first place: which amino acid(s) are sensed and how? Toward this end, we characterize in detail the amino acids required to fully activate mTORC1 signaling in HEK-293T cells and identify SLC38A9 as a Rag-interacting amino acid transporter that may serve as an arginine sensor at the lysosome. Finally, we discuss the many open questions that remain to be studied regarding the mechanisms and logic behind amino acid sensing by the mTORC1 pathway.

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Table of Contents

Chapter 1

Introduction

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I. Introduction

All eukaryotic cells, in unicellular and multicellular organisms alike, need to determine when it is appropriate to grow and divide. This critical decision hinges on the cell's ability to coordinate the detection of conditions favorable for growth and the execution of the growth program and, conversely, to halt growth-related processes when growth cues, nutrient stores, and energy levels fall below threshold. The mechanistic target of rapamycin (mTOR) anchors a conserved signaling pathway that fulfills these functions in many species, enabling yeast, worms, flies, and mammals to thrive amidst fluctuating environmental conditions.

mTOR is a large serine/threonine protein kinase that serves as the catalytic subunit of two functionally distinct but complementary multi-protein complexes, mTORC1 and mTORC2. mTORC1 is uniquely responsive to changes in the availability of amino acids and glucose, while both complexes are activated by insulin and other growth factors. When active, mTORC1 promotes anabolic processes such as ribosome biogenesis, protein synthesis, and lipid and nucleotide biosynthesis, while suppressing catabolic ones like autophagy. Under starvation conditions, mTORC1 reverses course, limiting translation and stimulating autophagy to replenish the sparse pool of amino acids.

Given its crucial role in coordinating metabolic pathways vital for cell health, it is thus unsurprising that aberrant mTOR signaling is frequently found in cancer, diabetes, and aging (Zoncu, Efeyan, & Sabatini, 2011). Negative regulators of mTOR activity have been found to be tumor suppressors across a wide range of cancer types, which is consistent with the idea that unrestrained mTOR activity leads to cellular growth and proliferation (Guertin & Sabatini, 2007). Additionally, the insulin resistance phenotype characteristic of type 2 diabetes is thought to be related to overloading the nutrient input to mTORC1 and activating maladaptive feedback pathways (Zoncu, Efeyan, et al., 2011). Most recently, a series of studies has demonstrated that chemical inhibition of mTORC1 activity prolongs lifespan in a variety of model organisms, including mice (Harrison et al., 2009; Jia, Chen, & Riddle, 2004; Kaeberlein et al., 2005; Kapahi et al., 2004; R. W. Powers, 3rd, Kaeberlein, Caldwell, Kennedy, & Fields, 2006; Vellai et al., 2003). Hence, a better understanding of the nutrient-sensing pathways upstream of mTOR activity, in addition to being a subject of longstanding and fundamental interest, may shed light on mechanisms that contribute to common diseases.

II. mTOR and its complexes
A. Discovery of mTOR

Interest in TOR emerged from attempts to understand the anti-proliferative properties of rapamycin, a macrolide isolated from *Streptomyces hygroscopicus*, which is a bacterial strain found in soil samples harvested from Easter Island. Although rapamycin had no observable effects on bacteria, it caused profound growth arrest in yeast as well as mammalian cancer cells (Abraham & Wiederrecht, 1996; Eng, Sehgal, & Vezina, 1984). Due to its chemical resemblance to FK506, a natural product found in screens for inhibitors of immune function, rapamycin was also discovered to have robust anti-inflammatory effects (Abraham & Wiederrecht, 1996). This finding paved the way for its widespread and enduring use as an immunosuppressant to prevent organ rejection in the post-transplant setting (Groth et al., 1999; Webster, Lee, Chapman, & Craig, 2006).

Genetic screens in yeast identified several classes of mutants that were resistant to rapamycin treatment (Heitman, Movva, & Hall, 1991; Koltin et al., 1991). The most frequently occurring mutations were found in FPR1, the yeast orthologue of mammalian FKBP12, a protein intermediary also required for the anti-immune effects of FK506. However, as deletion of FPR1 failed to recapitulate rapamycin's cytostatic effect on yeast, FPR1 was deemed not to be the cytostatic target of rapamycin. Two additional clusters of mutations were dominant gain-of-function mutations in two highly homologous yeast genes named TOR1 and TOR2 (Cafferkey, McLaughlin, Young, Johnson, & Livi, 1994; Heitman et al., 1991; Koltin et al., 1991).

Final confirmation that TOR is the direct cellular target of rapamycin emerged from the work of several groups that took biochemical approaches in mammalian cells, which identified a large protein with homology to yeast TOR that bound the FKBP12/rapamycin effector complex (Brown et al., 1994; Sabatini, Erdjument-Bromage, Lui, Tempst, & Snyder, 1994; Sabers et al., 1995). This fundamental discovery gave birth to what has since become the large and sprawling field of mTOR biology, which is notable for its intricate upstream and downstream signaling pathways as well as untold numbers of physiological connections and consequences.

B. Two distinct complexes: mTORC1 and mTORC2

Studies of yeast and mammalian systems have identified two architecturally and functionally distinct mTOR-anchored multi-protein complexes – mTOR complex 1, which is

rapamycin-sensitive, and mTOR complex 2, which is not (Fig. 1) (Loewith et al., 2002). In addition to mTOR itself, these two complexes also share mLST8, DEPTOR (a negative regulator), and the Tti1/Tel2 scaffold proteins (Laplante & Sabatini, 2012). Otherwise, these two complexes feature different protein constituents and, as such, are subject to divergent modes of regulation and phosphorylate distinct sets of substrates.
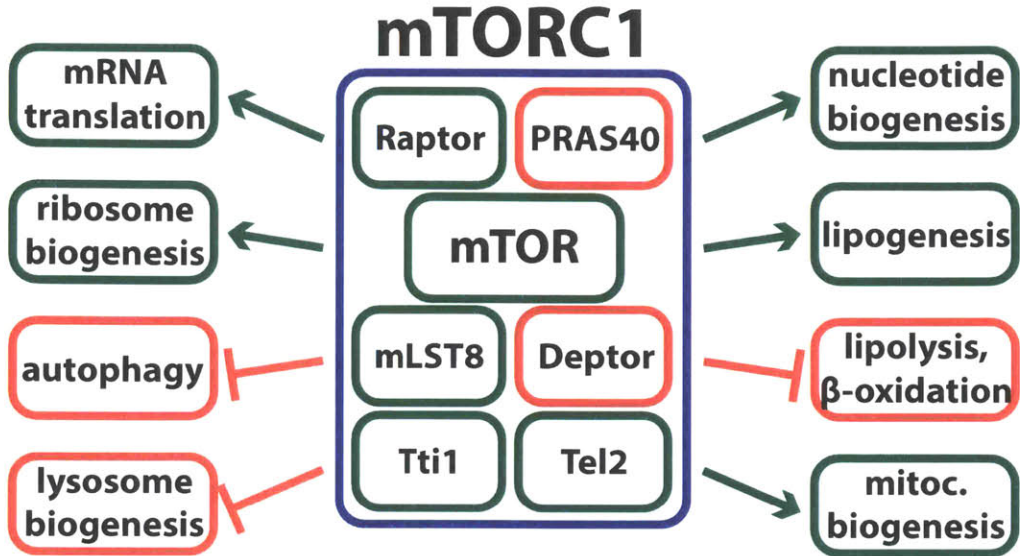
The defining member of mTORC1 (Fig. 1A) is the regulatory-associated protein of mTOR (raptor), a large scaffold protein of 150 kDa that regulates the assembly, localization, and substrate binding of the catalytic complex (Hara et al., 2002; D. H. Kim et al., 2002; Loewith et al., 2002; Shinozaki-Yabana, Watanabe, & Yamamoto, 2000). Knockdown of raptor (an essential gene) in cells by RNAi leads to signaling effects similar to those resulting from rapamycin treatment, which is consistent with the fact that only mTORC1, but not mTORC2, is sensitive to rapamycin. Finally, mTORC1 also contains PRAS40, a negative regulator of pathway activity (Sancak et al., 2007).

By contrast, mTORC2 (Fig. 1B) is distinguished by the presence of rapamycin-insensitive companion of mTOR (rictor), a scaffold protein that regulates the assembly and substrate binding of mTORC2 (Sarbassov et al., 2004). Without rictor, mTORC2 does not properly assemble into a functional complex. Likewise, mSin1 binds to rictor and is required for mTORC2 assembly, stability, and activity (Frias et al., 2006; Jacinto et al., 2006). mSin1 has three different isoforms which contribute to three mTORC2 variants that respond with differential sensitivity to insulin, a major input into mTORC2 signaling (Frias et al., 2006). The final component of mTORC2 is protor1/2, which is required for the activation of a specific subset of SGK1 substrates (Pearce et al., 2007).

Much of the remaining introduction will be devoted to the upstream and downstream pathways linked to mTORC1, the better understood complex by far, with a particular focus on the mechanisms of amino acid sensing (the core interest of our lab). A brief discussion of mTORC2 will follow toward the end.

Fig. 1

A

mTORC1

mRNA translation

ribosome biogenesis

autophagy

lysosome biogenesis

Raptor | PRAS40

mTOR

mLST8 | Deptor

Tti1 | Tel2

nucleotide biogenesis

lipogenesis

lipolysis, β-oxidation

mitoc. biogenesis

B

mTORC2

Protor

Rictor | mSin1

mTOR

mLST8 | Deptor

Tti1 | Tel2

cellular survival

metabolism

actin cytoskeleton
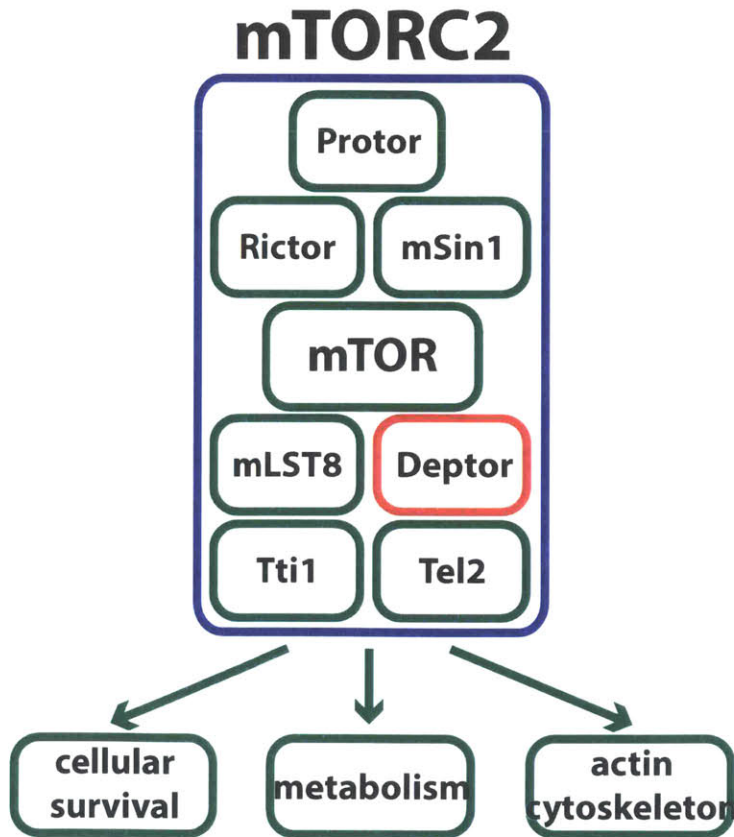
III. Downstream outputs: mTORC1 controls many metabolic processes

mTORC1 has long been appreciated to play a pivotal role in the regulation of protein synthesis and degradation (Laplante & Sabatini, 2012). In recent years, it has become evident that mTORC1 governs a much broader swath of the cellular metabolic space than protein metabolism alone (Fig. 1A). This pathway is now thought to regulate the biogenesis of lysosomes and mitochondria as well as lipid and nucleotide metabolism. From a mechanistic perspective, phosphorylation of mTORC1 substrates carries important enzymatic and transcriptional consequences, and it is worth noting that this regulatory switch is dictated by the rate of phosphorylation (by mTORC1) rather than by that of dephosphorylation. This section briefly explores the mechanisms and functional outputs of mTORC1 signaling.

A. Protein synthesis

When active, mTORC1 drives protein synthesis by coordinately boosting the production of protein synthesis machinery (ribosomes) and increasing the efficiency of its rate-limiting step (translation initiation). Conversely, inhibition of mTORC1 activity with ATP-competitive Torin1 suppresses incorporation of ^{35}S-Cys/Met into protein by 65% and shifts ribosomes away from polysomes (multiple ribosomes per mRNA molecule) to monosomes (one ribosome per mRNA molecule), an indication of reduced translation initiation (Thoreen et al., 2012). mTORC1 orchestrates these anabolic effects through the phosphorylation of two key substrates: 4E-BP and S6K (Brunn et al., 1997; Burnett, Barrow, Cohen, Snyder, & Sabatini, 1998; Hara et al., 1997; Isotani et al., 1999). Indeed, even before the discovery of TOR, both of these substrates were already recognized as important downstream targets of a rapamycin-sensitive pathway (Beretta, Gingras, Svitkin, Hall, & Sonenberg, 1996; Chung, Kuo, Crabtree, & Blenis, 1992; Kuo et al., 1992; von Manteuffel, Gingras, Ming, Sonenberg, & Thomas, 1996).

Initiation of translation and assembly of the small ribosomal subunit around the mRNA substrate involves recognition of the 7-methylguanosine 5' cap on the mRNA molecule by the eIF4F protein complex, which consists of eIF4E, eIF4G, and eIF4A (Ma & Blenis, 2009). Of these proteins, eIF4E binds the 5' cap most proximally and recruits other components of eIF4F to form a competent initiation complex. In cells with inactive mTORC1, hypophosphorylated 4E binding protein (4E-BP) binds tightly to eIF4E and sequesters it from interacting with eIF4G (Ma & Blenis, 2009). Upon mTORC1 activation, 4E-BP becomes hyperphosphorylated and

releases eIF4E, which goes on to recruit eIF4G to the 5'cap. This sets the stage for the recruitment of several additional initiation factors, ultimately resulting in mRNA translation.

The subset of mRNAs whose translation is most closely regulated by mTORC1 all share 5' terminal oligopyrimidine (TOP) motifs, or a sizeable tract of cytosine and uracil bases (Jefferies et al., 1997). These 5' TOP motifs feature prominently in the 5' UTR of many genes encoding ribosomal proteins, which is consistent with the observation that mTORC1 activity increases ribosome numbers (Meyuhas, 2000). In the setting of 4E-BP deletion, chemical inhibition of mTORC1 activity no longer affects the translation of mRNA with 5' TOP motifs, suggesting that the 4E-BP axis is sufficient to explain the specificity of mRNAs targeted by mTORC1 (Thoreen et al., 2012). Lastly, there are also lines of evidence to suggest that mTORC1 activity stimulates the transcription of rRNA components of the ribosome (Hardwick, Kuruvilla, Tong, Shamji, & Schreiber, 1999; Mahajan, 1994; T. Powers & Walter, 1999).

S6K is a very well established substrate of mTORC1 and the knockout of S6K1 causes a 20% size reduction relative to wild-type mice (Shima et al., 1998). However, the mechanism(s) by which S6K affects protein synthesis is not entirely clear. Upon phosphorylation by mTORC1, S6K1 phosphorylates 40S ribosomal protein S6 (Holz, Ballif, Gygi, & Blenis, 2005; Jeno, Ballou, Novak-Hofer, & Thomas, 1988; Sturgill & Wu, 1991), although it is debatable whether this modification substantially affects the rate of translation. Additionally, active S6K1 phosphorylates a number of components of the mRNA translation machinery to promote protein synthesis, including eIF4B, S6K1 Aly/RE-like substrate (SKAR), programmed cell death 4 (PDCD4), eukaryotic elongation factor 2 kinase (eEF2K), eukaryotic initiation factor 3 (eIF3), and 80 kDa nuclear cap-binding protein (CBP80) (Dann & Thomas, 2006; Dorrello et al., 2006; Magnuson, Ekim, & Fingar, 2012).

B. Autophagy

In addition to promoting anabolic processes like protein synthesis, active mTORC1 also suppresses catabolic ones like autophagy. Autophagy refers to the process by which cells recover molecular building blocks such as amino acids, sugars, and lipids through the enzyme-mediated degradation of damaged or otherwise dispensable proteins and organelles in autophagosome-lysosome networks. These building blocks can be mobilized for energy production during times of starvation or again incorporated into macromolecules. First, mTORC1 directly phosphorylates

and suppresses the activity of ULK1, a kinase required in the early stages of autophagosome formation (Dunlop & Tee, 2014; Mizushima, 2010). Second, mTORC1 indirectly inhibits ULK1 by phosphorylating and inactivating AMBRA1, which along with E3-ligase TRAF6 is required for the stabilization of ULK1 (Nazio et al., 2013). It should be noted that ULK1 activation is also sensitive to other forms of cellular stress and deprivation, including signaling of glucose starvation through the AMPK pathway (Dunlop & Tee, 2014; J. Kim, Kundu, Viollet, & Guan, 2011).

C. Lysosome biogenesis

mTORC1 not only governs ULK1-mediated initiation of autophagy, as described above, but also the cell's total capacity for autophagic flux through the control of lysosomal biogenesis. TFEB, a basic helix-loop-helix transcription factor, is a master regulator of lysosomal biogenesis under the control of mTORC1 (Sardiello et al., 2009; Settembre et al., 2011; Settembre et al., 2012). Active mTORC1 phosphorylates TFEB and renders it susceptible to cytoplasmic sequestration by 14-3-3. Inactivation of mTORC1 with a potent ATP-competitive inhibitor like Torin1 enables hypophosphorylated TFEB to escape the cytoplasm and accumulate in the nucleus (Settembre et al., 2012), where it induces the expression of lysosomal hydrolases, lysosomal membrane proteins, and components of the v-ATPase complex (Sardiello et al., 2009). Moreover, these transcriptional events also promote the formation of autophagosomes and their fusion with lysosomes. With these enhanced degradative abilities, cells can both clear up damaged molecules and generate energy from newly liberated nutrient building blocks.

Since the clearance of damaged and dysfunctional macromolecules and organelles is an important antidote to aging processes, it may be advantageous to suppress mTORC1 activity where there exists a large load of cellular aggregates, as is indeed the case in many forms of aging-associated neurodegeneration (Nixon, 2013; Rubinsztein, 2006). It is therefore tempting to speculate that chronic rapamycin treatment may be delaying aging in organisms ranging from yeast to mice in part through the enhancement of autophagy and the turnover of damaged biomolecules.

D. Mitochondria biogenesis

mTORC1 also plays a role in mitochondrial biogenesis and function, although aspects of these mechanisms remain controversial. It has been proposed that nuclear mTORC1 controls the transcriptional activity of mitochondria-regulating PPARγ coactivator-1 (PGC1α) by altering its physical interaction with another transcription factor, yin-yang 1 (YY1) (Cunningham et al., 2007). However, the notion that mTORC1 translocates to and functions within the nucleus has not been widely accepted. Indeed, most reports show mTORC1 shuttling between an unspecified cytoplasmic compartment and the lysosomal membrane, depending on the degree of cellular deprivation or stress (Sancak et al., 2010). There is additional evidence to suggest that mTORC1, through 4E-BP, promotes the translation of mitochondrial mRNAs with the effect of augmenting the energy-generating capacity of a cell (Morita et al., 2013). As mTORC1-driven anabolism – and protein synthesis in particular – is energetically costly, the idea that mTORC1 drives concurrent energy production is thus an attractive model.

E. Lipid metabolism

mTORC1 also serves as a central regulator of lipid homeostasis. When active, mTORC1 promotes *de novo* lipogenesis by enhancing the proteolytic processing and nuclear accumulation of sterol regulatory element binding proteins (SREBP1/2), transcription factors that stimulate the expression of the entire suite of lipogenic enzymes (Lamming & Sabatini, 2013; Laplante & Sabatini, 2009; Ricoult & Manning, 2013). This process requires the phosphorylation of mTORC1 substrates S6K, 4E-BP, and Lipin1 (Duvel et al., 2010; Huffman, Mothe-Satney, & Lawrence, 2002; Li et al., 2011; Owen et al., 2012; Peterson et al., 2011; B. T. Wang et al., 2011). The molecular events linking the phosphorylation of S6K or 4E-BP to SREBP maturation have not yet been well characterized, although the action of transcription factor Lipin1 is thought to involve restructuring of the nuclear lamina (Santos-Rosa, Leung, Grimsey, Peak-Chew, & Siniossoglou, 2005). The genes encoding SREBP themselves have sterol regulatory elements, enabling a positive transcriptional feedback loop whereby mTORC1 activity stimulates SREBP activity and, consequently, further SREBP induction (Amemiya-Kudo et al., 2000; Duvel et al., 2010; Sato et al., 1996). Conversely, mTORC1 suppresses lipolysis: in mice, serum lipid levels rise with both chemical inhibition of mTORC1 by rapamycin and adipose-specific genetic deletion of mTORC1 effectors S6K1 and 4E-BP1/2 (Chakrabarti, English, Shi, Smas, & Kandror, 2010; Le Bacquer et al., 2007; Soliman, Acosta-Jaquez, & Fingar, 2010; Um et al.,

2004; Zhang, Yoon, & Chen, 2009). Mice that are incapable of autophagy also fail to trigger lipolysis, which suggests that the autophagic pathway is required for the liberation of free lipids (Y. Zhang et al., 2009). Moreover, mTORC1 activity suppresses beta-oxidation of fatty acids, the canonical mechanism by which fatty acids are further broken down into acetyl-CoA units, and may also influence systemic lipid transport (Aguilar et al., 2007).

F. Nucleotide metabolism

Lastly, recent work has demonstrated that mTORC1 also governs the biosynthesis of pyrimidines and purines, which collectively serve as the building blocks for DNA and RNA synthesis. mTORC1 signaling activates S6K1, which in turn phosphorylates and activates CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, dihydroorotase), the enzyme responsible for catalyzing the first three steps of *de novo* pyrimidine biosynthesis (Ben-Sahra, Howell, Asara, & Manning, 2013). Under these conditions, flux through the pyrimidine pathway increases, leading to a larger pool of available nucleotides for ribosome biogenesis and anabolic growth. mTORC1 signaling also stimulates the production of purine nucleotides through the eIF2α-independent, rapamycin-sensitive activation of the ATF4 transcription factor (Ben-Sahra, Hoxhaj, Ricoult, Asara, & Manning, 2016). It now appears that the ATF4 consensus DNA binding motif is found in the promoters of many genes under mTORC1 transcriptional control, including MTHFD2 (methylenetetrahydrofolate dehydrogenase 2). The induction of MTHFD2 expression boosts the production of formyl units that then feed into the purine biosynthetic pathway.

