Discovering regulators of the amino acid sensing pathway of mTORC1

by

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Dedicated to my parents
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ABSTRACT

The mechanistic target of rapamycin complex I (mTORC1) protein kinase functions as a master regulator of growth, and its deregulation is common in human disease, including cancer and diabetes. mTORC1 integrates multiple environmental cues to control anabolic and catabolic processes. A key input is amino acids, which function to promote the translocation of mTORC1 to the lysosomal surface, its site of activation. Necessary for this recruitment are the Rag GTPases and several distinct factors that modulate their nucleotide state in response to amino acid availability. Despite these advances, several key questions remain. The components that mediate mTORC1 inhibition upon amino acid deprivation and the identities of the amino acid sensors upstream of mTORC1 are both unknown.

To provide insight into these questions, we undertook an unbiased proteomics approach to discover novel mTORC1 regulators. Here, we describe the identification of GATOR2 as a pentameric complex that positively regulates mTORC1 and functions upstream of or in parallel to GATOR1, a GTPase activating protein complex for the Rags and a negative regulator of the mTORC1 pathway. KICSTOR, a four-membered protein complex, is necessary to localize GATOR1 to the lysosome to enable it to suppress mTORC1 activity. GATOR1 components are mutated in cancer and may identify tumors that respond to clinically approved mTORC1 inhibitors. Furthermore, we describe the identification of Sestrin2 and CASTOR1 as GATOR2-interacting proteins that function as leucine and arginine sensors, respectively, for the mTORC1 pathway. Both sensors are required to signal the absence of leucine and arginine to mTORC1, and the amino acid-binding capacity of both sensors is necessary for amino acids to activate mTORC1. Altogether, the identification of these mTORC1 regulators furthers our understanding of the mechanisms by which amino acid availability controls cellular growth.
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CHAPTER 1

INTRODUCTION

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This section is partially adapted from the following publication:
Nutrient-sensing mechanisms throughout evolution. Chantranupong L*, Wolfson RL*, Sabatini

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Nutrient availability strongly influences the growth of all organisms and starvation conditions can alter developmental programs in both unicellular and multicellular organisms (Oldham, 2000). When faced with nutritional limitation, *S. cerevisiae* exit the mitotic cycle and enter a stationary phase (Zaman et al., 2008), *C. elegans* persist for several months in a state of stasis known as dauer larvae (Klass and Hirsh, 1976) and *Drosophila* postpone their development (Edgar, 2006).

Despite the diversity of these organisms, one common pathway, anchored by the target of rapamycin (TOR) serine/threonine protein kinase, regulates entry into these alternative states in response to environmental cues. The TOR pathway is unique in that it integrates many environmental cues, including nutrient, energy, and growth factor levels, as well as multiple forms of stress, to regulate key anabolic and catabolic processes in cells (Howell et al., 2013; Kim et al., 2013; Laplante and Sabatini, 2012). Here, we focus on how one critical input - nutrients - controls the mTOR pathway.

1. Discovery of mTOR

The study of TOR began several decades ago with the isolation of a potent antifungal compound from the soils of Rapa Nui, more commonly known as Easter Island. This bacterially-produced macrolide, named rapamycin in deference to its site of discovery, garnered clinical and research interest because of its powerful anti-proliferative effects on yeast and mammals and potent immunosuppressive activity in humans (Morris, 1992; Segall et al., 1986). Genetic studies in yeast led to the identification of TOR1 and TOR2 as key genes mediating rapamycin sensitivity (Cafferkey et al., 1993; Kunz et al., 1993). Subsequent biochemical work in mammals revealed the mTOR (mechanistic target of rapamycin) protein as the direct target of rapamycin (Brown et al., 1994; Sabatini et al., 1994; Sabers et al., 1995).