G. Organ- and organism-wide metabolic effects

It is now known that mTORC1 exerts tight homeostatic control over a constellation of metabolic processes to ensure proper growth at the cellular level. These regulatory pathways are also the basis of organ- and organism-wide metabolic effects, including gluconeogenesis and glucose transport in the liver, glycogen synthesis in the liver, muscles, and kidneys, and adipogenesis and lipogenesis in white adipose tissue (Laplante & Sabatini, 2012; Zoncu, Efeyan, et al., 2011). The mTORC1 pathway has evolved to handle the interconnectedness of whole body metabolism by coordinately governing metabolic responses at cell, organ, and organism-wide levels.

IV. Upstream inputs: mTORC1 integrates diverse environment cues

mTORC1 is controlled by a constellation of signals that collectively reflect the metabolic status of the cell. In unicellular organisms like yeast, TORC1 acts as a sensor of nitrogen and carbon availability and is activated by amino acid and glucose sufficiency (De Virgilio & Loewith, 2006). In multicellular metazoans, the mTORC1 pathway also takes into account growth factors, energy levels, oxygen availability, and stress as indicators of readiness for growth (Sengupta, Peterson, & Sabatini, 2010). The list of environmental cues that mTORC1 is known to sense is impressively long, although it is curious to speculate why the pathway has evolved to sense some nutrients but not others. For example, mTORC1 is known to tune lipid homeostasis through a variety of mechanisms, yet it has not yet been shown to act as a lipid sensor. As our mechanistic understanding of the upstream inputs expands, there may be surprises yet to be revealed.

A. mTORC1 is a coincidence detector: Convergence of two upstream branches

mTORC1 integrates its upstream metabolic cues through two parallel signaling branches, one of which is anchored by the small GTPase Rheb (Manning & Cantley, 2003) and the other by the obligate heterodimeric Rag GTPases (Fig. 2A) (E. Kim, Goraksha-Hicks, Li, Neufeld, & Guan, 2008; Sancak et al., 2008). Rheb is regulated by the large heterodimeric TSC complex, which aggregates all inputs with the exception of the nutrient signal (amino acids and glucose), and in its GTP-bound state strongly stimulates the kinase activity of mTORC1(Manning & Cantley, 2003). By contrast, the Rag GTPases respond to changes in amino acid abundance and, when sufficient, recruit mTORC1 from the cytoplasm to the lysosomal membrane (Sancak et al., 2010; Sancak et al., 2008), where Rheb normally resides. Thus, the Rheb and the Rag GTPases form two halves of a coincidence detector, ensuring that mTORC1 becomes activated only when all of its upstream inputs are satisfied.

B. Inputs to TSC/Rheb axis

The TSC complex is comprised of tuberin (TSC1), hamartin (TSC2), and now a third core subunit TBC1D7 (Dibble et al., 2012). Even before being implicated in the mTORC1 pathway, TSC1 or TSC2, when lost (typically autosomal dominant inheritance with loss of

heterozygosity), was known to lead to the development of tuberous sclerosis complex, a tumor syndrome characterized by the appearance of benign tumors containing large cells in many tissue types (Kwiatkowski & Manning, 2005; Manning & Cantley, 2003). As a GTPase activating protein (GAP) for Rheb, the TSC complex (with the catalytic core residing in TSC2) blocks mTORC1 activation by promoting the conversion of Rheb^{GTP} into inactive Rheb^{GDP} (Inoki, Li, Xu, & Guan, 2003; Tee, Anjum, & Blenis, 2003). As growth conditions improve, the GAP activity of the TSC complex is repressed and so Rheb^{GTP} resumes mTORC1 activation. The activity of the TSC complex depends on its phosphorylation status, which is regulated by the pathways that transmit growth factor, energy, oxygen, and stress signals (Fig. 2A).

1. Growth factors

Growth factors such as insulin and IGF-1 reflect the fed status of the organism and are secreted into the bloodstream when food is plentiful. The binding of these growth factors to their cognate tyrosine kinase receptors activates the PI3K-Akt signaling cascade (Potter, Pedraza, & Xu, 2002; Tee et al., 2003). Phosphorylation of TSC2 by Akt causes the TSC complex to dissociate from the lysosome and thus from Rheb, enabling Rheb to activate mTORC1 (Menon et al., 2014). This mechanism is supported by the fact that chemical inhibition of Akt activity by MK2206 prevents this dissociation event even in the presence of growth factors (Menon et al., 2014). Akt also directly phosphorylates PRAS40, relieving its repression of mTORC1 (Oshiro et al., 2007; Sancak et al., 2007; Thedieck et al., 2007; Vander Haar, Lee, Bandhakavi, Griffin, & Kim, 2007; L. Wang, Harris, & Lawrence, 2008; L. Wang, Harris, Roth, & Lawrence, 2007).

2. Energy

Energy stress, which is characterized by an elevated AMP/ATP ratio, activates AMP-activated protein kinase (AMPK) to phosphorylate the TSC complex (Corradetti, Inoki, Bardeesy, DePinho, & Guan, 2004; Inoki, Zhu, & Guan, 2003; Shaw et al., 2004). These sites are distinct from those targeted by Akt, as are the consequences of these phosphorylation events. Phosphorylation by AMPK, through an unknown mechanism, enhances the GAP activity of the TSC complex and suppresses mTORC1 signaling. Moreover, AMPK has also been shown to directly phosphorylate raptor in an mTORC1-inhibitory manner (Gwinn et al., 2008).

3. Hypoxia

Hypoxia suppresses mTORC1 signaling through multiple mechanisms. First, low oxygen levels stall the mitochondrial electron transport chain and thus reduce the cell's capacity for ATP generation. The resultant rise in AMP/ATP activates AMPK, which inhibits mTORC1 directly via raptor phosphorylation and indirectly via the TSC complex. Second, hypoxia also induces the expression of Redd1, which encodes a 232 amino acid cytoplasmic protein with no recognizable domains (Brugarolas et al., 2004; Reiling & Hafen, 2004). Redd1 activates the TSC complex through an as yet undetermined mechanism and in fact turns out to be transcriptionally induced under a variety of cellular stresses, including DNA damage, glucocorticoids, and oxidizing agents (Ellisen et al., 2002; Z. Wang et al., 2003; Yoshida et al., 2010).

C. Inputs to Rag GTPase axis

The Rag GTPases are the founding members of a much more recently discovered arm of the sensing machinery upstream of mTORC1, through which the long-elusive amino acid signal is transmitted (Fig. 2B) (E. Kim et al., 2008; Sancak et al., 2008). This obligate heterodimer of small GTPases consists of RagA or RagB bound to RagC or RagD and can adopt one of four possible nucleotide configurations. The consequences of two such states on signaling are well-established: expression of a mutant heterodimer in which the Rags are locked into the $RagA^{GTP}/RagC^{GDP}$ configuration constitutively activates mTORC1 even in the absence of amino acids, while expression of $RagA^{GDP}/RagC^{GTP}$ suppresses signaling despite amino acid stimulation. The remaining two states – $RagA^{GTP}/RagC^{GTP}$ and $RagA^{GDP}/RagC^{GDP}$ – may represent transient intermediates that readily resolve to one of the two stable nucleotide configurations. Why the Rag proteins exist as an obligate heterodimer (as opposed to a simple monomer) and how their nucleotide binding states are regulated remain poorly understood. It has been hypothesized, but not proven, that the nucleotide state of one Rag protein can influence that of its binding partner and that this heterodimeric setup, with all of its attendant complexities, affords many opportunities for extrinsic regulation.

1. Ragulator complex

The identification of the Rag GTPases afforded the long-sought-after biochemical handle to find additional protein components of the amino acid sensing pathway, with the ultimate goal

of discovering and characterizing the sensor(s) themselves. In recent years, work from the Sabatini lab has uncovered several multiprotein complexes that influence both Rag nucleotide binding state and mTORC1 signaling in response to amino acid stimulation. First, mass spectrometric analyses of anti-Rag immunoprecipitates from mammalian cells that stably express Rag proteins identified the pentameric Ragulator complex (Bar-Peled, Schweitzer, Zoncu, & Sabatini, 2012; Sancak et al., 2010). This complex consists of two sets of roadblock-containing dimers – p14/MP1 and HBXIP/c7orf59 (Bar-Peled et al., 2012) – bound to p18, whose N-terminal myristoyl or palmitoyl tail anchors the entire complex as well as the Rag proteins to the outer leaflet of the lysosomal membrane. Ragulator was initially shown to act *in vitro* as a guanosine nucleotide exchange factor (GEF) for RagA (Bar-Peled et al., 2012). Consistent with the notion that RagA^{GTP} promotes mTORC1 translocation and activation, deletion of any Ragulator component impairs mTORC1 activation upon amino acid stimulation.

2. v-ATPase

The vacuolar H$^+$-ATPase (v-ATPase), a membrane-bound megacomplex that acidifies the lysosomal lumen, is also necessary for mTORC1 activation. It engages in extensive amino acid-regulated interactions with the Ragulator complex and may influence mTORC1 activity via its ability to enhance the GEF activity of Ragulator (Zoncu, Bar-Peled, et al., 2011). It is not yet clear to what degree or how the canonical function of the v-ATPase may affect its ability to transmit the amino acid signal and vice versa, although *in vitro* experiments suggest that ATP hydrolysis is a necessary step in both processes. Furthermore, amino acids appear to be sensed in an inside-out fashion, with the signal originating from within the lysosomal lumen before being transmitted across the lysosomal membrane (Zoncu, Bar-Peled, et al., 2011). Cautious interpretation of these results is critical, however. Since many lysosome-centric activities are pH-dependent, perturbation of v-ATPase function can trigger a cascade of indirect sequelae. Blocking pH homeostasis could, among other things, alter the efficiency of enzymatic reactions within the lumen or transporter behavior at the membrane, which in turn could dramatically affect the amino acid composition of the lumen or the flux across the membrane. To better understand the role of the v-ATPase in amino acid sensing and the validity of the inside-out model, it would be necessary to reconstitute the system *in vitro*, to devise a knock-in system to

study the effects of specific genetic mutations, and/or to develop a method to monitor the metabolic contents of the lysosomal lumen.

3. GATOR1 and GATOR2

The Rag GTPases are also regulated by the cytoplasmic GATOR1 complex, which was named to reflect its ability to act as a GTPase-activating protein (*GA*P) *to*ward *R*agA (Bar-Peled et al., 2013; Panchaud, Peli-Gulli, & De Virgilio, 2013). GATOR1 is comprised of Nprl2, Nprl3, and DEPDC5 proteins and promotes the hydrolysis of $RagA^{GTP}$ to $RagA^{GDP}$. Since cells with nonfunctional GATOR1 exhibit constitutive mTORC1 activity despite amino acid deprivation, it is perhaps not surprising that all three genes are documented tumor suppressors. A separate pentameric complex, called GATOR2 (Mios, WDR24, WDR59, Sec13, Seh1L), negatively regulates GATOR1 according to epistasis analyses conducted in Drosophila and mammalian cells alike (Bar-Peled et al., 2013; Panchaud et al., 2013). Accordingly, deletion of any component of GATOR2 impairs mTORC1 activation upon amino acid stimulation. Mios, WDR24, and WDR59 all have RING domains characteristic of E3 ligases, but the molecular function of GATOR2 remains unclear. The complexity of the GATOR1 and GATOR2 complexes – eight proteins in all – hints at manifold possibilities for higher-order regulation, as will be discussed in section V.

4. Folliculin

The folliculin/FNIP1/2 complex stimulates the GTP hydrolysis of RagC (Tsun et al., 2013). As $RagC^{GDP}$ forms one-half of the active Rag heterodimer and is capable of binding the raptor component of mTORC1, active folliculin facilitates the translocation of mTORC1 to the lysosomal surface and its subsequent activation by Rheb. There exists, however, an as yet unresolved paradox as folliculin is a frequently deleted tumor suppressor in Birt-Hogg-Dubé syndrome. To date, this is the only example in which a positive regulator of mTORC1 signaling at the cellular level behaves as a tumor suppressor in organisms (Tsun et al., 2013).

5. Rag axis mutations in disease

As with components of the TSC/Rheb pathway, mutations in components of the amino acid sensing arm upstream of mTORC1 have been increasingly implicated in disease states.

Exome analysis of 24 follicular lymphoma tumor samples from five patients uncovered recurrent RagC mutations that both activate mTORC1 signaling and render cells resistant to amino acid starvation (Okosun et al., 2016). Likewise, homozygous loss of GATOR1 components has been observed in tumors from diverse tissues of origin (Bar-Peled et al., 2013). GATOR1 mutations have also been implicated in several familial cases of focal epilepsy, which are thought to have arisen from loss of heterozygosity at the affected GATOR1 locus (Dibbens et al., 2013; Ishida et al., 2013; Ricos et al., 2016). This disease phenotype may be explained by the finding that focal overgrowth, such as that commonly seen in tuberous sclerosis complex, is highly associated with neuronal hyperexcitability. Conversely, a hypomorphic allele of Ragulator component p14 was discovered in a large Mennonite family in which four of fifteen children showed a pronounced growth defect (below 3rd percentile in age-adjusted height), hypopigmentation, and primary immunodeficiency (Bohn et al., 2007). It is plausible that this phenotype could be linked to hypoactive mTORC1 signaling. From these examples and more, it has become quite clear that both gain- and loss-of-function mutations in the amino acid sensing arm of the mTORC1 pathway can have deleterious consequences, prompting an urgent need to better understanding this pathway and its potential physiological implications.

**V. Amino acid sensing by the mTORC1 pathway**

Up until 2014, much of the scientific developments in the field of amino acid sensing involved the identification of protein complexes that relay the amino acid signal to mTORC1. However, there was very little insight into the most proximal amino acid binding event that would initiate this signaling cascade. Understanding this important protein/metabolite interface as well as how it connects with the rest of the characterized signaling cascade will be the subject of discussion in this section.

Fig. 2
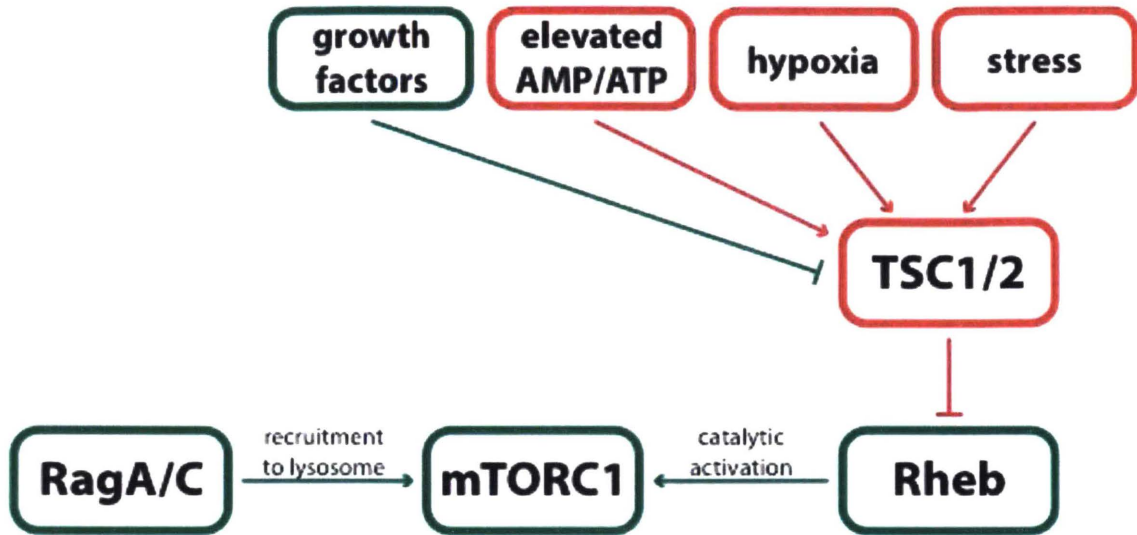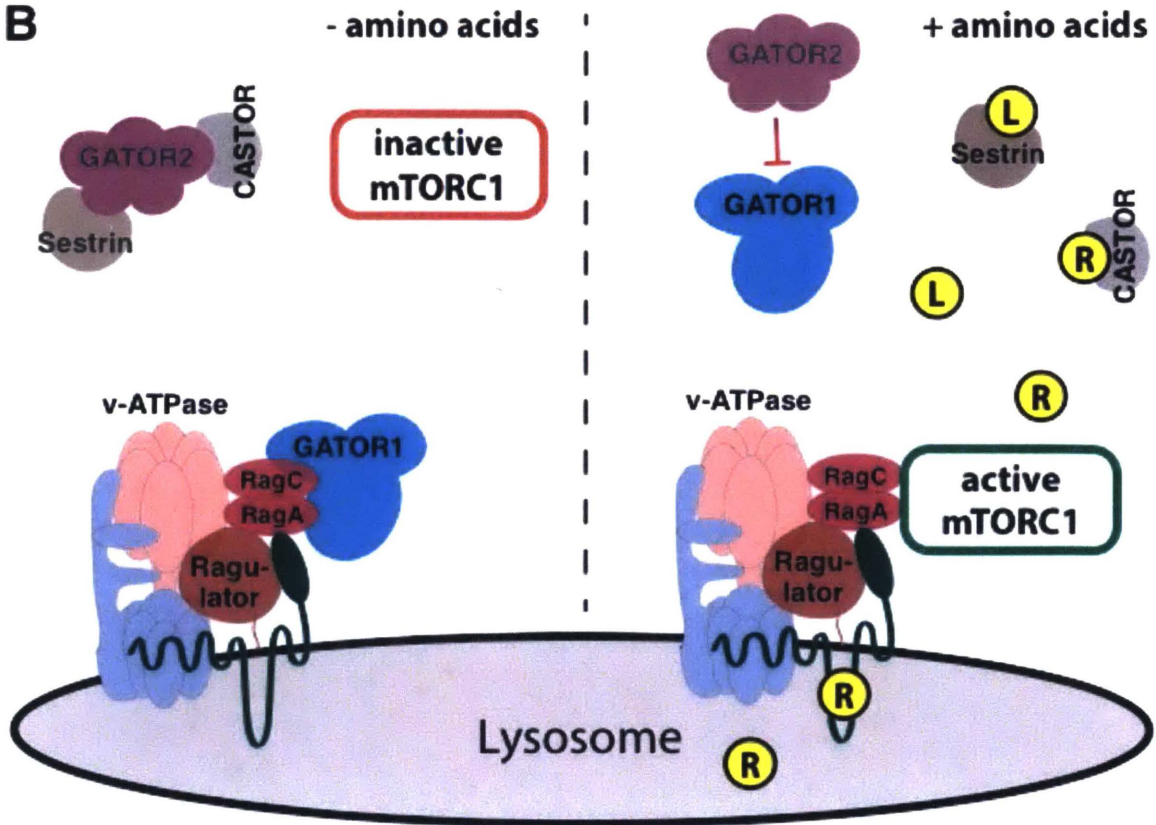
A. Which amino acids are sensed?

It is widely believed that the amino acid signal originates from within the cell and is relayed through intracellular signaling pathways to mTORC1. The best evidence for this is that inhibition of translation with cycloheximide can activate mTORC1 even in the absence of extracellular amino acids, presumably by introducing a surplus of intracellular amino acids (Beugnet, Tee, Taylor, & Proud, 2003). Although significant progress over the past decade has revealed a network of proteins that transmit the amino acid signal (see section IV), it remained unknown, until recently, which particular metabolite(s) – be it one amino acid, a subset of amino acids, all amino acids, degradation product(s) thereof, or even a non-side-chain feature shared among bulk amino acids – is actually sensed, as well as where and how this process occurs.

There are 20 proteogenic amino acids, of which only a subset, called the non-essential amino acids, are synthesized *de novo* in metazoans. The remaining essential amino acids are acquired through nutritional intake or turnover of existing proteins. It would seem reasonable to speculate that the ability to detect essential amino acids could be advantageous for metazoans, as these molecules are more likely to be limiting under starvation conditions.

Previous work indicated that leucine and arginine, both essential amino acids, play a privileged role in mTORC1 activation (Hara et al., 1998). Leucine is known to stimulate protein synthesis in animals, and many have argued that this is partly due to activation of the mTORC1 pathway (Kimball & Jefferson, 2006). In cell culture, withdrawal of either leucine or arginine inhibits mTORC1 signaling, but re-addition of the two in tandem does not substitute for stimulation with the full complement of amino acids. It may be that the mTORC1 pathway requires one or more additional amino acids, which, in combination with leucine and arginine, can fully activate signaling. Another possibility is that leucine and arginine are not sensed themselves but rather enable the sensing of other amino acids. There are many mechanisms by which this could occur, including – but not limited to – potentiating the transport of other amino acids into the compartment where sensing occurs or promoting their conversion into a metabolite capable of binding the sensor. In the absence of a defined subset of amino acids that is fully sufficient for activating mTORC1, none of these alternative possibilities could be ruled out.

Through a series of signaling experiments described in chapter 2, we determined that leucine, arginine, and lysine are necessary and sufficient for activating the mTORC1 pathway in HEK-293T cells. These amino acids are not co-dependent for cell entry and cannot substitute for

one another in signaling experiments. It is not yet known, however, if these amino acids affect one another's transport across internal membranes. Our work demonstrated for the first time that lysine is necessary for mTORC1 signaling in HEK-293T cells and, even more interestingly, suggested that there may exist direct sensors of these three amino acids or their downstream metabolites.

Many groups have explored the possibility that leucine or one of its catabolic derivatives is responsible for activating mTORC1. Leucine is primarily degraded in the mitochondria, beginning with an initial reversible deamination by branched chain amino acid transferase (BCAT) to produce keto-isocaproate (KIC). KIC is then irreversibly dehydrogenated by the rate-limiting branched chain alpha-keto acid dehydrogenase (BCKDH) complex and further oxidized. Addition of KIC alone has been shown to potently activate mTORC1, but the introduction of a BCAT chemical inhibitor negates this effect, indicating that KIC depends on its conversion back to leucine rather than its catabolic degradation (Fox, Pham, Kimball, Jefferson, & Lynch, 1998). The fact that cells derived from BCAT-knockout mice no longer support KIC induction of mTORC1 signaling further supports this conclusion (She et al., 2007).

B. Mechanisms of sensing

Efforts to understand amino acid sensing by mTORC1 have been complicated by the sheer multidimensional complexity of the problem: not only does there appear to be a need to sense multiple amino acids within appropriate physiological concentration ranges, there may also be multiple compartments in the cell that require simultaneous monitoring in order to optimally regulate growth. These requirements raise interesting questions about the most proximal amino acid sensing interactions as well as how these binding events are integrated into the greater Rag-dependent signaling network.

It had been hypothesized early on that leucine starvation or stimulation could alter mTORC1 activity through indirect pathways. For example, mTORC1 might be inhibited by the accumulation of uncharged tRNAs, although subsequent studies have shown that inhibition of leucyl-tRNA synthetases using leucyl alcohol analogues had no effect on leucine stimulation of mTORC1 activity (Iiboshi et al., 1999).

1. Transceptors: Transporters as sensors

Over the course of evolution, it is conceivable that direct amino acid sensors evolved by co-opting protein folds or even whole proteins that have known amino acid binding capabilities, such as transporters and enzymes. This has been demonstrated in yeast, in which transporter-like proteins behave as sensors, despite in some cases retaining little to no capacity for transport. For example, methylamine permease 2 (MEP2) in yeast imports ammonium as a key nitrogen source (Lorenz & Heitman, 1998). Although MEP2 has retained some transport activity, there exist transport-capable but signaling-defective mutants that cannot relay the presence of ammonium to activate downstream processes (Rutherford, Chua, Hughes, Cardenas, & Heitman, 2008). Additionally, the Ssy1-Ptr3-Ssy5 (SPS) pathway in yeast senses extracellular amino acids, with Ssy1 being the transport-inactive sensor/receptor/transceptor in the plasma membrane (Klasson, Fink, & Ljungdahl, 1999; Poulsen, Gaber, & Kielland-Brandt, 2008). As a final example, Snf3 and Rgt2 are transporter-like extracellular glucose sensors in yeast with high and low affinity for glucose, respectively; signaling from these proteins promotes the transcription of high affinity and low affinity glucose transporters (Bisson, Neigeborn, Carlson, & Fraenkel, 1987; Ozcan, Dover, Rosenwald, Wolfl, & Johnston, 1996).

The notion that high-affinity, low-capacity transporter-like proteins might additionally serve as sensors has been explored in multicellular organisms as well. It has been proposed that the *Drosophila* proton-assisted transporter PATH is a high-affinity, low-capacity amino acid transporter essential for dTORC1 activity (Goberdhan, Meredith, Boyd, & Wilson, 2005). The PATH protein shows sequence homology to the SLC36 family of proton-assisted transporters, which have also been linked to mTORC1 activity in cancer cell lines (Heublein et al., 2010). Additionally, the transporter slimfast has been shown to regulate drosophila body size: downregulation of this gene within the fat body causes a systemic growth defect reminiscent of nutrient deprivation (Colombani et al., 2003). In mammalian cells, SLC38A2 has been hypothesized to act as a transceptor upstream of mTORC1 (Hundal & Taylor, 2009). However, it is not entirely clear if these transporters signal directly to the mTORC1 pathway or indirectly govern mTORC1 activity by altering compartment-specific amino acid availability.

Through our own mass spectrometry experiments using the Rag GTPases and Ragulator proteins as immunoprecipitation handles, we consistently detected peptides from SLC38A9, an uncharacterized amino acid transporter with structural homology to the SLC36 and SLC38 families mentioned above. Our work demonstrates that SLC38A9 is a lysosome-bound

transporter with a critically important signaling role, leading us to propose that SLC38A9 is a candidate sensor for arginine in the mTORC1 pathway (described in detail in chapter 3) (S. T. Wang, Z.; Wolfson, R.; Shen, K.; Wyant, G. A.; Plovanich, M. E.; Yuan, E. D.; Jones, T.J.; Chantranupong, L.; Comb, W.; Wang, T.; Bar-Peled, L.; Zoncu, R.; Straub, C.; Kim, C.; Park, J.; Sabatini, B. L.; Sabatini, D. M., 2014).

2. Lysosomal origins of amino acid sensing

The vast majority of proteins in the mTORC1 amino acid sensing pathway are localized to the lysosomal membrane, either constitutively or in an amino acid-dependent fashion. This naturally leads to the following question: why did the sensing apparatus evolve in this way? From a comparative standpoint, lysosomes are evolutionary descendants and, in certain respects, functional equivalents of the yeast vacuole. The yeast vacuole is an acidified compartment that performs autophagy and serves as a storage depot for amino acids, among other small molecules. One attractive hypothesis is that the yeast homologues of the Rag GTPases, called Gtr1 and Gtr2, evolved on the surface of the vacuole in order to monitor vacuolar amino acid contents. This might be a particularly critical function for unicellular organisms like yeast because during times of nutrient limitation, there are no alternative nutrient reserves to draw from. Even though multicellular organisms can rely on the mobilization of nutrients from one tissue to another as a stopgap measure to maintain metabolic homeostasis, the function and location of this sensing apparatus may have been conserved during evolution. Moreover, as the site of autophagy, the lysosome is a key source of freshly liberated amino acids (the other sources being proteasomal degradation in the cytosol and import from the extracellular space) and has even been proposed to serve as a quality control center, enabling only undamaged metabolites to reenter the cellular circulation. Lastly, it is now clear that mTORC1 also plays a major role in controlling lysosome biogenesis, although it is unclear when in evolutionary time this regulatory function became coupled to the TORC1 machinery situated at the lysosome.

3. Amino acid sensing in the cytosol

It is not entirely clear to what extent amino acid contents in the cytosol and lysosome are in equilibrium. This has been a difficult question to tackle in the absence of accurate methods to detect amino acid concentrations in both compartments. If transport of amino acids across the

lysosomal membrane is carefully regulated, as is likely to be the case, then the lysosomal stores of amino acids may not be fully representative of the total availability within a cell. Thus it may be advantageous for the cell to devise a mechanism to monitor cytosolic contents as well, particularly since protein synthesis is a function of cytosolic amino acid availability. Indeed, recent studies from our lab have identified two cytosolic amino acid sensors that interact with the GATOR2 complex in a ligand-dependent fashion with signaling consequences for mTORC1. First, the Sestrin proteins, which were originally identified as stress-induced negative regulators of mTORC1 through the AMPK/TSC1-TSC2 axis (Budanov & Karin, 2008; Feng, Zhang, Levine, & Jin, 2005), have now been shown to be cytosolic sensors for leucine (Chantranupong et al., 2014; Saxton et al., 2016; Wolfson et al., 2016). Binding of leucine induces a conformational change in Sestrin, which results in its dissociation from and alleviates its inhibition of GATOR2. Knockout of the Sestrin proteins presumably activates GATOR2 and thus causes constitutive mTORC1 signaling. Similarly, the CASTOR proteins are cytosolic arginine sensors: the presence of arginine disrupts the CASTOR/GATOR2 interaction, presumably again activating GATOR2 (Chantranupong et al., 2016). The Sestrin proteins and the CASTOR proteins bind different parts of the pentameric GATOR2 complex, although the molecular details of these binding interactions and how they influence the as yet undiscovered function of GATOR2 are unknown. From an evolutionary standpoint, it is curious to note that although there exist orthologs of GATOR1 and GATOR2 in yeast, Sestrins orthologs are not detectable until *C. elegans* (weakly) and *Drosophila* and CASTOR orthologs do not appear until *Danio rerio* (despite having an ACT domain fold that originated in bacteria). Different species may have evolved different sensors in evolutionary time to respond to changing nutrient conditions and priorities.

4. Summary and questions

In summary, recent studies have shed much light on the sensing of several amino acids important for mTORC1 signaling. However, numerous diverse questions remain, ranging from atomic-level structural details to biochemical functions of protein complexes to the logic of sensing itself. Why do certain amino acids matter more than others? Why do there exist so many sensors? What is the physiological significance of these various sensing molecules for different tissues in the body, and what happens when they are disrupted?

VI. mTORC2: Inputs and Functions

In contrast to mTORC1, mTORC2 is a rapamycin-insensitive complex comprised of mTOR, rictor (the defining component), mSIN1, mLST8, and protor (Fig. 1B) (Laplante & Sabatini, 2012). mSIN1 is thought to dictate the responsiveness of mTORC2 to growth factors like insulin (Frias et al., 2006). mSIN1 has three different isoforms, two of which contain a putative C-terminal PH domain (Schroder et al., 2007). The third isoform, which lacks a PH domain, is unique in maintaining activity in the absence of PI3K signaling, hinting that it may have a distinct function (Frias et al., 2006). Besides the growth factor input, little remains known about the regulation of mTORC2 activity.

Depletion of mTORC2 components – or their yeast counterparts – results in defective actin cytoskeleton assembly in a PKC-dependent mechanism. More recently, Sarbassov *et al.* discovered that mTORC2 is also responsible for phosphorylating Akt at S473 (Sarbassov, Guertin, Ali, & Sabatini, 2005). Akt is the main downstream effector of the PI3K/PTEN pathway and mediates many of the effects of insulin signaling. This pathway is frequently hyperactivated in a diverse array of cancers, predominantly through loss-of-function mutations in PTEN or activating mutations in PI3K. Like S6K, Akt belongs to the AGC family of kinases, whose kinase activity is governed by a two-part mechanism: phosphorylation of the hydrophobic motif (S473 in Akt, T389 in S6K) is thought to poise the substrate for further phosphorylation by PDK1 at an internal "activation loop" site (T308 in Akt, T229 in S6K) (Biondi, Kieloch, Currie, Deak, & Alessi, 2001). However, it is not entirely clear if Akt follows this general pattern, as there are reports indicating that phosphorylation at S473 and T308 can occur independently (Alessi et al., 1996; Biondi et al., 2001). Indeed, genetic loss of mTORC2 ablates phosphorylation at S473 but does not alter levels of T308 phosphorylation (Guertin et al., 2006). Since this genetic perturbation affects only a subset of Akt substrates (Guertin et al., 2006), it is likely either that singly phosphorylated Akt maintains some degree of basal activity or that there exists an independent kinase capable of targeting this particular subset of Akt substrates in a redundant fashion.

The connection between PI3K and mTORC2 offers fertile ground for further exploration, given its potential clinical significance in light of the PI3K/PTEN-accented mutational landscape of cancers. Does hyperactivated PI3K signaling always route through and depend on mTORC2

signaling? Could mTORC2 inhibition serve as effective intervention for PI3K mutant cancers? Do activating mutations in mTOR itself affect mTORC2 signaling in addition to mTORC1 signaling?

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Chapter 2

Which amino acids matter?

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Experiments in all figures were conceived of and performed by SW.

Introduction

When nutrient and growth factor conditions are favorable, the mTORC1 pathway coordinates anabolic and catabolic processes to stimulate growth at cellular, organ, and organism levels. The ability to sense amino acids is a critical and long-appreciated feature of this pathway, yet despite considerable efforts by many groups, very little was known until recently about this fundamental process at the molecular level. Not only were the identity and mechanism of the sensor(s) unknown, there was also no consensus regarding which of the twenty proteogenic amino acid(s) – or perhaps indeed all of them – actually mattered for signaling to mTORC1.

Amino acids comprise a set of twenty small molecules that have in common, as their name implies, both amino and carboxy termini. The distinguishing feature of each amino acid is its side chain, and amongst the proteogenic amino acids, side chains vary significantly in size, charge, hydrogen-bonding ability, and hydrophobicity. This physicochemical diversity enables the generation of proteins with incredibly diverse functionality, but what does it mean for sensing of amino acids as a collective entity by the mTORC1 pathway? Does mTORC1 detect a generic feature like the amino or carboxy terminus, and thus sense amino acids in bulk? Or does mTORC1 detect specific side chains of specific amino acids?

It could very well be that different organisms, depending on their complexity and environment, evolved different strategies for detecting nutrient availability. For example, unicellular organisms like yeast have the ability to synthesize all amino acids *de novo* and thus need not salvage intact amino acids from their surroundings. Moreover, being unicellular, they also directly interface with the external environment, which can undergo dramatic fluctuations with respect to nutrient types and availability. Given their intrinsic biosynthetic capacity as well as this extrinsic unpredictability, it is conceivable that yeast might not care so much about specific, intact amino acids as about a more fundamental element for growth: total nitrogen (De Virgilio & Loewith, 2006). There is some evidence in the literature consistent with this hypothesis. First, budding yeast can sustain growth on a wide variety of nitrogenous compounds, although there is a hierarchy of preferred substrates. Second, nitrogen starvation elicits a response that closely resembles rapamycin treatment or TORC1 deficiency, suggesting that TORC1 is regulated by the abundance and/or the quality of the nitrogen source. The mechanism of nitrogen sensing has not been fully fleshed out, although glutamine, which is generally considered a key indicator of the cell's overall nitrogen status, has been proposed to be a critical

intermediate (De Virgilio & Loewith, 2006). What remains unclear, however, is whether or not glutamine is directly sensed by the TORC1 pathway, since it is a fundamental building block for many biosynthetic processes and can be rapidly converted into TCA cycle intermediates, precursors for other amino acids, and nucleotides. Thus, although yeast TORC1 likely cares about intracellular nitrogen content, there is little evidence to indicate that it senses specific amino acids.

Metazoans cannot, unlike yeast, synthesize all amino acids *de novo* and thus may benefit from being able to sense the availability of these molecules. In 1998, Hara *et al.* demonstrated that deprivation of leucine or arginine, but not of other amino acids, potently inhibited mTORC1 signaling in CHO cells, suggesting privileged roles for these amino acids in mammalian systems (Hara et al., 1998). However, in the setting of complete amino acid withdrawal, restimulation with just leucine and arginine was insufficient to fully activate the mTORC1 pathway. Complete pathway activation may require additional specific amino acids or perhaps surpassing a bulk threshold of all amino acids. Without a subset of amino acids that is necessary and sufficient for mTORC1 signaling, it is difficult to conclude that there are specific sensors for specific amino acids as well as to draw larger conclusions about the logic of sensing.

In this chapter, we demonstrate that leucine, arginine, and lysine constitute the set of amino acids that are necessary and sufficient for signaling to mTORC1 in HEK-293T cells. We rule out several hypotheses that seek to explain why these three amino acids are needed in combination and explore how this knowledge might facilitate our search for amino acid sensors.

Results

All of the signaling experiments shown in this chapter, unless otherwise noted, were conducted in HEK-293T cells, the workhorse cell line that our lab has used to identify many of the protein players in the amino acid sensing pathway. The typical experiment consisted of two phases: cells were transiently deprived of all amino acids in the cell media to inactivate mTORC1 signaling and then briefly stimulated with a defined combination of amino acids at RPMI concentrations. Cell lysates were probed for S6K phosphorylation, a standard indicator of mTORC1 activity.

Previous work indicated that mTORC1 activity depends on the presence of a variety of amino acids in the cell media, including leucine and arginine, although there are some cell type-

specific requirements (Hara et al., 1998). In HEK-293T cells, a mixture of essential amino acids (F, H, I, K, L, M, R, T, V, W) activated mTORC1 as well as all 20 proteogenic amino acids, whereas a mixture of nonessential amino acids (A, C, D, E, G, N, P, Q, S, Y) had no effect on pathway activity (Fig. 1A). Of the essential amino acids, only in the absence of leucine, arginine, or lysine did the remaining nine fail to fully activate mTORC1, indicating that each of these three amino acids is necessary for signaling (Fig. 1B). Remarkably, the combination of all three (LRK), but not of any two, was sufficient to stimulate mTORC1 activity to a similar degree as a mixture of all amino acids (Fig. 1C). When amino acids were added to the media at concentrations that more closely reflect serum levels, LRK still emerged as the three most important amino acid inputs for mTORC1 signaling (data not shown). In a slightly modified experimental paradigm, starvation of any one of these three amino acids is sufficient to inhibit mTORC1, while re-addition of the missing amino acid completely restores signaling (data not shown).

Each amino acid within LRK appears to have a unique role in the activation of mTORC1. Combinations of isoleucine, valine, and methionine at RPMI concentrations could not substitute for leucine, nor could arginine and lysine substitute for each other (Fig. 1D). Moreover, LRK are not co-dependent on one another for cell entry: the addition of dropout mixes that lacked one amino acid at a time did not affect the degree of intracellular accumulation of the amino acids that remained (Fig. 1E). Since the molarity of a mixture of all amino acids greatly exceeds that of LRK, it is unlikely that mTORC1 simply relies on a model of bulk amino acid sensing. Taken together, these results suggest that there may be multiple sensors, at least in HEK-293T cells, each recognizing a distinct feature of leucine, arginine, or lysine rather than characteristics common to all amino acids.

Figure 1. Leucine, arginine, and lysine are necessary and sufficient to signal to mTORC1 in HEK-293T cells. **(A)** Essential amino acids are sufficient to fully activate mTORC1 signaling, whereas non-essential amino acids have no stimulatory effect. **(B)** Leucine, arginine, and lysine are necessary for signaling to mTORC1. **(C)** The combination of leucine, arginine, and lysine, but not of any one, can activate mTORC1 to the same degree as all amino acids. **(D)** Isoleucine and valine cannot substitute for leucine (lanes 4-5) and arginine and lysine cannot substitute for each other (lanes 8,11). **(E)** Cells were starved for all amino acids for 50 min. and restimulated with the indicated set for 10 min. prior to harvest for metabolite profiling. The absence of any one of these three amino acids does not affect the cellular entry of the other two.

Fig. 1
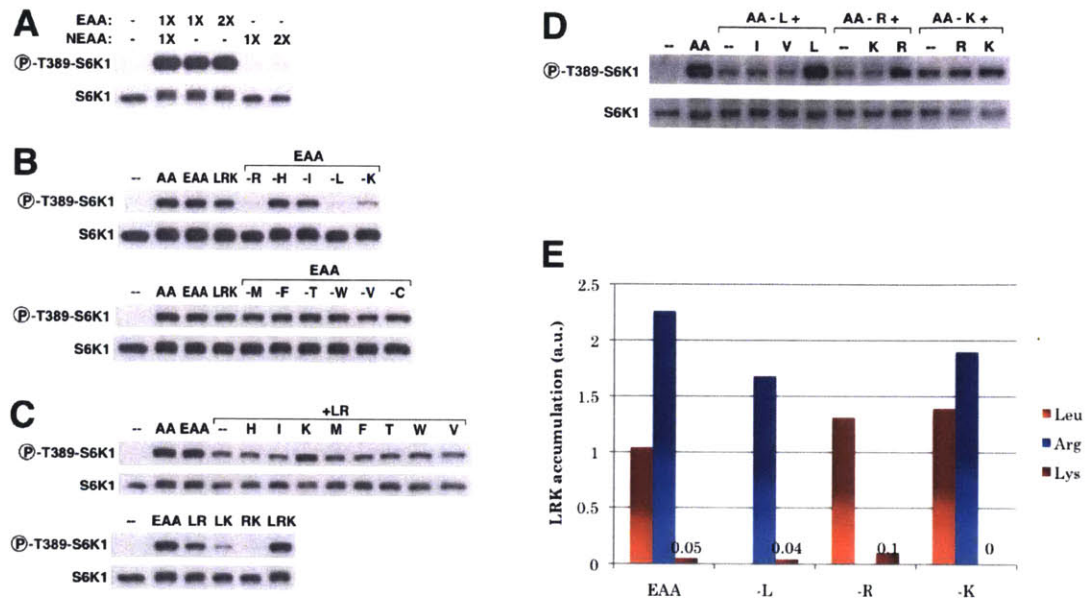
Next, a study of structure-activity relationship was initiated to determine which structural features of leucine, arginine, and lysine were obligatory, modifiable, or dispensable for signaling to mTORC1. Ultimately, we hoped to use modified amino acids as chemical probes to uncover the identities of their cognate sensors (Fig. 2A). Many amino acid analogues were tested in the minimalist LRK context for their abilities to substitute for (agonist) and to prevent activation by (antagonist) the native counterpart, as success in either test may indicate engagement with the sensor. For example, canavanine, an analogue of arginine, was added alongside LK or LRK to test for agonist and antagonist activity, respectively. The signaling effects of all the tested analogues are summarized in Fig. 2B-D. One important caveat of these results is that not all amino acid analogues were confirmed to gain entry into the cell, and it is indeed possible that some of them, particularly those with bulky appendages, may have failed to cross the plasma membrane. Thus, for some analogues, the absence of a signaling outcome may reflect a deficiency in transport rather than an inability to engage with the sensor.
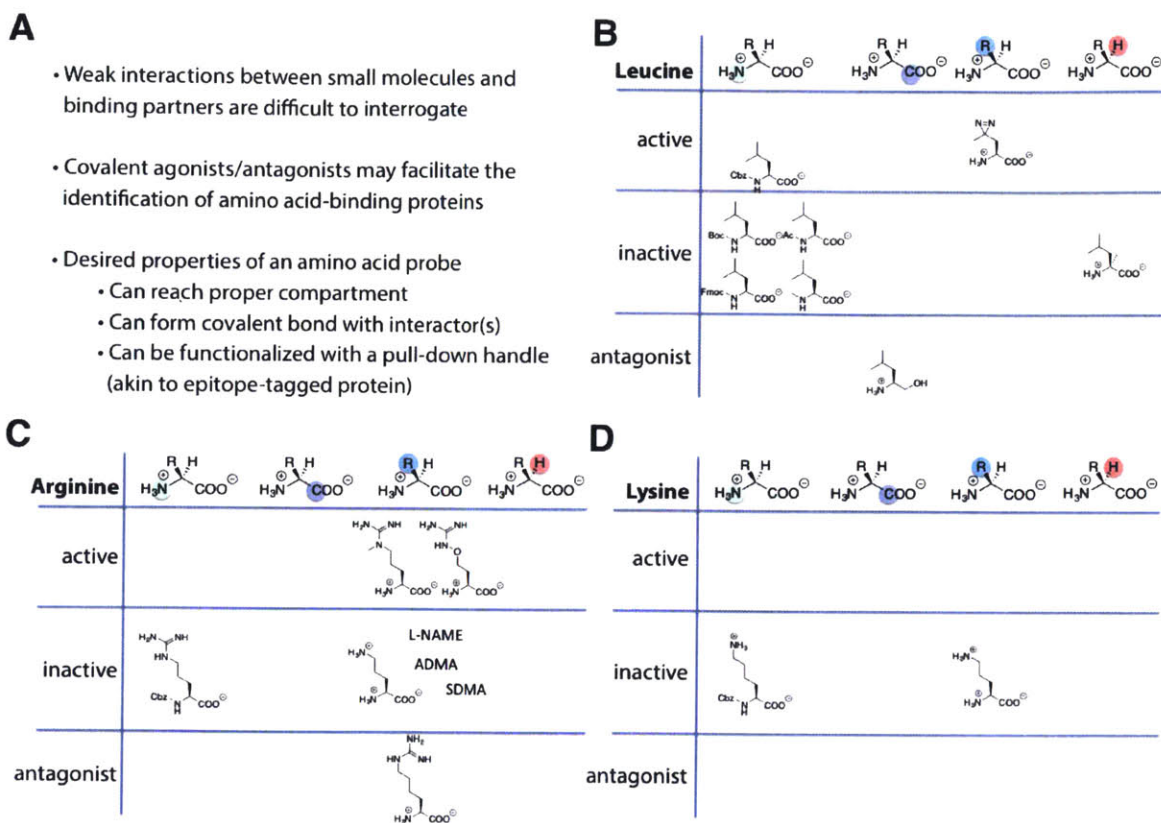
Fig. 2

A

- Weak interactions between small molecules and binding partners are difficult to interrogate

- Covalent agonists/antagonists may facilitate the identification of amino acid-binding proteins

- Desired properties of an amino acid probe
  - Can reach proper compartment
  - Can form covalent bond with interactor(s)
  - Can be functionalized with a pull-down handle (akin to epitope-tagged protein)

B

C

D

Figure 2. Exploration of the structure-activity relationship for leucine, arginine, and lysine in the mTORC1 signaling assay. **(A)** Rationale for studying which amino acid sites may be dispensable or otherwise modifiable. **(B-D)** Tables of the leucine, arginine, and lysine analogs tested for agonist and antagonist properties in the mTORC1 signaling assay.