2. The TOR pathway is evolutionarily conserved from yeast to mammals

Yeast, unlike most eukaryotes encode two different TOR proteins, Tor1 and
Tor2, which nucleate distinct multi-protein complexes (Helliwell et al., 1994; Loewith et al., 2002). TOR complex 1 (TORC1) consists of Tor1 bound to Kog1, Lst8, and Tco89 and promotes ribosome biogenesis and nutrient uptake under favorable growth conditions. Inhibition of TORC1 by nutrient starvation or rapamycin treatment, leads to the activation of macroautophagy and nutrient and stress-responsive transcription factors like GLN3, which is required for the use of secondary nitrogen sources (Jacinto et al., 2004; Wullschleger et al., 2006). TORC2 contains Tor2 bound to Avo1-3, Bit61, and Lst8, is largely rapamycin insensitive, and is thought to regulate spatial aspects of growth, such as cytoskeletal organization (Loewith et al., 2002; Reinke et al., 2004; Wedaman et al., 2003).

Mammals also have two mTOR-containing complexes but unlike yeast, only one gene encoding mTOR. mTOR complex 1 (mTORC1) consists of mTOR, regulatory-associated protein of mammalian target of rapamycin (raptor), mammalian lethal with sec-13 protein 8 (mLST8), proline-rich Akt substrate 40 kDa (PRAS40), and DEP-containing mTOR-interacting protein (DEPTOR) (Laplante and Sabatini, 2012). The mTORC1 kinase complex modulates mass accumulation through the control of many anabolic and catabolic processes, including protein, lipid, and nucleotide synthesis; energy metabolism; lysosome biogenesis; and autophagy (Laplante and Sabatini, 2012). The mechanisms by which mTORC1 regulates these processes will be discussed in further detail below.

Like mTORC1, mTORC2 also consists of mTOR, mLST8, and DEPTOR. In addition, it contains unique components rapamycin-insensitive companion of mTOR (rictor), mammalian stress-activated map kinase-interacting protein 1 (mSIN1), and protein observed with rictor 1 and 2 (Protor1/2) (Laplante and Sabatini, 2012). mTORC2 controls cell proliferation, cell survival, and actin cytoskeletal organization and migration (Jacinto et al., 2004; Oh and Jacinto, 2011; Sarbassov et al., 2004). Of the two mTOR complexes, the regulation and output of mTORC1 is more completely understood.
3. mTORC1 controls diverse anabolic and catabolic processes

mTORC1 integrates diverse environmental inputs to control the balance of anabolic and catabolic processes in the cell. When nutrients are plentiful, mTORC1 promotes the accumulation of mass and thus cellular growth, but upon nutrient starvation, mTORC1 must conserve resources until conditions are more favorable.

3.1 Translation

Translation, an energy and resource intensive process, is a major downstream pathway under extensive mTORC1 control. Reflecting the importance of mTORC1 in regulating translation, complete mTORC1 inhibition by the specific kinase inhibitor Torin1 suppresses global protein synthesis by 65% (Thoreen et al., 2012). The initiation of cap-dependent translation requires the binding of eIF4E, an essential initiation factor, to the m\(^7\)GpppN cap of the mRNA. The eukaryotic translation initiation factor 4E 1 (4EBP1) is a major inhibitor of eIF4E. Under mTORC1 inhibition, 4EBP1 is hypophosphorylated and binds to eIF4E, which prevents eIF4E from binding to the mRNA cap (Ma and Blenis, 2009). Under normal growth conditions, translation resumes when mTORC1 phosphorylates 4EBP1 and inhibits its ability to sequester eIF4E (Mamane et al., 2006).

In addition to directly controlling the initiation of cap-dependent translation, mTORC1 also regulates other aspects of protein synthesis. mTORC1 phosphorylates and activates S6 kinase 1 (S6K1), which in turn phosphorylates diverse translation regulators. These include S6, a protein that resides in the 40S subunit of the ribosome and modulates ribosome function through incompletely understood mechanisms, S6K1 Aly/REF-like substrate (SKAR) and 80 kDa nuclear cap-binding protein (CBP80), which both regulate mRNA processing, programmed cell death 4 (PDCD4) and eIF4B, which regulate cap-dependent translation initiation in parallel with 4EBP1, and eEF2 kinase, which controls translation elongation (Magnuson et al., 2012).
Furthermore, mTORC1 also promotes ribosomal RNA (rRNA) synthesis by phosphorylating and activating the regulatory element tripartite motif-containing protein-24 (TIF-1A), which promotes its interaction with RNA Polymerase I (Pol I) (Mayer, 2004). Conversely, mTORC1 phosphorylates and inhibits the Pol III repressor Maf1 to promote the production of 5S rRNA and transfer RNA (tRNA) (Kantidakis et al., 2010; Shor et al., 2010). It is clear that the mTORC1 pathway plays a key role in regulating the capacity of the cell to synthesize new proteins.