One intriguing analogue is photoleucine, a photo-crosslinkable leucine analogue that can be recognized by leucyl-tRNA synthetase and incorporated into nascent proteins (Fig. 3A) (Suchanek, Radzikowska, & Thiele, 2005). mTORC1 signaling experiments indicate that photoleucine has some agonist activity at the leucine sensor as well, particularly when added at high concentrations in combination with RK (Fig. 3B). Since the diazirine moiety built into the modified side chain is capable of covalently crosslinking with C-H, N-H, or O-H bonds (Fig. 3C), photoleucine is a promising candidate for capturing its cognate sensor through a stable covalent linkage. This functional characteristic greatly enhances its usefulness as a chemical probe for the leucine sensor, since the putative sensor would not be expected to have a high
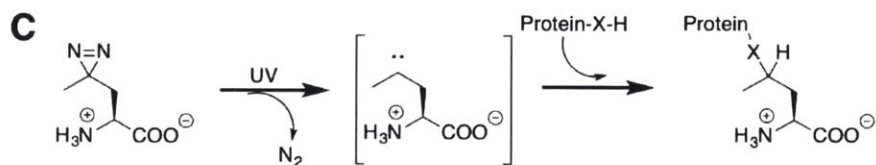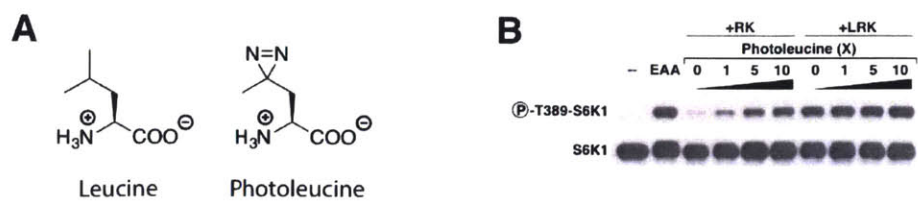
affinity for a ligand that it needs to bind and unbind within physiological ranges (likely micromolar).

To ensure that the photocrosslinking protocol is functional, we mixed photoleucine with purified leucyl-tRNA synthetase, performed crosslinking, and, in collaboration with Prof. Alan Saghatelian (then at Harvard University) and the Harvard FAS proteomics facility, attempted to detect leucyl-tRNA synthetase peptide fragments with a defined mass gain corresponding to one crosslinked photoleucine molecule. We noted putative crosslinking at several sites along the extended leucyl-tRNA synthetase polypeptide (Appendix A), although without a crystal structure we could not easily confirm if these points of contact formed a contiguous three-dimensional binding surface for leucine.
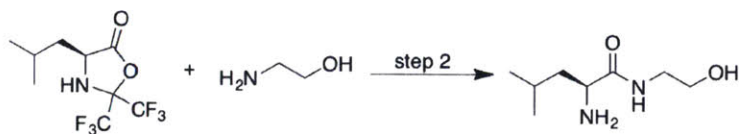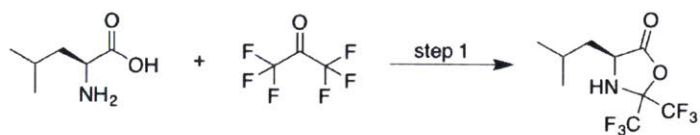
The last step is to enrich for the photocrosslinked photoleucine through sites on the photoleucine that may remain accessible – the amino and carboxy terminus. We had devised a chemical strategy to achieve this enrichment, based on the reported ability of hexafluoroacetone to cyclize proximal amino and carboxy groups (see schematic in Fig. 3D) (Spengler, Bottcher, Albericio, & Burger, 2006). However, this step was never fully optimized (data not shown).

Figure 3. Photoleucine can stimulate mTORC1 signaling in lieu of leucine and may serve as a useful handle to identify the leucine sensor. **(A)** Structures of leucine and photoleucine, which only differ at a terminal methyl group on the leucine side chain. **(B)** Photoleucine can stimulate mTORC1 activity when added at moderately high levels but does not block leucine's activating potential. **(C)** Photoleucine undergoes crosslinking with X-H bonds via a highly reactive carbine intermediate when photolyzed with 345 nm light. **(D)** Hexafluoroacetone-based condensation of alpha-amino carboxylic acids may serve as a method to enrich for photoleucine-crossed proteins in the cell, since there should not be simultaneous exposure of both amino and carboxy termini of all other protein-linked amino acids.
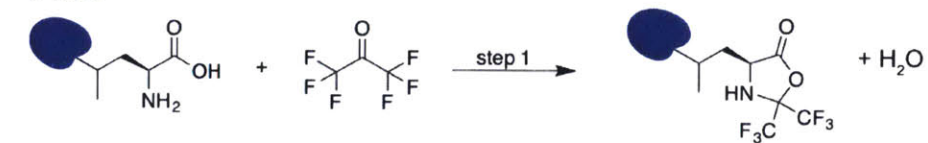
45

Fig. 3

A

Leucine   Photoleucine

B

C

D Literature

step 1

step 2

Plan

step 1   + H₂O

step 2

Proteomics

46

Discussion

In summary, we determined, through a series of signaling experiments in HEK-293T cells, that leucine, arginine, and lysine are necessary and sufficient for signaling to mTORC1. Each amino acid appears to serve a distinct role during pathway activation, as any one amino acid cannot substitute for the others. This also led us to hypothesize that there are distinct intracellular sensors for these amino acids as well as to attempt using a chemical biology strategy to uncover the identity of the leucine sensor. (N.b. After I had stopped working on optimizing this strategy, my colleagues in the Sabatini Lab identified the Sestrin proteins to be cytosolic sensors of leucine.)

The fact that HEK-293T cells strictly requires only three amino acids for mTORC1 activation naturally leads us to question whether or not other cell lines and tissue types share the same requirements. Preliminary characterization of several cancer cell lines and primary hepatocytes indicates that while most types of cells require leucine and arginine to maintain mTORC1 activity, the requirement for lysine is far less universal (Appendix B). It is plausible that differential expression of amino acid transporters and/or sensors across cell types may account for the observed heterogeneity in the amino acid requirement. What remains a mystery, however, is why over the course of evolution these amino acids – particularly leucine – have been singled out as indicators of amino acid availability.

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Acknowledgements

Materials and Methods

Materials

Reagents were obtained from the following sources: HRP-labeled anti-rabbit secondary antibodies from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, S6K1 from Cell Signaling Technology; Xtremegene 9 and Complete Protease Cocktail from Roche; amino acid-free RPMI from US Biological; native amino acids and most amino acid analogs and hexafluoroacetone from Sigma; photoleucine from ThermoFisher.

Cell lines and tissue culture

HEK-293T cells were cultured in DMEM supplemented with 10% inactivated fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 µg/mL) and maintained at 37°C and 5% CO_2. In HEK-293E, but not HEK-293T, cells the mTORC1 pathway is strongly regulated by serum and insulin (Sancak et al., 2007).

Amino acid or individual amino acid starvation and stimulation of cells

Almost confluent cell cultures in 10 cm plates were rinsed twice with amino acid-free RPMI, incubated in amino acid-free RPMI for 50 min, and stimulated for 10 min with a water-solubilized amino acid mixture added directly to the amino acid-free RPMI. For leucine or arginine starvation, cells in culture were rinsed with and incubated in leucine- or arginine-free RPMI for 50 min, and stimulated for 10 min with leucine or arginine added directly to the starvation media. After stimulation, the final concentration of amino acids in the media was the same as in RPMI. Cells were processed for biochemical assays as described below. The 10X amino acid mixture and the 300X individual stocks were prepared from individual amino acid powders.

Cell lysis and Western blotting

HEK-293T cells were rinsed once with ice-cold PBS and lysed in ice-cold lysis buffer (40 mM HEPES pH 7.4, 1% Triton X-100, 10 mM β-glycerol phosphate, 10 mM pyrophosphate, 2.5 mM $MgCl_2$ and 1 tablet of EDTA-free protease inhibitor (Roche) per 25 ml buffer). The soluble fractions from cell lysates were isolated by centrifugation at 13,000 rpm for 10 min in a microcentrifuge and normalized by total protein content prior to loading onto a Tris-glycine gel (mostly 8% for S6K and phosphor-S6K detection).

Appendix B
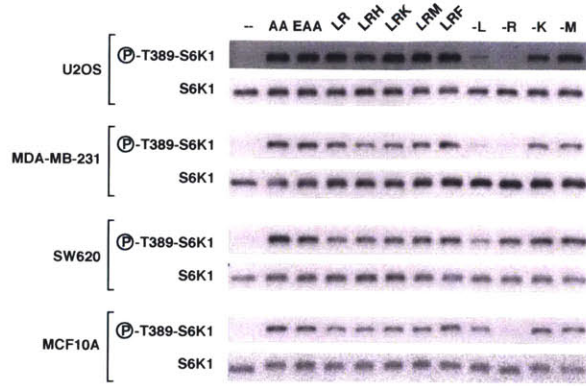
Chapter 3

The amino acid transporter SLC38A9 is a key component of a lysosomal membrane complex that signals arginine sufficiency to mTORC1

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Experiments in Figure 1C and 1D were performed by SW
Experiments in Figure 1E and 1F were performed by RW, based on findings by SW
Experiments in Figure 2A were performed by ZT
Experiments in Figure 2B were performed by LC, based on findings by SW
Experiments in Figure 3 were performed by SW
Experiments in Figure 4A and 4C were performed by SW
Experiments in Figure 4B were performed by ZT
Experiments in Figure 5A-E were performed by ZT
Experiments in Figure 5F were performed by SW or GW, based on findings by SW

Abstract

The mTOR complex 1 (mTORC1) protein kinase is a master growth regulator that responds to multiple environmental cues. Amino acids stimulate, in a Rag-, Ragulator-, and v-ATPase-dependent fashion, the translocation of mTORC1 to the lysosomal surface, where it interacts with its activator Rheb. Here, we identify SLC38A9, an uncharacterized protein with sequence similarity to amino acid transporters, as a lysosomal transmembrane protein that interacts with the Rag GTPases and Ragulator in an amino acid-sensitive fashion. SLC38A9 transports arginine with a high K_m and loss of SLC38A9 represses mTORC1 activation by amino acids, particularly arginine. Overexpression of SLC38A9 or just its Ragulator-binding domain makes mTORC1 signaling insensitive to amino acid starvation but not to Rag activity. Thus, SLC38A9 functions upstream of the Rag GTPases and is an excellent candidate for being an arginine sensor for the mTORC1 pathway.

Introduction

The mechanistic target of rapamycin complex 1 (mTORC1) protein kinase is a central controller of growth that responds to the nutritional status of the organism and is deregulated in several diseases, including cancer (Howell & Manning, 2011; Jewell, Russell, & Guan, 2013; Zoncu, Efeyan, & Sabatini, 2011). Upon activation, mTORC1 promotes anabolic processes, including protein and lipid synthesis, and inhibits catabolic ones, such as autophagy (Sengupta, Peterson, & Sabatini, 2010). Environmental cues such as nutrients and growth factors regulate mTORC1, but how it senses and integrates these diverse inputs is unclear.

The Rag and Rheb GTPases have essential but distinct roles in mTORC1 pathway activation, with the Rags controlling the subcellular localization of mTORC1 and Rheb stimulating its kinase activity (Laplante & Sabatini, 2012). Nutrients, particularly amino acids, activate the Rag GTPases, which then recruit mTORC1 to the lysosomal surface where they are concentrated (Kim, Goraksha-Hicks, Li, Neufeld, & Guan, 2008; Sancak et al., 2008). Rheb also localizes to the lysosomal surface (Buerger, DeVries, & Stambolic, 2006; Menon et al., 2014; Saito, Araki, Kontani, Nishina, & Katada, 2005; Sancak et al., 2008) and, upon growth factor withdrawal, the tuberous sclerosis complex (TSC) tumor suppressor translocates there and inhibits mTORC1 by promoting GTP hydrolysis by Rheb (Menon et al., 2014). Thus, the Rag and Rheb inputs converge at the lysosome, forming two halves of a coincidence detector that ensures that mTORC1 activation occurs only when both are active.

There are four Rag GTPases in mammals and they form stable, obligate heterodimers consisting of RagA or RagB with RagC or RagD. RagA and RagB are highly similar and functionally redundant, as are RagC and RagD (Jewell et al., 2013; Sancak et al., 2008). The function of each Rag within the heterodimer is poorly understood and their regulation is likely complex as many distinct factors play important roles. A lysosome-associated molecular machine containing the multi-subunit Ragulator and vacuolar ATPase (v-ATPase) complexes regulates the Rag GTPases and is necessary for mTORC1 activation by amino acids (Zoncu, Bar-Peled, et al., 2011). Ragulator anchors the Rag GTPases to the lysosome and also has nucleotide exchange activity for RagA/B (Bar-Peled, Schweitzer, Zoncu, & Sabatini, 2012; Sancak et al., 2010), but the molecular function of the v-ATPase in the pathway is unknown. Two GTPase activating protein (GAP) complexes, which are both tumor suppressors, promote GTP hydrolysis by the Rag GTPases, with GATOR1 acting on RagA/B (Bar-Peled et al., 2013)

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and Folliculin-FNIP2 on RagC/D (Tsun et al., 2013). Lastly, a distinct complex called GATOR2 negatively regulates GATOR1 through an unknown mechanism (Bar-Peled et al., 2013). Despite the identification of many proteins involved in signaling amino acid sufficiency to mTORC1, the actual amino acid sensors remain unknown.

Results
SLC38A9 Interacts with Ragulator and the Rag GTPases

We have proposed that amino acid sensing initiates at the lysosome and requires the presence of amino acids in the lysosomal lumen (Zoncu, Bar-Peled, et al., 2011). Thus, we sought to identify, as candidate sensors, proteins that interact with known components of the pathway and also have transmembrane domains. Mass spectrometric analyses of non-heated immunoprecipitates of several Ragulator components and, to a lesser extent, RagB, revealed the presence of isoform 1 of SLC38A9 (SLC38A9.1), a previously unstudied protein with sequence similarity to the SLC38 class of sodium-coupled amino acid transporters (Sundberg et al., 2008) (Fig. 1A). SLC38A9.1 is predicted to have eleven transmembrane domains, a cytosolic N-terminal region of 119 amino acids, and three N-linked glycosylation sites in the luminal loop between transmembrane domains 3 and 4 (Fig. 1B and fig. S1, A and B). When stably expressed in human embryonic kidney (HEK)-293T cells, SLC38A9.1 migrated on SDS-PAGE as a smear that collapsed to near its predicted molecular weight of 63.8 kDa after treatment with Peptide-*N*-Glycosidase F (PNGase F) (Fig. 1C). Isoforms 2 (SLC38A9.2) and 4 (SLC38A9.4) lack the first 63 or 124 amino acids of SLC38A9.1, respectively (Fig. 1B).

As expected from the mass spectrometry results, immunoprecipitates of stably expressed FLAG-tagged SLC38A9.1, but not of three other lysosomal membrane proteins –LAMP1 (Carlsson, Roth, Piller, & Fukuda, 1988), SLC36A1 (Sagne et al., 2001), and SLC38A7 (Chapel et al., 2013) – contained Ragulator (as detected by its p14 and p18 components), RagA, and RagC (Fig. 1D and fig. S2A). Indicative of the strength of the Ragulator-SLC38A9.1 interaction, the amounts of endogenous Ragulator that coimmunoprecipitated with SLC38A9.1 were similar to those associated with the RagB-RagC heterodimer (Fig. 1D). In contrast, SLC38A9.2, SLC38A9.4 or a mutant of SLC38A9.1 lacking its first 110 amino acids (SLC38A9.1 Δ110) did not associate with Ragulator (fig. S2, B and C). The N-terminal region of SLC38A9.1 is sufficient for it to interact with Ragulator-Rag because on its own the first 119 amino acids of SLC38A9.1 coimmunoprecipitated similar amounts of Ragulator and Rag GTPases as did the

full-length protein (Fig. 1D and fig. S2C). Using alanine scanning mutagenesis of residues in the N-terminal region conserved to the SLC38A9.1 homolog in *C. elegans* (F13H10.3), we identified I68, Y71, L74, P85, and P90 as required for the Ragulator-SLC38A9.1 interaction (Fig. 1E).

The v-ATPase and its activity are necessary for amino acid sensing by the mTORC1 pathway and, like SLC38A9.1, it coimmunoprecipitated with stably expressed FLAG-tagged Ragulator (Li et al., 2013; Xu et al., 2012; Zoncu, Bar-Peled, et al., 2011). Indicating the existence of a supercomplex, stably expressed SLC38A9.1, but not LAMP1, associated with endogenous components of the v-ATPase in addition to Ragulator and the Rag GTPases (Fig. 1F). Although SLC38A9.2 does not interact with Ragulator, it did co-immunoprecipitate the v-ATPase, albeit at lesser amounts than SLC38A9.1 (Fig. 1F). This suggests that the interaction between SLC38A9.1 and the v-ATPase is not mediated through Ragulator but directly or indirectly through the region of SLC38A9.1 that contains its transmembrane domains. Concordant with this interpretation, the N-terminal domain of SLC38A9.1, which interacts strongly with Ragulator, did not coimmunoprecipitate the v-ATPase (Fig. 1F).

Figure 1. Interaction of SLC38A9.1 with Ragulator and the Rag GTPases. (A) The spectral counts of SLC38A9-derived peptides detected by mass spectrometry in immunoprecipitates prepared from HEK-293T cells stably expressing the indicated FLAG-tagged proteins. (B) Schematic depicting SLC38A9 isoforms and truncation mutants. Transmembrane domains predicted by the TMHMM (transmembrane hidden Markov model) algorithm (http://www.cbs.dtu.dk/services/TMHMM/) are shown as blue boxes. (C) Effects of PNGase F treatment of SLC38A9.1 on its electrophoretic migration. (D) Interaction of full-length SLC38A9.1 or its N-terminal domain with endogenous Ragulator (p18 and p14) and RagA and RagC GTPases. HEK-293T cells were transfected with the indicated cDNAs in expression vectors and lysates were prepared and subjected to FLAG immunoprecipitation followed by immunoblotting for the indicated proteins. (E) Identification of key residues in the N-terminal domain of SLC38A9.1 required for it to interact with Ragulator and the Rag GTPases. Experiment was performed as in (D) using indicated SLC38A9.1 mutants. (F) Interaction of SLC38A9.1 with v-ATPase components V0d1 and V1B2. HEK-293T cells stably expressing the indicated FLAG-tagged proteins were lysed and processed as in (D).

Fig. 1
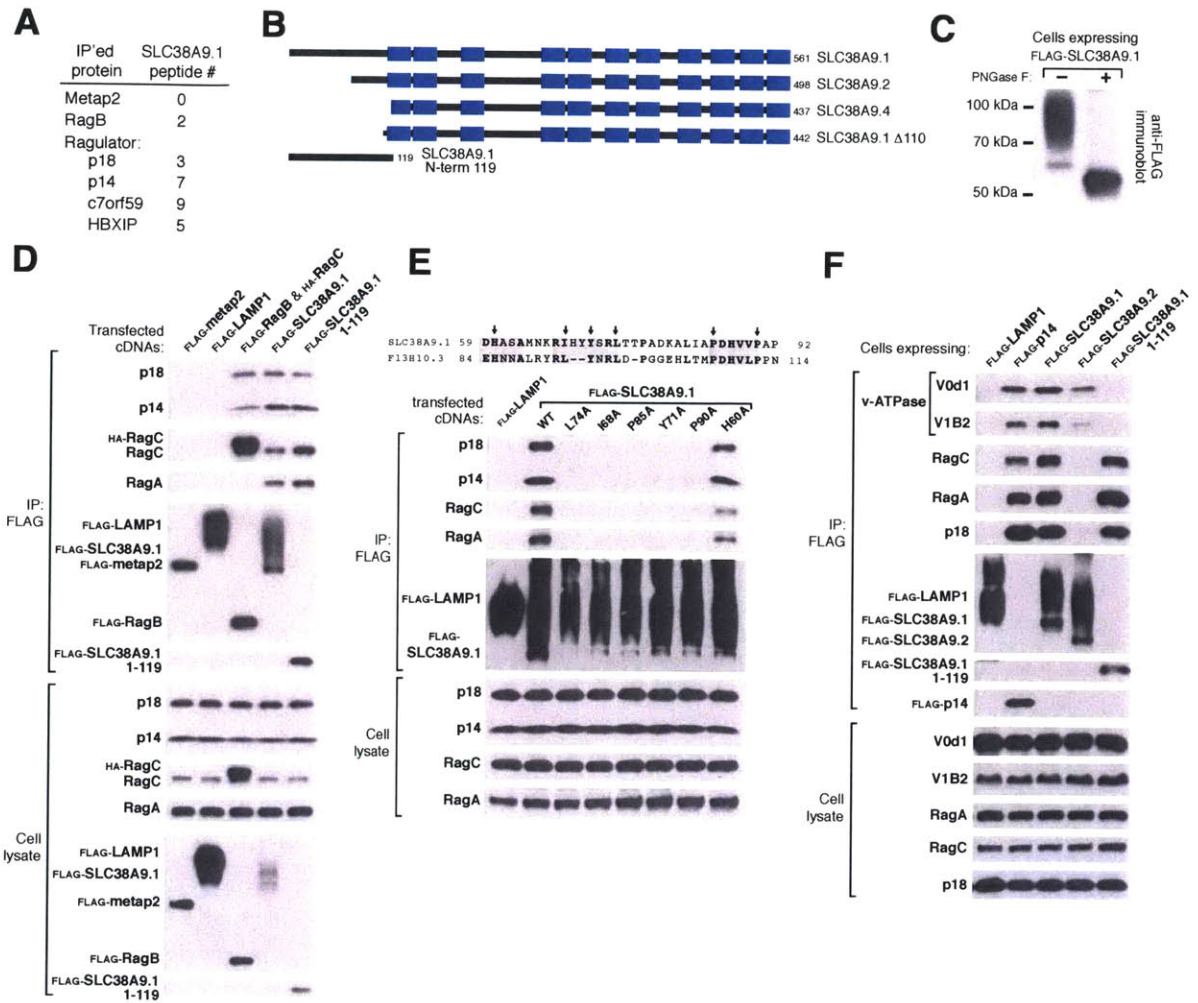
SLC38A9 is a Lysosomal Membrane Protein Required for mTORC1 Activation

Well-characterized members of the SLC38 family of amino acid transporters (SLC38A1-5) localize to the plasma membrane (Mackenzie & Erickson, 2004) but at least one member, SLC38A7, is a lysosomal membrane protein (Chapel et al., 2013). This is also the case for SLC38A9.1, SLC38A9.2, and SLC38A9.4 as in HEK-293T cells all three isoforms co-localized with LAMP2, an established lysosomal membrane protein (Fig. 2A and fig. S3, A and B). Amino acids did not affect the lysosomal localization of SLC38A9.1 (Fig. 2A). As would be expected if SLC38A9.1 binds to Ragulator at the lysosome, a Ragulator mutant that does not localize to the lysosomal surface because its p18 component lacks lipidation sites (Nada et al., 2009) did not interact with SLC38A9.1 (fig. S3C).

ShRNA- or siRNA-mediated depletion of SLC38A9 in HEK-293T cells suppressed activation of mTORC1 by amino acids, as detected by the phosphorylation of its established substrate ribosomal protein S6 Kinase 1 (S6K1) (Fig. 2B and fig. S3D). Thus, like the five known subunits of Ragulator (Bar-Peled et al., 2012; Sancak et al., 2010), SLC38A9.1 is a positive component of the mTORC1 pathway. We conclude that SLC38A9.1 is a lysosomal membrane protein that interacts with Ragulator and the Rag GTPases through its N-terminal 119 amino acids ('Ragulator-binding domain') and is required for mTORC1 activation.
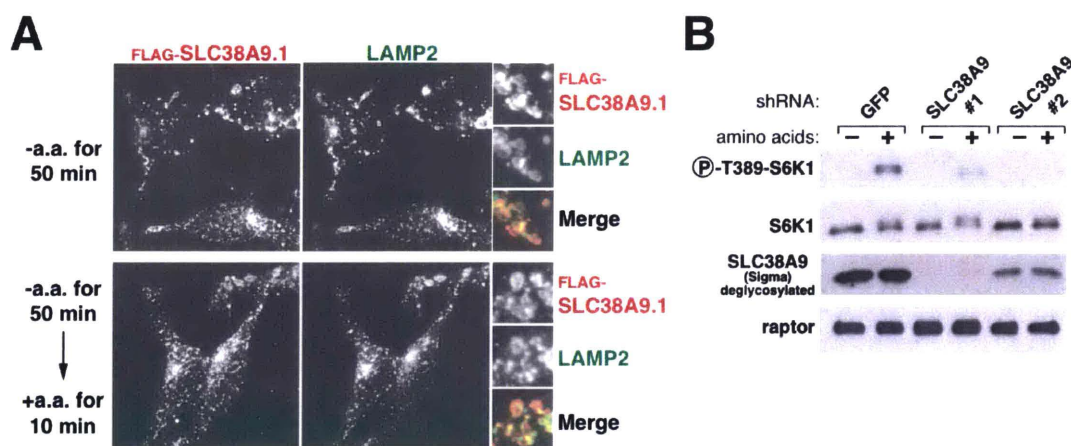
Fig. 2

Figure 2. Localization of SLC38A9.1 to the lysosomal membrane in an amino acid-independent fashion and requirement of SLC38A9 for mTORC1 pathway activation by amino acids. (**A**) SLC38A9.1 localization in cells deprived of or replete with amino acids. HEK-293T cells stably expressing FLAG-SLC38A9.1 were starved and stimulated with amino acids for the indicated times. Cells were processed and immunostained for LAMP2 and FLAG-SLC38A9.1. (**B**) Requirement of SLC38A9 for the activation of the mTORC1 pathway by amino acids. HEK-293T cells expressing indicated short hairpin RNAs (shRNAs) were deprived of amino acids for 50 min or deprived of and then re-stimulated with amino acids for 10 min. Cell lysates were analyzed for the levels of indicated proteins and the S6K1 phosphorylation state.

SLC38A9.1 Overexpression Makes mTORC1 Signaling Insensitive to Amino Acids

Given the similarity of SLC38A9.1 to amino acid transporters, we reasoned that it might act in conveying amino acid sufficiency to Ragulator and the Rag GTPases. In accord with this expectation, stable or transient overexpression in HEK-293T cells of SLC38A9.1, but not of several control proteins, rendered mTORC1 signaling resistant to total amino acid starvation or

to just that of leucine or arginine, two amino acids that regulate mTORC1 activity in many cell types (Ban et al., 2004; Hara et al., 1998; Yao et al., 2008) (Fig. 3A and fig. S4A). Overexpression of SLC38A9.1 did not affect the regulation of mTORC1 by growth factor signaling (fig. S4, D and E). Commensurate with its effects on mTORC1, SLC38A9.1 overexpression suppressed the induction of autophagy caused by amino acid starvation (fig. S4C), a phenotype shared with activated alleles of RagA and RagB (Efeyan et al., 2013; Efeyan, Zoncu, & Sabatini, 2012; Kim et al., 2008; Sancak et al., 2008). Overexpression of variants of SLC38A9 that do not interact with Ragulator and the Rag GTPases, including SLC38A9.2, SLC38A9.4, and the SLC38A9.1 Δ110 and SLC38A9.1 I68A mutants, failed to maintain mTORC1 signaling after amino acid withdrawal (Fig. 3, B and C, and fig. S4A). Thus, even in cells deprived of amino acids, some of the overexpressed SLC38A9.1 protein appears to be in an active conformation that confers amino acid insensitivity on mTORC1 signaling in a manner dependent on its capacity to bind Ragulator and Rags. SLC38A9.1 overexpression also activated mTORC1 in the absence of amino acids in HEK-293E, HeLa, and LN229 cells, as well as in mouse embryonic fibroblasts (MEFs), with the degree of activation proportionate to the amount of SLC38A9.1 expressed (fig. S4B). Interestingly, overexpression of just the Ragulator-binding domain of SLC38A9.1 mimicked the effects of the full-length protein on mTORC1 signaling (Fig. 3D), indicating that it can adopt an active state when separated from the transmembrane portion of SLC38A9.1.

The gain of function phenotype caused by SLC38A9.1 overexpression offered an opportunity to test its relation to the Rag GTPases, mTORC1, and the v-ATPase. The Rag GTPases and mTORC1 both clearly function downstream of SLC38A9.1 as expression of the dominant negative Rag heterodimer (RagB^{T54N}-RagC^{Q120L}) or treatment with the mTOR inhibitor Torin1 (Thoreen et al., 2009) completely inhibited mTORC1 activity, irrespective of whether SLC38A9.1 was overexpressed or not (Fig. 3, E and F). In contrast, the v-ATPase has a more complex relationship with SLC38A9.1. Its inhibition with concanamycin A eliminated mTORC1 signaling in the control cells but only partially blocked it in cells overexpressing SLC38A9.1 (Fig. 3F). These results suggest a model in which SLC38A9.1 and the v-ATPase represent parallel pathways that converge upon the Ragulator-Rag GTPase complex.
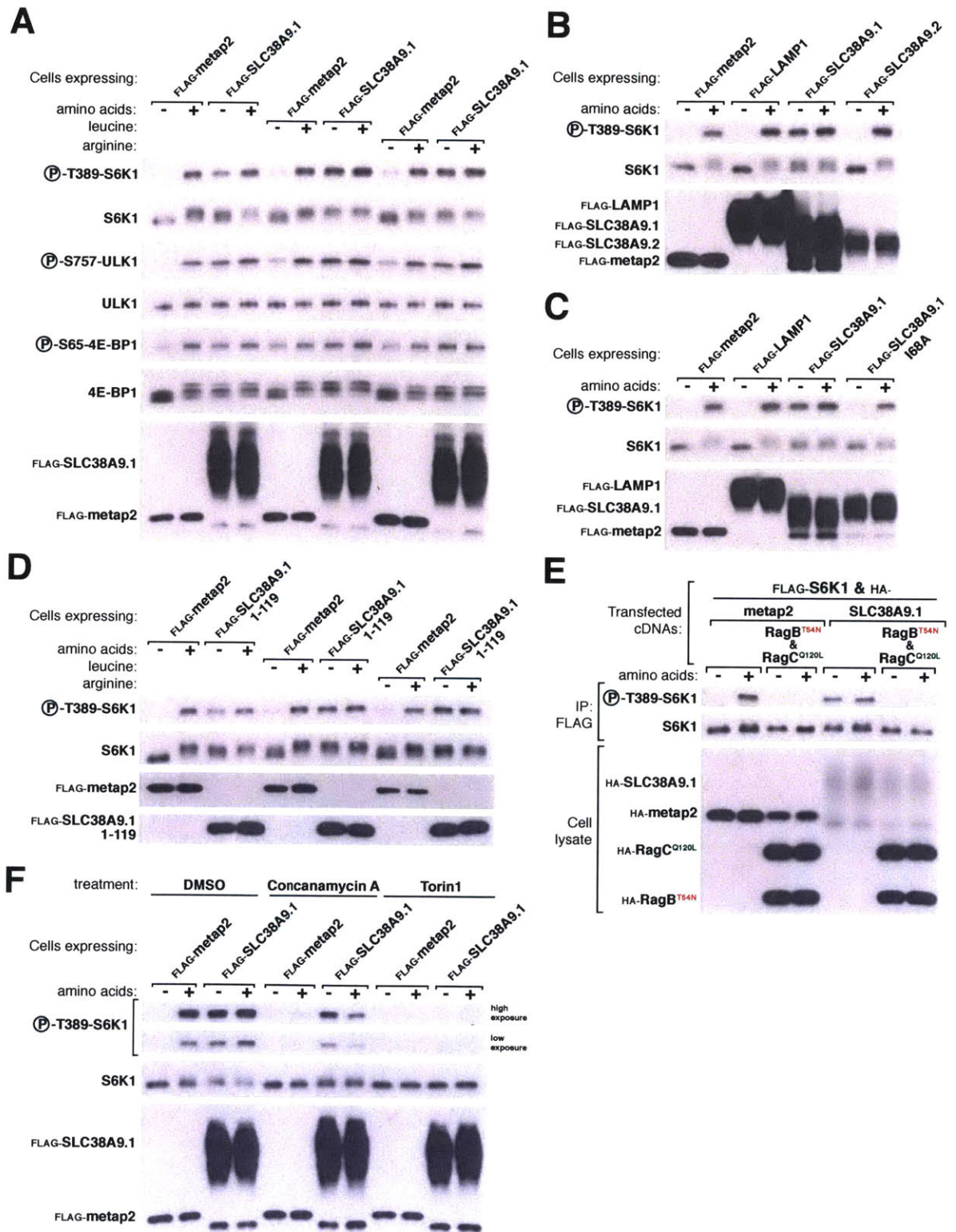
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Fig. 3

Figure 3. Stable overexpression of full-length SLC38A9.1 or its N-terminal Ragulator-binding domain makes the mTORC1 pathway insensitive to amino acid deprivation. (**A**) Stable overexpression of FLAG-SLC38A9.1 largely restores mTORC1 signaling during total amino acid starvation and completely restores it upon deprivation of leucine or arginine. HEK-293T cells transduced with lentiviruses encoding the specified proteins were deprived for 50 min of all amino acids, leucine, or arginine and, where indicated, re-stimulated for 10 min with the missing amino acid(s). Cell lysates were analyzed for the levels of the specified proteins and the phosphorylation states of S6K1, ULK1, and 4E-BP1. (**B and C**) Overexpression of neither SLC38A9.2 nor a point mutant of SLC38A9.1 that fails to bind Ragulator rescues mTORC1 signaling during amino acid starvation. Experiment was performed as in (A) except that cells were stably expressing SLC38A9.2 (B) or SLC38A9.1 I68A (C). (**D**) Stable overexpression of the Ragulator-binding domain of SLC38A9.1 largely restores mTORC1 signaling during total amino acid starvation and completely rescues it upon deprivation of leucine or arginine. Experiment was performed as in (A) except cells were stably expressing FLAG-SLC38A9.1 1-119. (**E**) The ability of SLC38A9.1 overexpression to rescue mTORC1 signaling during amino acid starvation is eliminated by co-expression of $RagB^{T54N}$-$RagC^{Q120L}$, a Rag heterodimer locked in the nucleotide configuration associated with amino acid deprivation. Effects of expressing the indicated proteins on mTORC1 signaling were monitored by the phosphorylation state of co-expressed FLAG-S6K1. (**F**) Effects of concanamycin A and Torin1 on mTORC1 signaling in cells stably expressing SLC38A9.1. HEK-293T cells stably expressing the indicated FLAG-tagged proteins were treated with the DMSO vehicle or the specified small molecule inhibitor during the 50 min starvation for and, where indicated, the 10 min stimulation with amino acids.

## Modulation of the SLC38A9-Rag-Ragulator Interactions by Amino Acids

Amino acids modulate the interactions between many of the established components of the amino acid sensing pathway, so we tested if this was also the case for the SLC38A9.1-Ragulator-Rag complex. Indeed, amino acid starvation strengthened the interaction between stably expressed or endogenous Ragulator and endogenous SLC38A9 (Fig. 4A, fig. S5) and between stably expressed SLC38A9.1 and endogenous Ragulator and Rags (Fig. 4B). We obtained similar results when cells were deprived of and stimulated with just leucine or arginine (Fig. 4A). Curiously, although the N-terminal domain of SLC38A9.1 readily bound Ragulator, the interaction was insensitive to amino acids (Fig. 4B), suggesting that the transmembrane region is required to confer amino acid responsiveness.

As amino acid starvation alters the nucleotide state of the Rag GTPases (Kim et al., 2008; Sancak et al., 2008), we tested whether SLC38A9 interacted differentially with mutants of the Rags that lock their nucleotide state. Heterodimers of epitope-tagged RagB-RagC containing $RagB^{T54N}$, which mimics the GDP-bound state (Kim et al., 2008; Sancak et al., 2008), were associated with more endogenous SLC38A9 than heterodimers containing wild-type RagB (Fig.

4C). In contrast, heterodimers containing RagB^{Q99L}, which lacks GTPase activity and so is bound to GTP (Kim et al., 2008; Sancak et al., 2008; Tsun et al., 2013), interacted very weakly with SLC38A9 (Fig. 4C). Thus, like Ragulator, SLC38A9 interacts best with Rag heterodimers in which RagA/B is GDP-loaded, which is consistent with SLC38A9 binding to Ragulator and with Ragulator being a GEF for RagA/B. These results suggest that amino acid modulation of the interaction of SLC38A9.1 with Rag-Ragulator largely reflects amino acid-induced changes in the nucleotide state of the Rag GTPases. Because the RagB mutations had greater effects on the interaction of the Rag GTPases with SLC38A9 than with Ragulator (in Figure 4C compare the SLC38A9 blots with those for p14 and p18), it is very likely that the Rag heterodimers make Ragulator-independent contacts with SLC38A9 that affect the stability of Rag-SLC38A9 interaction.

Figure 4. Modulation of the interaction between SLC38A9 and Ragulator and the Rag GTPases by amino acids. (**A**) Effects of amino acids on interaction between the Ragulator complex and endogenous SLC38A9. HEK-293T cells stably expressing the indicated FLAG-tagged Ragulator components were deprived of total amino acids, leucine, or arginine for 1 hour and, where indicated, re-stimulated with amino acids, leucine, or arginine for 15 min. After lysis, samples were subject to FLAG immunoprecipitation and immunoblotting for the indicated proteins. Quantification of SLC38A9 levels in the stimulated state relative to starved state, p14 IP: 0.75 (+AA), 0.79 (+L), 0.74 (+R); p18 IP: 0.56 (+AA), 0.57 (+L), 0.49 (+R). (**B**) Effects of amino acids on the interaction between full-length or truncated SLC38A9.1 and endogenous Ragulator and the Rag GTPases. Experiment was performed as in (A) except that cells stably expressed the indicated SLC38A9 isoforms or its N-terminal domain (SLC38A9.1 1-119). Quantification of indicated protein levels in the stimulated state relative to starved state, SLC38A9.1 IP: 0.43 (p18), 0.51 (p14), 0.61 (RagC), 0.58 (RagA); SLC38A9.1 1-119 IP: 0.99 (p18), 1.05 (p14), 1.04 (RagC), 1.09 (RagA). (**C**) Effects of the RagB^{T54N} mutation on association with endogenous SLC38A9. HEK-293T cells were transfected with the indicated cDNAs in expression vectors and lysates were prepared and subjected to FLAG immunoprecipitation followed by immunoblotting for the indicated proteins. Two different antibodies were used to detect endogenous SLC38A9.

Fig. 4
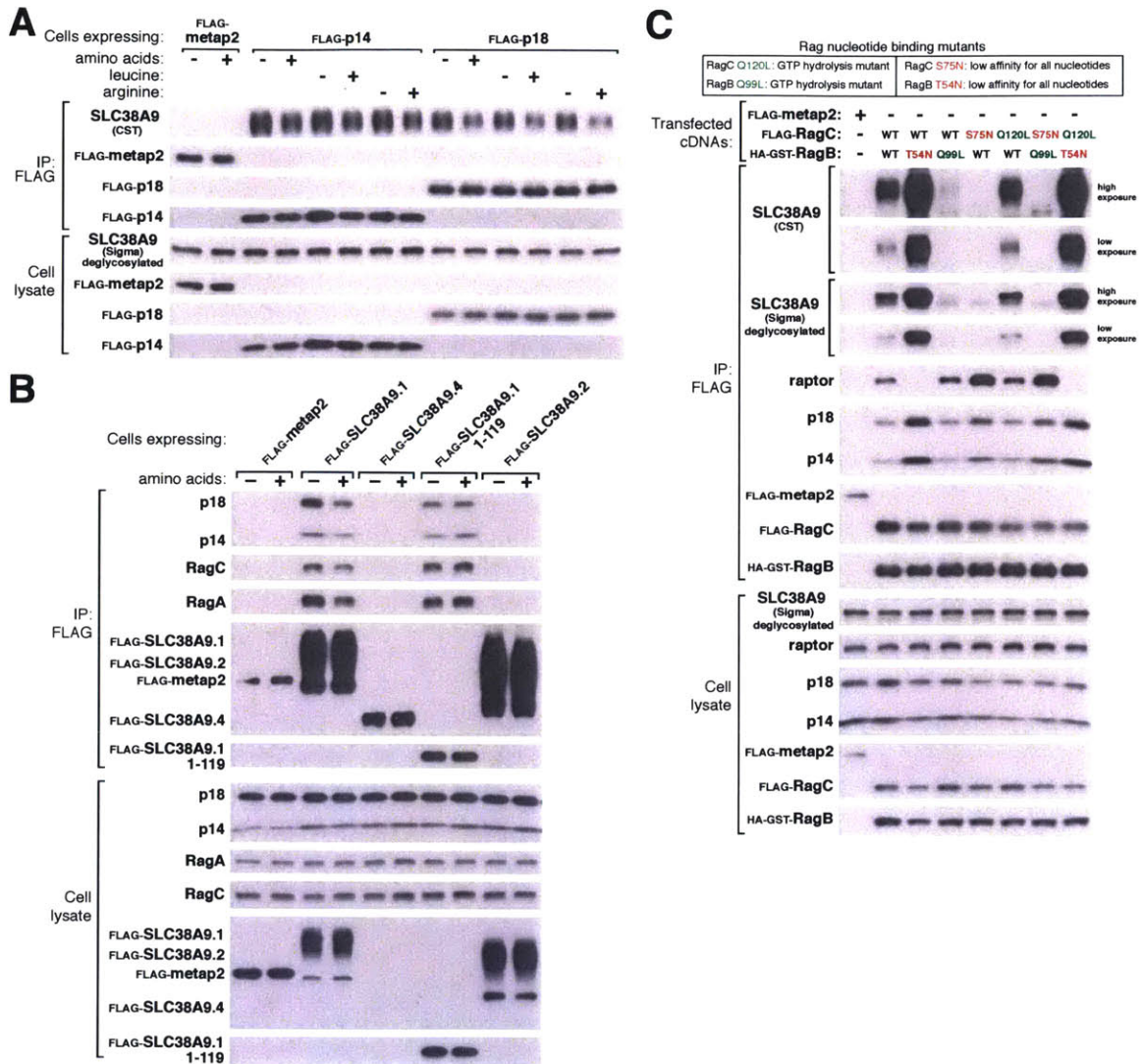
SLC38A9.1 is an Amino Acid Transporter

We failed to detect SLC38A9.1-mediated amino acid transport or amino acid-induced sodium currents in live cells in which SLC38A9.1 was so highly overexpressed that some reached the plasma membrane (fig. S6, A-E). Because these experiments were confounded by the presence of endogenous transporters or relied on indirect measurements of transport, respectively, we reconstituted SLC38A9.1 into liposomes to directly assay the transport of radiolabelled amino acids. Affinity-purified SLC38A9.1 inserted unidirectionally into liposomes so that its N-terminus faced outward in an orientation analogous to that of the native protein in

lysosomes (fig. S6, F-H). We could not use radiolabelled L-leucine in transport assays because it bound non-specifically to liposomes so we focused on the transport of L-arginine, which had low background binding (fig. S6I). The SLC38A9.1-containing proteoliposomes exhibited time-dependent uptake of radiolabelled arginine while those containing LAMP1 interacted with similar amounts of arginine as liposomes (Fig. 5A, fig. S6I). Steady-state kinetic experiments revealed that SLC38A9.1 has a Michaelis constant (K_m) of ~39 mM and a catalytic rate constant (k_{cat}) of ~1.8 min^{-1} (Fig. 5B), indicating that SLC38A9.1 is a low-affinity amino acid transporter. SLC38A9.1 can also efflux arginine from the proteoliposomes (Fig. 5C), but its orientation in liposomes makes it impossible to obtain accurate K_m and k_{cat} measurements for this activity. It is likely that by having to assay the transporter in the 'backwards' direction we are underestimating its affinity for amino acids during their export from lysosomes.

To assess the substrate specificity of SLC38A9.1, we performed competition experiments using unlabeled amino acids (Fig. 5D). The positively charged amino acids histidine and lysine competed radiolabelled arginine transport to similar degrees as arginine, while leucine had a modest effect and glycine was the least effective competitor. Thus, it appears that SLC38A9.1 has a relatively non-specific substrate profile with a preference for polar amino acids.

Given the preference of SLC38A9.1 for the transport of arginine and that arginine is highly concentrated in rat liver lysosomes (Harms, Gochman, & Schneider, 1981) and yeast vacuoles (Kitamoto, Yoshizawa, Ohsumi, & Anraku, 1988), we asked whether SLC38A9.1 may have an important role in transmitting arginine levels to mTORC1. Towards this end we examined how mTORC1 signaling responded to a range of arginine or leucine concentrations in HEK-293T cells in which we knocked out SLC38A9 using CRISPR-Cas9 genome editing (Fig. 5E). Interestingly, activation of mTORC1 by arginine was strongly repressed at all arginine concentrations while the response to leucine was only blunted so that high leucine concentrations activated mTORC1 equally well in null and control cells (Fig. 5F).
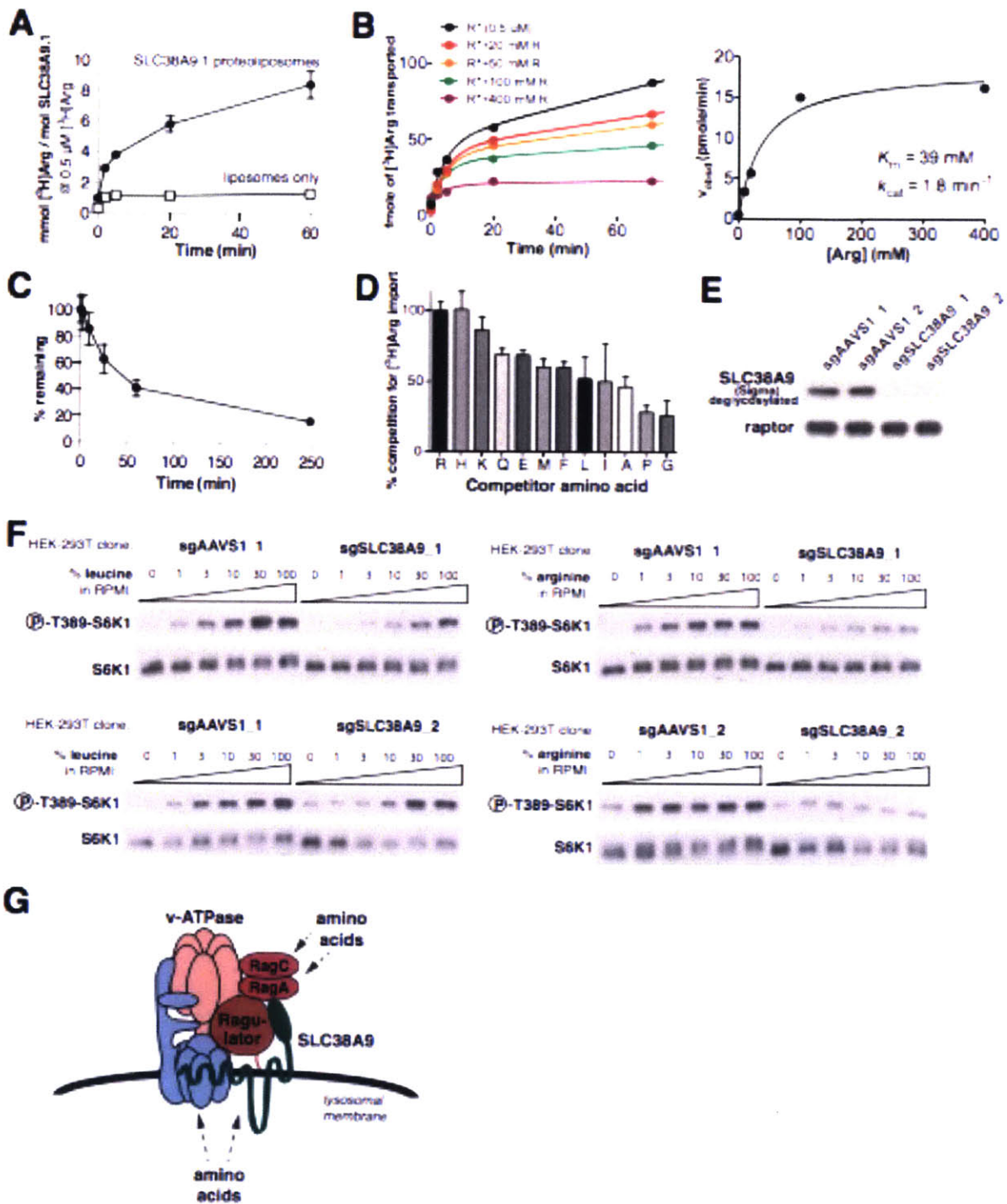
Fig. 5

Figure 5. SLC38A9.1 is a low affinity amino acid transporter and is necessary for mTORC1 pathway activation by arginine. (**A**) Time-dependent uptake of [³H]arginine at 0.5 μM by proteoliposomes containing 22.4 pmol of SLC38A9.1. To recapitulate the pH gradient across the lysosomal membrane, the lumen of the proteoliposomes is buffered at pH 5.0, while the external buffer is pH 7.4. (**B**) Steady-state kinetic analysis of SLC38A9.1 uptake activity reveals a

Michaelis constant (K_m) of ~39mM and catalytic rate constant (k_{cat}) of ~1.8min^{-1}. (Left) Time course of [^{3}H]arginine (R*) uptake, given fixed [^{3}H]arginine (0.5 μM) and increasing concentrations of unlabeled arginine. (Right) Velocity, calculated from left panel, as a function of total arginine concentration. Data were fitted to the Michaelis-Menton equation. Experiment was repeated over 4 times with similar results and a representative one is shown. (**C**) Time-dependent efflux of SLC38A9.1 proteoliposomes following 1.5 hr loading with 0.5 μM [^{3}H]arginine. (**D**) Competition of 0.5 μM [^{3}H]arginine transport by SLC38A9.1 using 100 mM of indicated unlabeled amino acids. In A-D, error bars represent standard deviation derived from at least 3 measurements. (**E**) HEK-293T cells null for SLC38A9 were generated using CRISPR-Cas9 genome editing using two different guide sequences and isolated by single cell cloning. The AAVS1 locus was targeted as a negative control. (**F**) Impairment of arginine-induced activation of the mTORC1 pathway in SLC38A9-null HEK-293T cells. Cells were starved of the indicated amino acid for 50 minutes and stimulated for 10 minutes using the indicated amino acid concentrations. The leucine and arginine concentrations in RPMI are, respectively, 381 μM and 1.14 mM. (**G**) Model for distinct amino acid inputs to the Rag GTPases in signaling amino acid sufficiency to mTORC1.

Conclusions

Several properties of SLC38A9.1 are consistent with it functioning as an amino acid sensor for the mTORC1 pathway. Purified SLC38A9.1 transports and therefore directly interacts with amino acids. Overexpression of SLC38A9.1 or just its Ragulator-binding domain activates mTORC1 signaling even in the absence of amino acids. The activation of mTORC1 by amino acids, particularly arginine, is defective in cells lacking SLC38A9. Given these results and that arginine is highly enriched in lysosomes from at least one mammalian tissue (Harms et al., 1981), we suggest that SLC38A9.1 is a strong candidate for being a lysosome-based arginine sensor for the mTORC1 pathway. To substantiate this possibility it will be necessary to determine the actual concentrations of arginine and other amino acids in the lysosomal lumen and cytosol and compare them to the affinity of SLC38A9.1 for amino acids. If high arginine levels are a general feature of mammalian lysosomes it could explain why SLC38A9.1 appears to have a relatively broad amino acid specificity; perhaps no other amino acid besides arginine is in the lysosomal lumen at levels that approach its K_m.

The notion that proteins with sequence similarity to transporters function as both transporters and receptors (transceptors) is not unprecedented (Holsbeeks, Lagatie, Van Nuland, Van de Velde, & Thevelein, 2004; Hyde, Cwiklinski, MacAulay, Taylor, & Hundal, 2007). The transmembrane region of SLC38A9.1 might undergo a conformational change upon amino acid binding that is then transmitted to Ragulator through its N-terminal domain. What this domain does is unknown but it could regulate Ragulator nucleotide exchange activity or access to the

Rag GTPases by other components of the pathway. To support a role as a sensor, it will be necessary to show that amino acid binding regulates the biochemical function of SLC38A9.1.

Even if SLC38A9.1 is an amino acid sensor, additional sensors, even for arginine, are almost certain to exist as we already know that amino acid-sensitive events exist upstream of Folliculin (Petit, Roczniak-Ferguson, & Ferguson, 2013; Tsun et al., 2013) and GATOR1 (Chantranupong et al., 2014), which, like Ragulator, also regulate the Rag GTPases. An attractive model is that distinct amino acid inputs to mTORC1 converge at the level of the Rag GTPases with some initiating at the lysosome through proteins like SLC38A9.1 and others from cytosolic sensors that remain to be defined (Fig. 5G). Indeed, such a model would explain why the loss of SLC38A9.1 specifically affects arginine sensing but its overexpression makes mTORC1 signaling resistant to arginine or leucine starvation: hyperactivation of the Rag GTPases through the deregulation of a single upstream regulator is likely sufficient to overcome the lack of other positive inputs. A similar situation may occur upon loss of GATOR1, which, like SLC38A9.1 overexpression, causes mTORC1 signaling to be resistant to total amino acid starvation (Bar-Peled et al., 2013).

Modulators of mTORC1 have clinical utility in disease states associated with or caused by mTORC1 deregulation. The allosteric mTOR inhibitor rapamycin is used in cancer treatment (Benjamin, Colombi, Moroni, & Hall, 2011) and transplantation medicine (Kahan & Camardo, 2001). However, to date, there have been few reports on small molecules that activate mTORC1 by engaging known components of the pathway. The identification of SLC38A9.1—a protein that is a positive regulator of the mTORC1 pathway and has an amino acid binding site— provides an opportunity to develop small molecule agonists of mTORC1 signaling. Such molecules should promote mTORC1-mediated protein synthesis and could have utility in combatting muscle atrophy secondary to disuse or injury. Lastly, there is reason to believe that a selective mTORC1 pathway inhibitor may have better clinical benefits than rapamycin, which in long-term use inhibits both mTORC1 and mTORC2 (Lamming, Ye, Sabatini, & Baur, 2013). SLC38A9.1 may be an appropriate target to achieve this.

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Acknowledgements

Materials and Methods

Materials

Reagents were obtained from the following sources: HRP-labeled anti-mouse, anti-rabbit, and anti-goat secondary antibodies and the antibody to LAMP2 from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, S6K1, phospho-ULK1, ULK1, phospho-S65 4E-BP1, 4E-BP1, RagA, RagC, p14 (LAMTOR2), p18 (LAMTOR1), mTOR, and the FLAG epitope (rabbit antibody) from Cell Signaling Technology; the antibody to the HA epitope from Bethyl laboratories; the antibody to ATP6V1B2 from Abcam; RPMI, FLAG M2 affinity gel, FLAG-M2 (mouse) and ATP6V0d1 antibodies, and amino acids from Sigma Aldrich; the PNGase F from NEB; Xtremegene 9 and Complete Protease Cocktail from Roche; AlexaFluor-labeled donkey anti-rabbit, anti-mouse, and anti-rat secondary antibodies from Invitrogen and Inactivated Fetal Calf Serum (IFS) from Invitrogen; amino acid-free RPMI and Leucine or Arginine-free RPMI from US Biological; siRNAs targeting indicated genes and siRNA transfection reagent from Dharmacon; Concanamycin A from A.G. Scientific; Torin1 from Nathanael Gray (DFCI); [^{14}C]-labeled amino acids and Opti-Fluor scintillation fluid from PerkinElmer; [^{3}H]-labeled amino acids from American Radiolabeled Chemicals; Egg phosphatidylcholine (840051C) from Avanti lipids; Bio-beads SM-2 from Bio-Rad; and PD-10 columns from GE Healthcare Life Sciences. The antibody to SLC38A9 from Sigma (HPA043785) was used to recognize the deglycosylated protein (according to NEB instructions except without the boiling step) in cell lysates and immunopurifications. A distinct antibody to SLC38A9.1 was generated in collaboration with Cell Signaling Technology and was used to detect the glycosylated protein in Ragulator immunopurifications but is not sensitive enough to detect it in cell lysates.

Cell lines and tissue culture

HEK-293T cells were cultured in DMEM supplemented with 10% inactivated fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 μg/mL) and maintained at 37°C and 5% CO_2. In HEK-293E, but not HEK-293T, cells the mTORC1 pathway is strongly regulated by serum and insulin (Sancak et al., 2007).

Mass spectrometric analyses

Immunoprecipitates from 30 million HEK-293T cells stably expressing FLAG-metap2, FLAG-p18, FLAG-p14, FLAG-HBXIP, FLAG-c7orf59, and FLAG-RagB were prepared as described below. Proteins were eluted with the FLAG peptide (sequence DYKDDDDK) from the anti-FLAG affinity beads, resolved on 4-12% NuPage gels (Invitrogen), and stained with SimplyBlue SafeStain (Invitrogen). Each gel lane was sliced into 10-12 pieces and the proteins in each gel slice digested overnight with trypsin. The resulting digests were analyzed by mass spectrometry as described (Sancak et al., 2008).

Amino acid or individual amino acid starvation and stimulation of cells

Almost confluent cell cultures in 10 cm plates were rinsed twice with amino acid-free RPMI, incubated in amino acid-free RPMI for 50 min, and stimulated for 10 min with a water-solubilized amino acid mixture added directly to the amino acid-free RPMI. For leucine or arginine starvation, cells in culture were rinsed with and incubated in leucine- or arginine-free RPMI for 50 min, and stimulated for 10 min with leucine or arginine added directly to the starvation media. After stimulation, the final concentration of amino acids in the media was the

same as in RPMI. Cells were processed for biochemical or immunofluorescence assays as described below. The 10X amino acid mixture and the 300X individual stocks were prepared from individual amino acid powders. When Concanamycin A (ConA) or Torin1 was used, cells were incubated in 5 μM Concanamycin or 250 nM Torin1 during the 50 min amino acid starvation and 10 min amino acid stimulation periods.

Cell lysis and immunoprecipitations

HEK-293T cells stably expressing FLAG-tagged proteins were rinsed once with ice-cold PBS and lysed in ice-cold lysis buffer (40 mM HEPES pH 7.4, 1% Triton X-100, 10 mM β-glycerol phosphate, 10 mM pyrophosphate, 2.5 mM $MgCl_2$ and 1 tablet of EDTA-free protease inhibitor (Roche) per 25 ml buffer). The soluble fractions from cell lysates were isolated by centrifugation at 13,000 rpm for 10 min in a microcentrifuge. For immunoprecipitates 30 uL of a 50% slurry of anti-FLAG affinity gel (Sigma) were added to each lysate and incubated with rotation for 2-3 hr at 4°C. Immunoprecipitates were washed three times with lysis buffer containing 500 mM NaCl. Immunoprecipitated proteins were denatured by the addition of 50 uL of sample buffer and incubation at RT for 30 min. It is critical that the samples containing SLC38A9 are neither boiled nor frozen prior to resolution by SDS-PAGE and analysis by immunoblotting. A similar protocol was employed when preparing samples for mass spectrometry.

cDNA manipulations and mutagenesis

The cDNAs for all human SLC38A9 isoforms, both native and codon-optimized, were gene-synthesized by GenScript. The cDNAs were amplified by PCR and the products were subcloned into Sal I and Not I sites of HA-pRK5 and FLAG-pRK5. The cDNAs were mutagenized using the QuikChange II kit (Agilent) with oligonucleotides obtained from Integrated DNA Technologies. All constructs were verified by DNA sequencing.

FLAG-tagged SLC38A9 isoforms and SLC38A9 N-terminal 1-119 were amplified by PCR and cloned into the Sal I and EcoR I sites of pLJM60 or into the Pac I and EcoR I sites of pMXs. After sequence verification, these plasmids were used, as described below, in cDNA transfections or to produce lentiviruses needed to generate cell lines stably expressing the proteins.

cDNA transfection-based experiments

For cotransfection-based experiments to test protein-protein interactions, 2 million HEK-293T cells were plated in 10 cm culture dishes. 24 hours later, cells were transfected with the pRK5-based cDNA expression plasmids indicated in the figures in the following amounts: 500 ng FLAG-metap2; 50 ng FLAG-LAMP1; 100 ng FLAG-RagB and 100 ng HA-RagC; 300 ng FLAG-SLC38A9.1; 600 ng FLAG-SLC38A9.1 Δ110; 200 ng FLAG-SLC38A9.4; 400 ng FLAG-N-terminal 119 fragment of SLC38A9.1; 200 ng FLAG-RagC; 200 ng FLAG-RagC S75N; 200 ng FLAG-RagC Q120L; 400 ng HAGST-RagB; 400 ng HAGST-RagB T54N; 400 ng HAGST-RagB Q99L. Transfection mixes were taken up to a total of 5 μg of DNA using empty pRK5.

For co-transfection experiments to test mTORC1 activity, 1 million HEK-293T cells were plated in 10 cm culture dishes. 24 hours later, cells were transfected with the pRK5-based cDNA expression plasmids indicated in the figures in the following amounts: 500 ng HA-metap2; 50 ng HA-LAMP1; 200 ng HA-SLC38A9.1; 500 ng HA-SLC38A9.1 Δ110; 200 ng HA-

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SLC38A9.4; 100 ng HA-RagB T54N and 100 ng HA-RagC Q120L; 2 ng FLAG-S6K1. 72 hours post-transfection, cells were washed once prior to 50-min incubation with amino acid-free RPMI. Cells were stimulated with vehicle or amino acids (to a final concentration equivalent to RPMI) prior to harvest.

Lentivirus production and lentiviral transduction

Lentiviruses were produced by co-transfection of the pLJM1/pLJM60 lentiviral transfer vector with the VSV-G envelope and CMV ΔVPR packaging plasmids into viral HEK-293T cells using the XTremeGene 9 transfection reagent (Roche). For infection of HeLa cells, LN229 cells, and MEFs, retroviruses were produced by co-transfection of the pMXs retroviral transfer vector with the VSV-G envelope and Gag/Pol packaging plasmids into viral HEK-293T cells. The media was changed 24 hours post-transfection to DME supplemented with 30% IFS. The virus-containing supernatants were collected 48 hours after transfection and passed through a 0.45 μm filter to eliminate cells. Target cells in 6-well tissue culture plates were infected in media containing 8 μg/mL polybrene and spin infections were performed by centrifugation at 2,200 rpm for 1 hour. 24 hours after infection, the virus was removed and the cells selected with the appropriate antibiotic.

Mammalian RNAi

Lentiviruses encoding shRNAs were prepared and transduced into HEK-293T cells as described above. The sequences of control shRNAs and those targeting human SLC38A9, which were obtained from The RNAi Consortium 3 (TRC3), are the following (5' to 3'):
SLC38A9 #1: GCCTTGACAACAGTTCTATAT (TRCN0000151238)
SLC38A9 #2: CCTCTACTGTTTGGGACAGTA (TRCN0000156474)
GFP: TGCCCGACAACCACTACCTGA (TRCN0000072186)

For siRNA-based experiments, 200,000 HEK-293T cells were plated in a 6-well plate. 24 hours later, cells were transfected using Dharmafect 1 (Dharmacon) with 250 nM of a pool of siRNAs (Dharmacon) targeting SLC38A9 or a non-targeting pool. 48 hours post-transfection, cells were transfected again but this time with double the amount of siRNAs. 24 hours following the second transfection, cells were rinsed with ice-cold PBS, lysed, and subjected to immunoblotting as described above. The following siRNAs were used:
Non-targeting: ON-TARGETplus Non-targeting Pool (D-001810-10-05)
SLC38A9: SMARTpool: ON-TARGETplus SLC38A9 (L-007337-02-0005)

Immunofluorescence assays

HEK-293T cells were plated on fibronectin-coated glass coverslips in 6-well tissue culture dishes, at 300,000 cells/well. 12-16 hours later, the slides were rinsed with PBS once and fixed and permeabilized in one step with ice-cold 100% methanol (for SLC38A9 detection) at -20°C for 15 min. After rinsing twice with PBS, the slides were incubated with primary antibody (FLAG CST 1:300, LAMP2 1:400) in 5% normal donkey serum for 1 hr at room temperature, rinsed four times with PBS, incubated with secondary antibodies produced in donkey (diluted 1:400 in 5% normal donkey serum) for 45 min at room temperature in the dark, and washed four times with PBS. Slides were mounted on glass coverslips using Vectashield with DAPI (Vector Laboratories) and imaged on a spinning disk confocal system (Perkin Elmer).

Whole-cell amino acid transport assay

HEK-293T cells (150,000/well) were plated onto fibronectin-coated 12-well dishes and transfected 12 hours later with the pRK5-based cDNA expression plasmids indicated in the figures in the following amounts using XtremeGene9: 400 ng LAMP1-FLAG, 400 ng FLAG-SLC38A9.1, 400 ng SLC38A2-FLAG, 150 ng PQLC2-FLAG, and 50 ng GFP. Transfection mixes were taken up to a total of 2 µg of DNA using empty pRK5. Cells were assayed 48 hours later by washing twice in transport buffer (140 mM NaCl, 5 mM KCl, 2 mM MgCl2, 2 mM CaCl$_2$, 30 mM Tris-HCl, pH 7.4, 5 mM glucose), incubating in transport buffer for 5 min. at 37°C before replacing the buffer with fresh buffer supplemented with amino acids (unlabeled and 0.1 µCi of [^{14}C]leucine at a total concentration of 380 µM, or unlabeled and 0.1µCi of [^{14}C]amino acid mix at total concentrations found in RPMI, or unlabeled and 0.2 µCi of [^{14}C]arginine at a total concentration of 3 mM) at the indicated pH (pH 5 buffered by MES, pH 8 buffered by Tris) for 10 minutes at 37°C. After uptake, cells were washed twice in ice-cold transport buffer and harvested in 0.5 mL of 1% SDS for scintillation counting. Protocol and amino acid concentrations used were informed by previous whole-cell assays to detect transport by SLC38A2 and PQLC2 (Liu, Du, Rutkowski, Gartner, & Wang, 2012; Zhang, Gameiro, & Grewer, 2008).

Electrophysiology

Whole-cell recordings were made from GFP-positive HEK-293T cells, prepared as described above, 48 to 72 hrs post transfection. Patch pipettes (open-tip resistance 3-4 MΩ) were filled with a solution containing (in mM) K-gluconate 153, MgCl$_2$ 2, CaCl$_2$ 1, EGTA 11, HEPES 10, pH 7.25 adjusted with KOH, and tip resistance was left uncompensated. Cells were continuously superfused (~ 2 ml/min) with extracellular solution containing (in mM) NaCl 150, KCl 3, CaCl$_2$ 2, MgCl$_2$ 1, Glucose 5, HEPES 10, pH adjusted to 7.4 with NaOH. Once whole-cell configuration was established, a homemade perfusion system consisting of several adjacent glass tubes (ID 252 µm) was used to locally perfuse extracellular solution pH 5.5 and to apply amino acids (in mM) leucine 1.6, arginine 2.4, glutamine 4. Membrane currents were amplified and low-pass filtered at 3 kHz using a Multiclamp 700B amplifier (Molecular Devices), digitized at 10 kHz and acquired using National Instruments acquisition boards and a custom version of ScanImage written in MATLAB (Mathworks) (Pologruto, Sabatini, & Svoboda, 2003). Data were analyzed offline using Igor Pro (Wavemetrics), and amino acid-induced currents were quantified as difference in the average membrane currents for the 5 s-windows right before and during application.

Proteoliposome Reconstitution

HEK-293T cells stably expressing FLAG-SLC38A9.1 were harvested as described above for immunoprecipitations, except cells were lysed in 40 mM HEPES pH 7.4, 0.5% Triton X-100, 1 mM DTT, and protease inhibitors. Following a 3 hr immunoprecipitation, FLAG-affinity beads were washed twice for 5 min each in lysis buffer supplemented with 500 mM NaCl. Beads were equilibrated with inside buffer (20 mM MES pH 5, 90 mM KCl 10 mM NaCl) supplemented with 10% glycerol by washing them 5 times. FLAG-affinity purified SLC38A9.1 protein was eluted in glycerol-supplemented inside buffer containing 1 mg/mL FLAG peptide by rotation for 30 min. Protein was concentrated using Amicon centrifuge filters to about 1 mg/mL and snap-frozen in liquid nitrogen and stored at -80°C.

Chloroform-dissolved phosphatidylcholine (PC, 50 mg) was evaporated using dry nitrogen to yield a lipid film in a round bottom flask and desiccated overnight under vacuum.

Lipids were hydrated in inside buffer at 50 mg/mL with light sonication in a water bath (Branson M2800H) and split into 100 µL aliquots in eppendorf tubes. Aliquoted lipids were clarified using water bath sonication and recombined and extruded through a 100 nm membrane with 15 passes (Avanti 61000). Reconstitution reaction (15 µg FLAG-SLC38A9.1 protein, 7.5 mg Triton X-100, 10 mg extruded PC, 1 mM DTT in inside buffer up to 700 µL) was initiated by rotating at 4°C for 30 min. Glycerol-supplemented inside buffer was used in lieu of SLC38A9.1 protein in liposome only controls. Bio-beads (200 mg/reaction) were prepared by washing 1 time in methanol, 5 times in water and 2 times in inside buffer. Reconstitution reaction was applied to Bio-beads for 1 hr, transferred to fresh Bio-beads overnight, and transferred again to fresh Bio-beads for 1 hr. Protocol was adapted from a recently reconstituted lysosomal transporter and a recent review (Rigaud & Levy, 2003; Zhao, Haase, Tampe, & Abele, 2008).

Floatation assay

A three-step sucrose gradient was generated by first adding 3.8 mL of the middle buffer (35% glycerol in inside buffer) to the ultracentrifuge tube, then applying 1 mL of the bottom buffer (50% glycerol in inside buffer) with 100 µL of SLC38A9.1 proteoliposomes (or protein only) using a 2 mL pipette to the bottom of the tube, and finally layering 1.2 mL of the top buffer (0% glycerol, inside buffer) on top. For assays containing urea, the proteoliposomes were rotated in bottom buffer containing 6 M urea for 30 min. at 4°C before generating the sucrose gradient with above buffers supplemented with 6 M urea. Gradients were topped with 2 mL paraffin oil and loaded into a SW32.1 rotor and centrifuged at 32,000 g for 24 hours. Fractions (500 µL, excluding the oil) were collected from the top and 20 µL of each subjected to anti-FLAG western analysis. Protocol was adapted from Wuu et al. (Wuu & Swartz, 2008).

Trypsin protection assay

Trypsin (1 µL of 0.05%, Invitrogen) was added to SLC38A9.1 proteoliposomes (15 µL) and incubated at 37°C for 30 min. As indicated, 1% Triton X-100 was added and rotated for 30 min. at 4°C before addition of trypsin. Reactions were subjected to anti-FLAG western analysis. Protocol was inspired by Brown and Goldstein (Nohturfft, Brown, & Goldstein, 1998).

In vitro amino acid transport assay

All buffers were chilled and assays performed in a 4°C cold room. For time course experiments, SLC38A9.1 proteoliposomes or liposome controls were applied to PD10 columns equilibrated with outside buffer (20 mM Tris pH 7.4, 100 mM NaCl) and eluted according to manufacturer's instructions. Amino acid uptake was initiated by the addition of 0.5 µM [^{3}H]arginine and incubated in a 30°C water bath. Time points were collected by taking a fraction of the assay reaction and applying it to PD10 columns pre-equilibrated with outside buffer. Columns were eluted in fractions or a single elution of 1.75 mL and added to 5 mL of scintillation fluid. To obtain accurate measures of amino acid concentrations, equal volumes of outside buffer was added to scintillation fluid in the standards.

For competition experiments with unlabeled amino acids, high concentrations of amino acids were required due to the high K_m (~39mM) of SLC38A9.1 import activity. SLC38A9.1 proteoliposomes or liposome controls were centrifuged at 100,000 g for 30 min. in a TLA-100.3 rotor and resuspended in a smaller volume of outside buffer such that they could be added to a larger volume of 100 mM unlabeled amino acid (final concentration) supplemented with outside buffer components. We had to resort to this procedure due to the solubility limit of leucine at

~130 mM. At such high concentrations, it is important to adjust all amino acid solutions to pH 7.4. Assays were initiated by addition of 0.5 μM [^{3}H]arginine to the amino acid buffer solution followed by the addition of SLC38A9.1 proteoliposomes or liposome controls. For steady-state kinetics experiments, time points were collected as described above and to assess substrate specificity, competition experiments were collected at 75 min.

For efflux experiments, SLC38A9.1 proteoliposomes or liposome controls were loaded with [^{3}H]arginine as described above for an import assay for 1.5 hrs. To remove external amino acids, the reactions were applied to PD10 columns pre-equilibrated with outside buffer, and time points were collected as described above. Scintillation counts from liposome controls were subtracted from that of SLC38A9.1 proteoliposomes.

Generation of knockout clones using CRISPR/Cas9

The CRISPR guide sequences designed to the N-terminus (1-119 a.a.) of SLC38A9 or the AAVS1 locus using http://crispr.mit.edu were cloned into pX459 (Ran et al., 2013).

AAVS1: GGGGCCACTAGGGACAGGAT
SLC38A9_1: GGCTCAAACTGGATATTCATAGG
SLC38A9_2: GGAGCTGGAACTACATGGTCTGG

HEK-293T cells (750,000/well) were plated into 6 well dishes and transfected 16 hours later with 1 μg of pX459 expressing above guides using XtremeGene9. Cells were trypsinized 48 hours later, 2 mg/mL puromycin was applied for 72 hours, and allowed to recover for a few days. When cells were approaching confluency, they were single-cell sorted into 96-well dishes containing 30% serum and conditioned media. Clones were expanded and evaluated for knockout status by western analysis for SLC38A9. These clones were evaluated for amino acid response as described above.

Supplementary Figures

Figure S1: Membrane topology of SLC38A9.1. **(A)** Representation of the TMHMM topology prediction for SLC38A9.1. **(B)** Visualization of SLC38A9.1 topology as generated by Protter.

Figure S2: Ragulator and the Rag GTPases do not interact with all lysosomal amino acid transporter-like proteins. **(A)** SLC38A9.1, but not SLC38A7 or SLC36A1, interacts with the Ragulator complex and the Rag GTPases. HEK-293T cells were transfected with the indicated cDNAs in expression vectors and lysates were prepared and subjected to FLAG immunoprecipitation followed by immunoblotting for the indicated proteins. **(B and C)** The interaction with Ragulator requires the presence of the intact N-terminal domain of SLC38A9.1, which is lacking in SLC38A9.2 (B), SLC38A9.1 Δ110 (C), and SLC38A9.4 (C). HEK-293T cells were transfected with the indicated cDNAs in expression vectors and processed as in (A).

Figure S3: Localization of SLC38A9 isoforms 2 and 4 and signaling effects of siRNA-mediated SLC38A9 knockdown. SLC38A9 isoforms lacking part **(A)** or all **(B)** of the N-terminal region of SLC38A9.1 still localize to the lysosomal membrane. HEK-293T cells stably expressing the indicated FLAG-tagged SLC38A9 isoforms were immunostained for FLAG and LAMP2. **(C)** The interaction between SLC38A9.1 and Ragulator occurs only when Ragulator is anchored at the lysosomal membrane through lipidation of the N-terminus of p18. Ragulator containing the lipidation-deficient p18^{G2A} mutant fails to interact with SLC38A9.1. HEK-293T cells were transfected with the indicated cDNAs in expression vectors and lysates prepared and subjected to FLAG immunoprecipitation followed by immunoblotting for the indicated proteins. **(D)** Knockdown of SLC38A9 in HEK293T cells with a pool of short interfering RNAs suppresses the phosphorylation of S6K1.
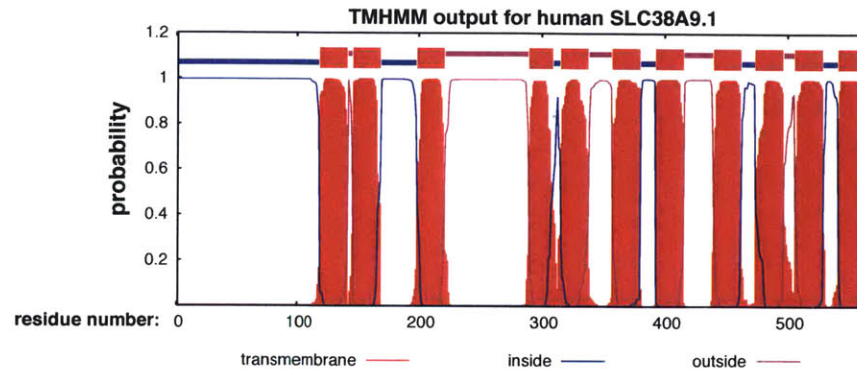
Figure S4: (A) Transient overexpression of SLC38A9.1, but not truncation mutants lacking the N-terminal Ragulator-binding domain, makes the mTORC1 pathway insensitive to amino acid starvation. Cell lysates were prepared from HEK-293T cells deprived for 50 min for amino acids and, then, where indicated, stimulated with amino acids for 10 min. Cell lysates and FLAG immunoprecipitates were analyzed for the levels of the specified proteins and for the phosphorylation state of S6K1. **(B)** Stable overexpression of SLC38A9.1 in HeLa cells, LN229 cells, and MEFs makes the mTORC1 pathway partially resistant to amino acid deprivation. Cells transduced with retroviruses encoding the specified proteins were deprived for 50 min of all amino acids and, where indicated, stimulated for 10 min with amino acids. Cell lysates were analyzed for the levels of the specified proteins and the phosphorylation state of S6K1. **(C)** Stable overexpression of SLC38A9.1 suppresses autophagy induction upon arginine starvation as indicated by detected by p62 accumulation and suppressed LC3 degradation. HEK-293T cells stably overexpressing FLAG-SLC38A9.1 were simultaneously deprived of arginine and, where indicated, treated with 30 uM chloroquine for the indicated time. Cell lysates were analyzed for the levels of the specified proteins and the phosphorylation state of S6K1. **(D)** Stable overexpression of SLC38A9.1 in HEK-293E cells does not perturb the response of mTORC1 signaling to serum starvation and insulin stimulation. **(E)** Stable overexpression of SLC38A9.1 does not protect mTORC1 signaling from the inhibitory effects of MK2206, which blocks growth factor signaling by allosterically inhibiting Akt.

Figure S5: Endogenous immunoprecipitation of Rag and Ragulator components recovers SLC38A9 in an amino acid-sensitive fashion. Cell lysates were prepared from HEK-293T cells deprived for 50 min for amino acids and, then, where indicated, stimulated with amino acids for 10 min. Cell lysates as well as control, p18, RagA, and RagC immunoprecipitates were analyzed for the levels of the indicated endogenous proteins.

Figure S6: SLC38A9.1 is a low-affinity amino acid transporter. (**A**) Immunostaining of HEK-293T cells transiently overexpressing SLC38A9.1 at levels that cause spillover to the plasma membrane. These cells were used for whole-cell amino acid transport assays and amino acid-induced current recordings. HEK-293T cells transiently expressing indicated cDNAs were incubated with [^{14}C]arginine (**B**), [^{14}C]amino acid mix (**C**), or [^{14}C]leucine (**D**) containing buffer at the indicated pH and washed before harvested for scintillation counting. (**E**) (Left) Whole-cell recordings from HEK-293T cells expressing indicated cDNAs at -80 mV. Quantified is the change in steady-state current following local application of 2.4 mM arginine, 1.6 mM leucine, and 4 mM glutamine (4x DMEM concentrations). All recordings were performed at pH 5.5. Statistical comparison was performed by Kruskall-Wallis test, followed by Dunn's test. (Right) Representative examples of individual recordings. Grey bars indicate application of amino acids. (**F**) Coomassie stain of FLAG-affinity purified LAMP1 or SLC38A9.1 from HEK-293T cells stably expressing respective protein. (**G**) Floatation assay shows successful insertion of SLC38A9.1 into proteoliposomes. Where indicated, 6 M urea was added following the reconstitution reaction. (**H**) SLC38A9.1 is unidirectionally inserted into proteoliposomes, with the N-terminus facing the outside of liposomes. Proteoliposomes containing N-terminally FLAG-tagged SLC38A9.1 were exposed to trypsin and immunoblotted for FLAG. The addition of 1% Triton X-100 did not reveal any protected FLAG-tagged fragments. (**I**) SLC38A9.1 proteoliposomes uptake [^{3}H]arginine. 0.5 μM [^{3}H]arginine was incubated with the indicated components for 60 min. and the reaction was applied to a column that traps free amino acids. Proteoliposomes pass through the column and fractions were subjected to scintillation counting and FLAG immunoblotting. To recapitulate the pH gradient across the lysosomal membrane, the lumen of the proteoliposomes is buffered at pH 5.0, while the external buffer is pH 7.4.
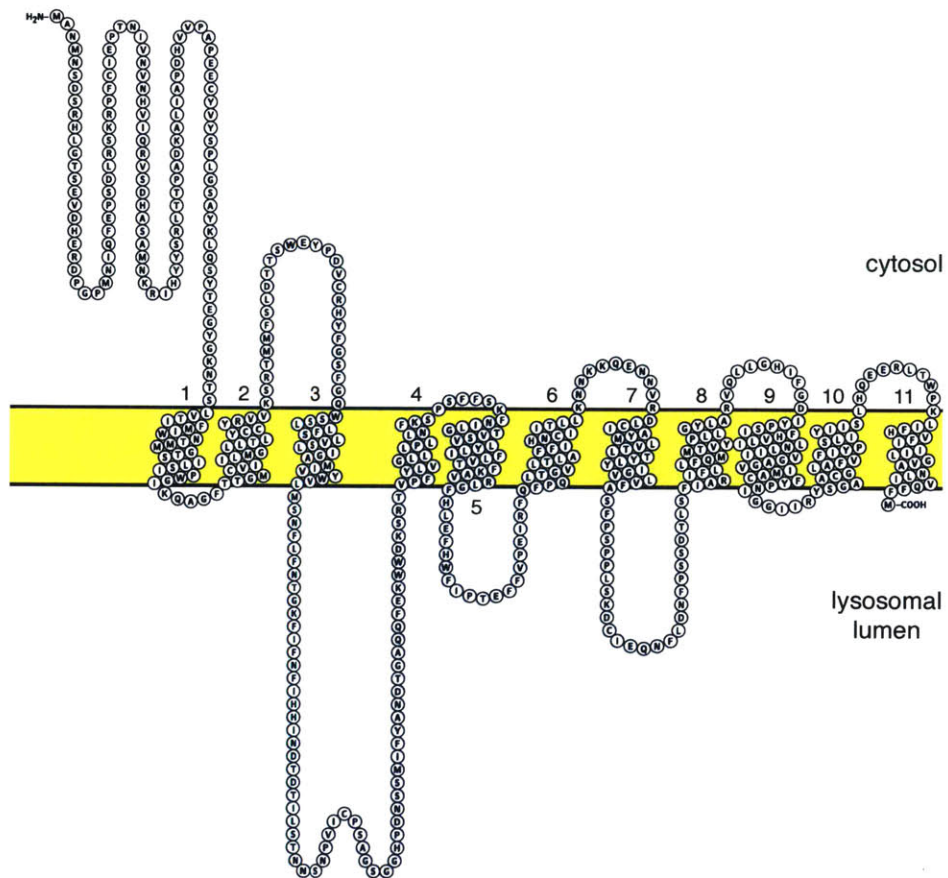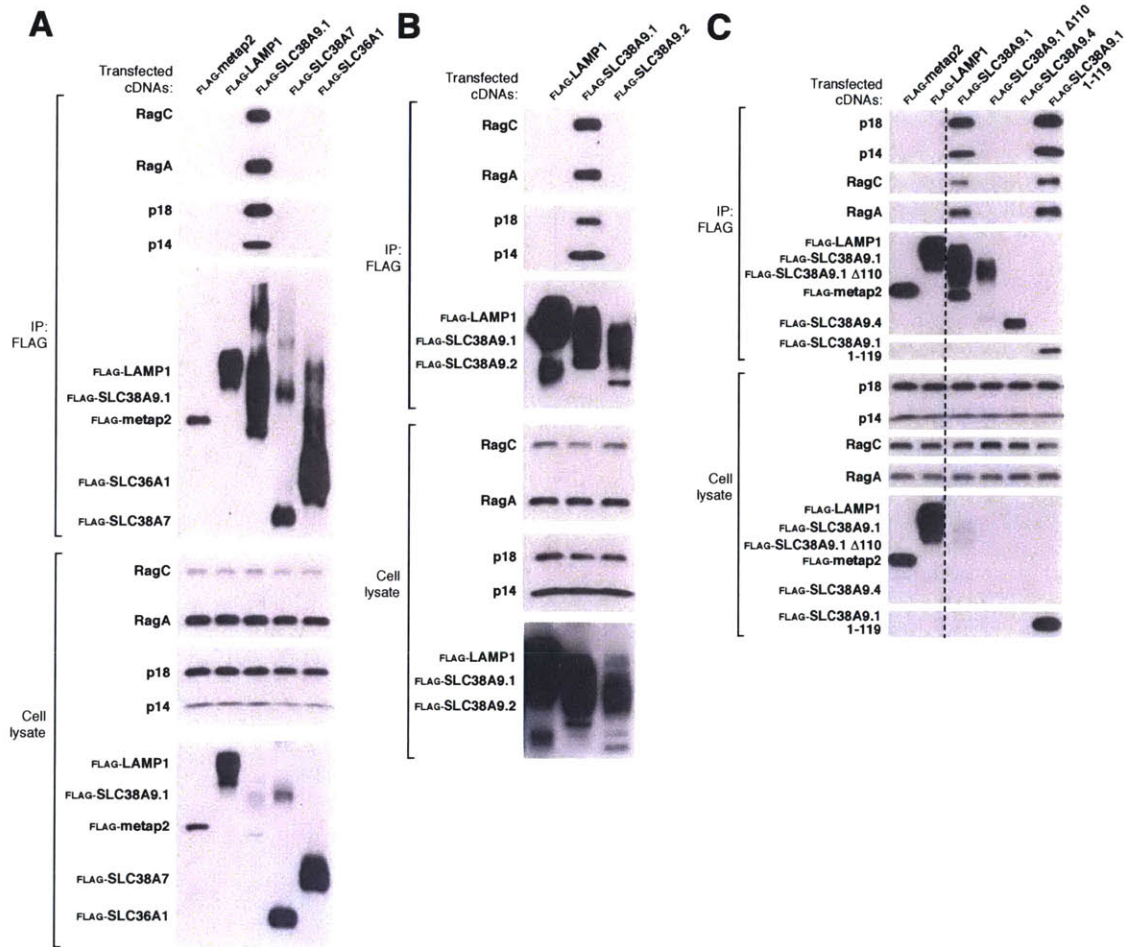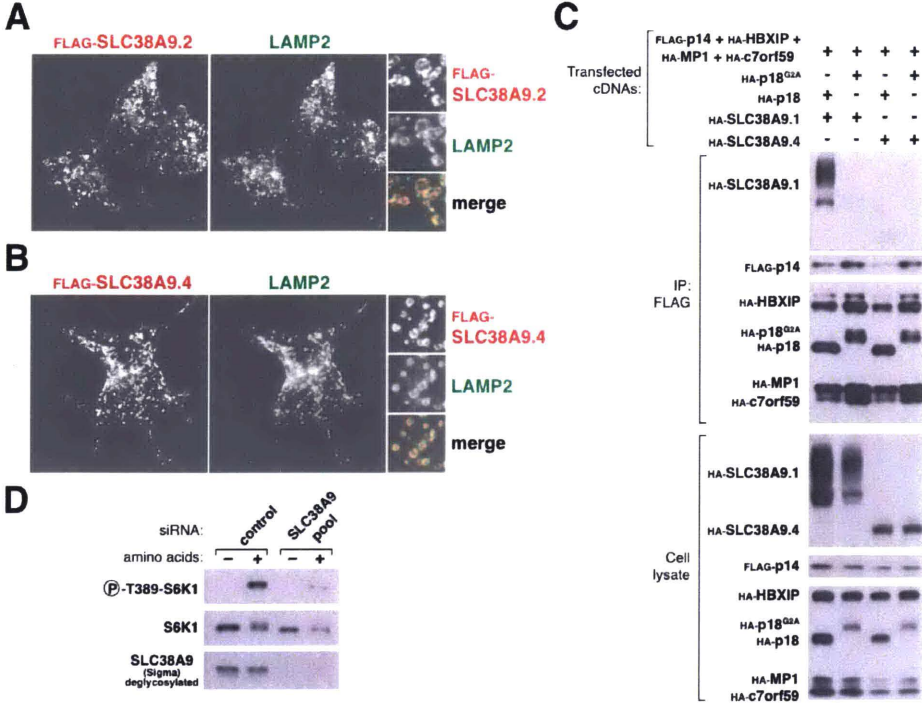
Fig. S1

A

TMHMM output for human SLC38A9.1

B

Fig. S2

Fig. S3

Fig. S4

Fig. S5

Fig. S6

A FLAG-SLC38A9.1

B ['14C]Arg cpm — Transfected cDNA: empty vector, LAMP1, PQLC2, SLC38A9.1 — pH 5

C ['14C]AA mix cpm — LAMP1, SLC38A2, SLC38A9.1 (pH 5), LAMP1, SLC38A2, SLC38A9.1 (pH 8)

D ['14C]Leu cpm — LAMP1, SLC38A9.1 — pH 5

E I_QRL (pA) — p < 0.001, n.s. — Transfected cDNA: empty vector, SLC38A9.1, SLC38A2 — empty vector, SLC38A9.1, SLC38A2 — 15 pA / 2 s

F FLAG-LAMP1, FLAG-SLC38A9.1 — LAMP1 / SLC38A9.1 — 230, 150, 100, 80, 60, 50, 40, 30, 25, 20, 15, 10

H trypsin: − + / +1% Triton − + — FLAG-SLC38A9.1

G fraction from top of sucrose gradient: 1 2 3 4 5 6 7 8 9 10 11 12 — FLAG-SLC38A9.1 protein only — FLAG-SLC38A9.1 + liposomes — FLAG-SLC38A9.1 + liposomes + 6M Urea

I FLAG-SLC38A9.1 proteoliposomes — FLAG-LAMP1 proteoliposomes — ['3H]Arg cpm vs Elution volume (mL) — ['3H]Arg + Buffer only, Liposomes only, SLC38A9.1 proteoliposomes, LAMP1 proteoliposomes
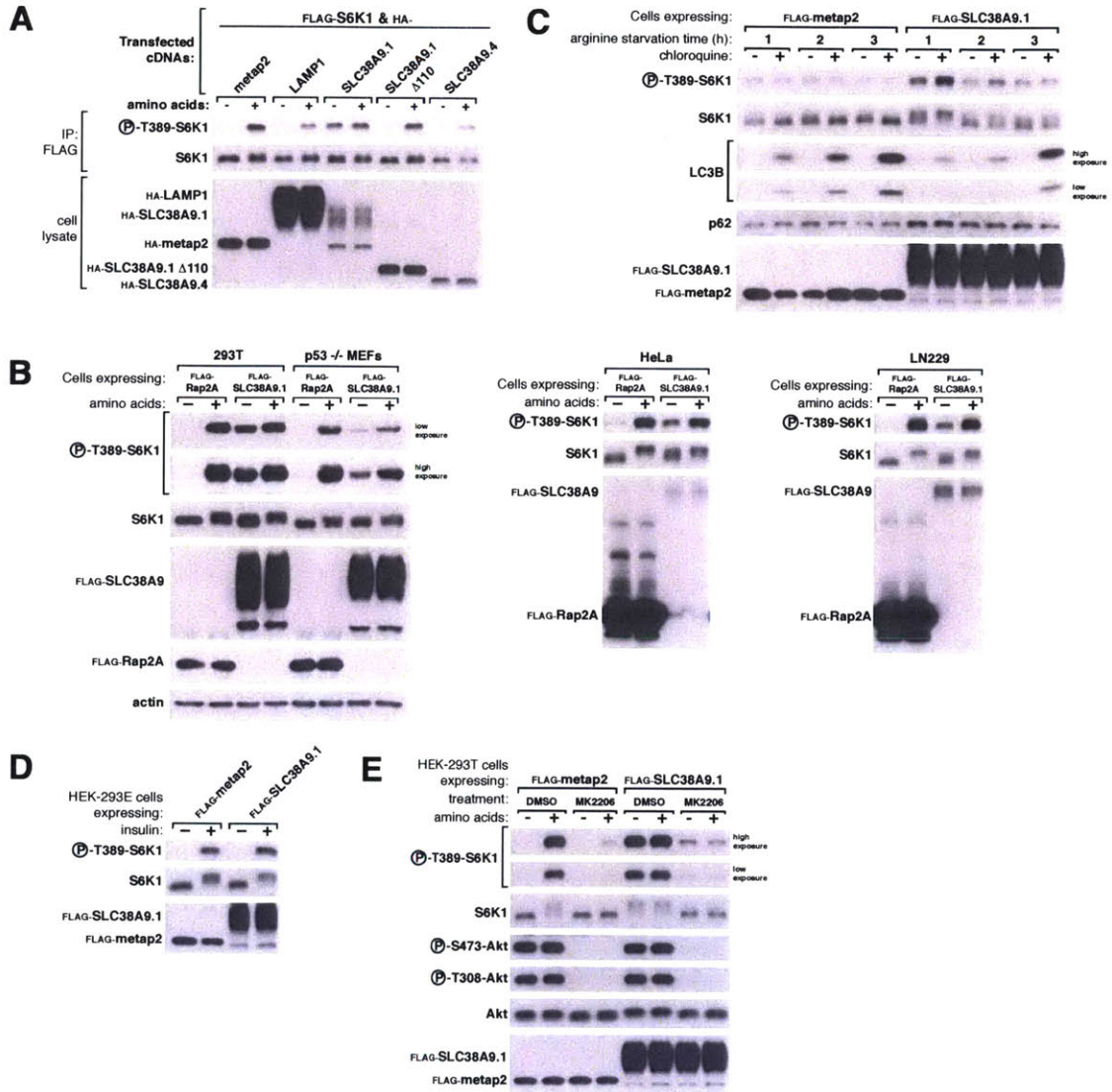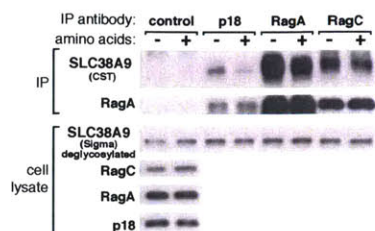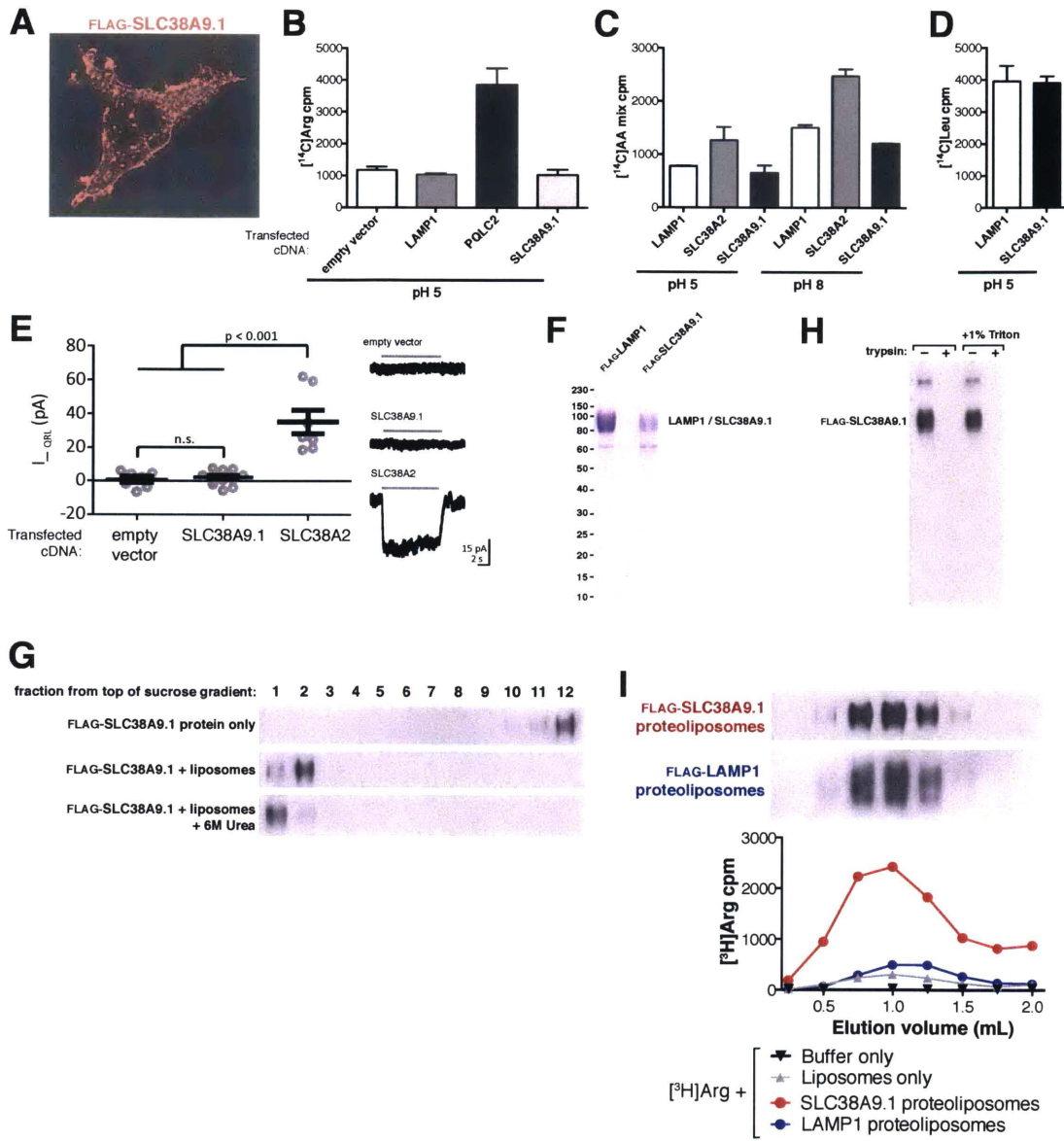
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Chapter 4

Conclusion and Future Directions

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Summary

The mTORC1 pathway is an important regulator of cellular growth and metabolism, responding to the nutrient status of the cell and organism to appropriately balance anabolic and catabolic processes. Although amino acids have long been known to be a major input regulating mTORC1 function, the mechanisms by which they are sensed were nebulous at best. Our lab has been studying this question for the better part of the past decade and, ever since the discovery of the Rag GTPases in 2008, has made steady progress, along with several other research groups, on the identification of proteins responsible for transmitting the amino acid signal to mTORC1. Developments in this area have accelerated dramatically over the past two years, during which the proteins that directly sense amino acids finally came into clearer focus (Fig. 1).

Here, we presented data characterizing the amino acid requirements of HEK-293T cells, the workhorse cell line with which our lab has identified many components of the amino acid signaling pathway. We demonstrated that a mixture of three amino acids – leucine, arginine, and lysine – is sufficient to fully activate mTORC1, which suggests that there are likely to be sensors for these specific amino acids at the top of this signaling cascade.

We also identified SLC38A9 as a Ragulator-interacting protein that putatively acts as an arginine sensor at the lysosomal membrane (Wang, 2014). SLC38A9 has a signaling domain at its N-terminus that when overexpressed is necessary and sufficient to activate mTORC1. It also has a series of 11 transmembrane passes that together act as a transporter for amino acids. Our working model posits that SLC38A9 senses amino acids through its C-terminal transmembrane domain and then relays this signal to its N-terminal signaling domain (Fig. 1b).

Despite the advances in this field over the last several years, including the data presented here, there remain many open questions concerning the detailed mechanisms of as well as the broader logic behind amino acid sensing by the mTORC1 pathway. A few questions closely related to what I presented above are described below.

Fig. 1

(a) Model in 2010   (b) Model in 2016
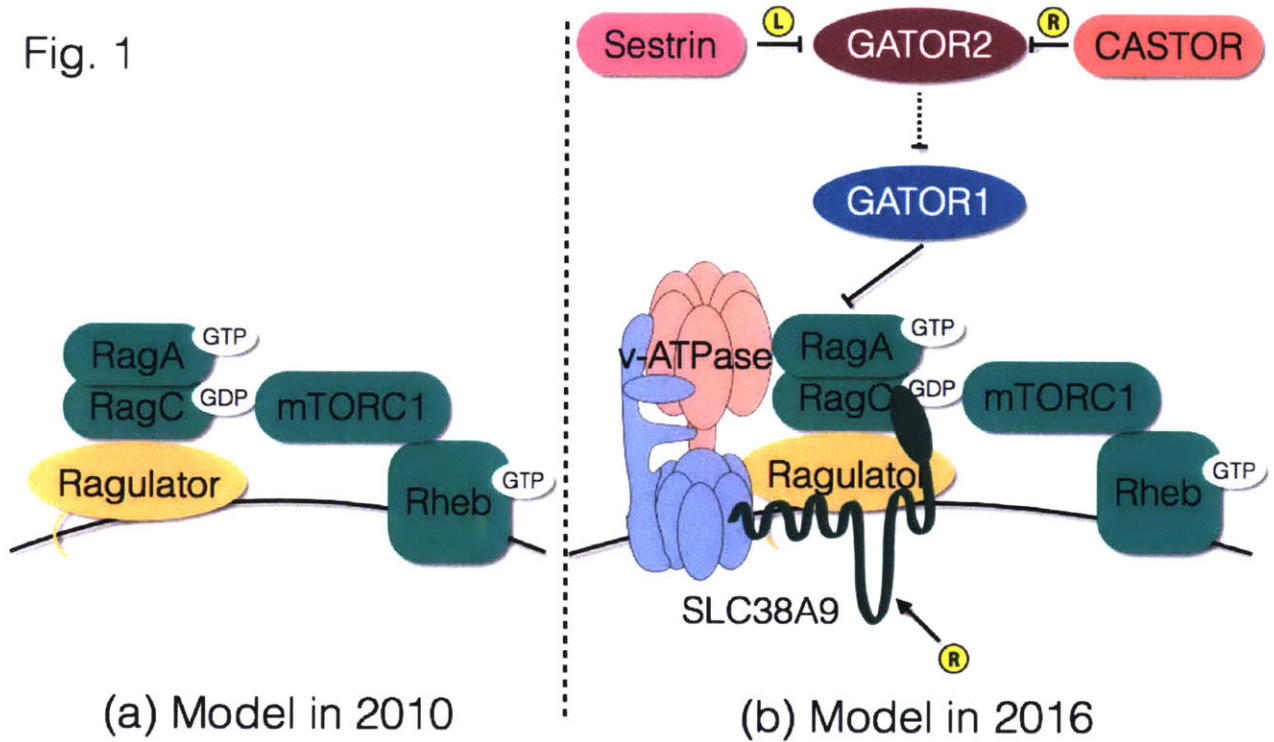
Outstanding Questions
What is the lysine sensor in HEK-293T cells?

We determined a few years ago that a cocktail consisting of leucine, arginine, and lysine is sufficient to fully activate mTORC1 in HEK-293T cells. This finding strongly suggested the possibility that there exist sensors for each of these amino acids – a prediction that has borne out thanks to a suite of recent discoveries from our lab. Since 2015, our lab has found that the Sestrin proteins act as a cytosolic sensor for leucine (Saxton et al., 2016; Wolfson et al., 2016), that the CASTOR proteins act as a cytosolic sensor for arginine (Chantranupong et al., 2016), and that SLC38A9 is a putative arginine sensor at the lysosomal membrane (Wang, 2014).

The identity of the lysine sensor(s), however, remains completely unknown. If prior work from our lab is any indication, a candidate sensor for lysine likely interacts, directly or indirectly, with known players of the amino acid sensing pathway. A careful examination of our proteomics datasets may reveal a protein that signals or interacts with binding partners in a lysine-sensitive fashion. From my preliminary analysis of different cancer cell lines, the requirement for lysine in activating mTORC1 is not nearly as widespread or stringent as that for leucine and arginine, although it is certainly not unique to HEK-293T cells. This may indicate that the expression of the lysine sensor may be more closely restricted to HEK-293T cells as well as other cell types for which lysine matters.

How is SLC38A9 acting as an arginine sensor at the lysosomal membrane?

Why the entire amino acid sensing machinery is anchored at the lysosomal membrane has been an enduring mystery. Most recently, SLC38A9, a protein that appears to signal to mTORC1 through Ragulator and the Rag GTPases, has been shown to localize to the lysosomal membrane independent of its interactions with the remainder of the sensing apparatus. So far, we have found that SLC38A9 has two business ends: its 11-transmembrane passes toward the C-terminal end form a pore that is capable of transporting a wide range of amino acids *in vitro* and its 119-amino acid N-terminal domain can signal directly to Ragulator and the Rag GTPases. Correspondingly, overexpression of either the full-length protein or the N-terminal domain – but not the transmembrane domain – is sufficient to activate mTORC1 signaling. However, the transmembrane domain is required for SLC38A9 to form amino acid-regulated contacts with Ragulator and the Rag GTPases. These data are consistent with a model in which the amino acid signal is detected by the transmembrane domain and then transmitted to the N-terminal domain for further propagation.

This model raises the following basic question: how does SLC38A9 couple its transport activity to its signaling role? One possibility is that amino acid binding and/or transport by the transmembrane domain can induce a global conformational change that renders the N-terminal domain capable of signaling. This hypothesis would be strengthened if transport-dead mutants were to fail at rescuing the signaling defect of SLC38A9-null cells. Alternatively, depending on the affinity of SLC38A9 for amino acids and their concentrations on either side of the lysosomal membrane, SLC38A9-mediated transport may shift key amino acid pools across the lysosomal membrane in a manner that promotes mTORC1 signaling. Interrogating this hypothesis would require the development of an improved liposome-based transport assay that can query both influx and efflux by SLC38A9 as well as a technique to measure the amino acid content of intact lysosomes in SLC38A9-replete and SLC38A9-null cells under different treatment conditions.

The ability to determine the concentrations of specific amino acids in key intracellular compartments may also shed some light on the following dilemma: how can one reconcile SLC38A9's broad substrate specificity in *in vitro* transport assays with the arginine-specific requirement of the mTORC1 pathway? It is possible that arginine is found at particularly high concentrations in the lysosome – a finding that has indeed been documented in yeast vacuoles

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(Harms, Gochman, & Schneider, 1981) – and thus plays a prominent role in signaling. Another possibility is that SLC38A9 may have multiple amino acid binding sites, perhaps one that is specific for arginine and another that is permissive for a broader range of amino acids. In this scenario, arginine acts as the requisite gatekeeper that enables the transport of other amino acids. Indeed, the LeuT protein, a bacterial transporter with structural homology to SLC38A9, has been proposed to have two amino acid binding sites based on computational modeling and binding studies (Shi, Quick, Zhao, Weinstein, & Javitch, 2008; Zhao et al., 2011). Confirmation of this hypothesis would require a crystal structure of SLC38A9 and the testing of structure-informed mutants.

How does SLC38A9 affect Ragulator and Rag GTPase activity?

At the time of its discovery, the Ragulator complex was initially reported to be a scaffold to anchor the Rag GTPases at the lysosomal membrane. Follow-up work proposed that Ragulator acts as a GDP exchange factor (GEF) for RagA/B, although the molecular basis for this function was elusive. Indeed, it now appears that the biochemical functions of these protein complexes are not completely settled. What makes this question challenging to address is that the Rag GTPases are an obligate heterodimer in which each half can theoretically not only receive independent inputs but also influence each other. Bar-Peled *et al.* first reported the use of mutants in which the nucleotide binding site on one half of the Rag heterodimer was altered to prefer xanthosine over guanosine. This allows the activity of both GTPases to be independently monitored *in vitro*, as they use different nucleotide substrates. Careful measurement and calculation of the on-rates, off-rates, binding affinities, and hydrolysis rates of different nucleotides should yield a more nuanced picture of the function and regulation of the Rag GTPases. A crystal structure of Gtr1/2 (Gong et al., 2011; Zhang, Peli-Gulli, Yang, De Virgilio, & Ding, 2012), the yeast orthologs of the mammalian Rag GTPases, already provides a helpful starting point to test specific mechanistic hypotheses, although crystal structures of their mammalian counterparts captured in different nucleotide binding configurations would no doubt facilitate the testing of more precise hypotheses.

Laying this robust foundation is important for all subsequent studies of Rag GTPase regulation, including by SLC38A9. SLC38A9 binds Ragulator as well as the Rag GTPases through Ragulator-independent contacts, collectively forming an eight-member supercomplex.

Deletion of either SLC38A9 or Ragulator appears to disrupt the stability of this supercomplex (data not shown). In cells, the signaling effect of SLC38A9 depends on the presence of Ragulator, although the trivial explanation for this finding is simply that proper Rag GTPase function itself depends on the presence of Ragulator. To uncover a regulatory role for SLC38A9 toward Ragulator and the Rags at the molecular level, it will be necessary to reconstitute this supercomplex *in vitro*. Since the signaling portion of SLC38A9 is its soluble N-terminal domain, reconstitution with this fragment – rather than with the much less wieldy full-length protein – would be expected to yield helpful hints. Biophysical assays designed in the vein above may enable differentiation between the following possible mechanisms: 1) SLC38A9 may alter Ragulator's activity toward the Rags, 2) SLC38A9 may directly impact Rag function, and 3) SLC38A9 may directly affect Rag function if and only if Ragulator has already acted.

Besides SLC38A9, the v-ATPase is the other lysosome-bound complex upstream of Ragulator that is required for the activation of mTORC1 by amino acids. Chemical inhibition of v-ATPase hydrolysis using concanamycin A partially suppresses the pathway hyperactivation associated with SLC38A9 overexpression (Wang, 2014). This suggests that SLC38A9 and the v-ATPase may employ different mechanisms and may act in parallel, rather than in series, toward Ragulator and the Rag GTPases. Lastly, it is not clear if the v-ATPase recognizes and transmits the signal of a specific amino acid, as SLC38A9 is thought to do.

Why do cells need both lysosomal and cytosolic sensing? Is there interplay between these two branches?

It has become increasingly apparent that eukaryotic organisms have evolved mechanisms to monitor amino acid availability in the cytosol as well as within the lysosome. The Sestrin and CASTOR proteins detect cytosolic leucine and arginine, respectively, and signal through GATOR2 and GATOR1 complexes to modulate RagA/B GTP hydrolysis (Fig. 1b). SLC38A9 and the v-ATPase, by contrast, are lysosome-bound proteins that transmit the amino acid signal through the Ragulator complex (Fig. 1b). The Rag GTPases are the point of convergence for these two branches, and under most physiological circumstances, signals from both branches are likely concordant. It is not obvious, however, how the Rags would process two discordant inputs – or even if this scenario ever arises. A better understanding of this might clarify how the two halves of a Rag heterodimer influence each other (and vice versa) and whether or not one half

ever dominates the other. A series of epistasis experiments involving both branches – for example, GATOR1 and SLC38A9 double knockout or GATOR1 knockout with v-ATPase inhibition or GATOR2 knockout and SLC38A9 overexpression – can simulate these circumstances, particularly when conducted over a wide range of nutrient conditions. There may also be feedback loops built into these branches that complicate interpretations of epistasis experiments.

It would be interesting and informative to catalog the expression levels of all genes in the amino acid sensing pathway across cell and tissues types in a systematic fashion. This analysis could even be extended across different species. We might find, for instance, that low expression of GATOR1, Sestrins, or CASTOR in certain cell types may result in the lysosomal branch delivering the dominant amino acid signal. Moreover, differential expression of sensors or their isoforms may contribute to the distinct amino acid requirements observed in different cell types.

The growing list of amino acid sensors hints at a complex map of interconnected pathways, each likely with different weights depending on the cell type and species under consideration. Achieving a better understanding of the amino acid sensing logic behind this complexity, particularly across heterogeneous tissues, will be a goal for years to come.

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