3.2 Lipid Synthesis, cellular metabolism and ATP production

A growing cell must not only accumulate mass, but it must also accumulate lipids to generate more membranes (Laplante and Sabatini, 2009). mTORC1 acts through multiple substrates to ensure that an increase in de novo synthesis of lipids coincides with cellular growth. The sterol regulatory element-binding protein 1/2 (SREBP1/2) transcription factors directly control the expression of fatty acid and cholesterol biosynthetic genes. Initially synthesized as inactive precursors, SREBP1/2 localize to the ER where a two-step proteolytic cleavage event frees an N-terminal fragment, which translocates to the nucleus to upregulate lipogenic genes (Rawson, 2003). mTORC1 positively regulates the expression and proteolytic processing of SREBP1/2 through multiple mechanisms, which includes the activation of S6K1 (Düvel et al., 2010; Li et al., 2010; Porstmann et al., 2008; Wang et al., 2011) and the inhibition of Lipin-1, a negative regulator of SREBP1/2 (Peterson et al., 2009). Finally, mTORC1 promotes the expression and activity of peroxisome proliferator-activated receptor γ (PPAR-γ), the master regulator of adipogenesis (Kim and Chen, 2004; Zhang et al., 2009).

To support these anabolic processes, mTORC1 signaling must also stimulate metabolic processes such as glucose uptake, glycolytic flux, and NADPH and ATP production, which is accomplished predominantly at the transcriptional level (Dibble and Manning, 2013). mTORC1 upregulates the activity and levels of hypoxia inducible factor 1α (HIF1α) to induce the expression of glycolytic enzymes and glucose transporters. Furthermore, mTORC1 activates the transcription factor Ying-Yang 1 to increase
mitochondrial DNA content and oxidative metabolism (Cunningham et al., 2007). Finally, mTORC1 promotes NADPH production by increasing the expression of genes in the oxidative, NADPH-producing branch of the pentose phosphate pathway (Düvel et al., 2010).

3.4 Protein Catabolism

3.4.1 Autophagy

Autophagy is a major intracellular degradation pathway that is under direct mTORC1 control. During autophagy, cytoplasmic proteins and organelles are engulfed in bulk by double membrane structures termed autophagosomes, and delivered to the lysosome where they are degraded by resident proteases (Jung et al., 2009; Laplante and Sabatini, 2012). Autophagy not only rid the cell of damaged organelles, but it also recycles essential cellular building blocks such as amino acids. In mammals, mTORC1 negatively regulates autophagy through multiple mechanisms. mTORC1 directly suppresses unc-51-like kinase 1 (ULK1), which is a core component of a kinase complex that includes mammalian autophagy-related gene 13 (Atg13) and focal adhesion kinase family-interacting protein of 200 kDa (FIP200), all of which are required to initiate autophagy (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009). In addition, mTORC1 activation also blocks the nuclear entry of the transcription factor EB (TFEB), which is a master regulator of lysosome and autophagosome biogenesis and fusion (Settembre et al., 2012).

3.4.2 Ubiquitin-Proteasome system

In addition to autophagy, the ubiquitin-proteasome system is another major route of protein degradation in cells. A multienzymatic cascade first conjugates ubiquitin - a small polypeptide - to proteins, and this tag is subsequently recognized by the proteasome, a protein complex of over 2.5 MDa that breaks down ubiquitylated proteins into heterogeneous peptide mixtures (Finley, 2009). It is clear the TORC1 controls the
proteasome, but the relationship between TORC1 and the control of proteasome function appears complex, as TORC1 acts through multiple mechanisms that depend on the particular cell context. While some studies find that acute, pharmacological mTORC1 inhibition in both yeast and mammalian cells upregulates protein degradation through the proteasome (Rousseau and Bertolotti, 2016; Zhao et al., 2015), another study reveals that there are nuances in the regulation of the proteasome by mTORC1. Under genetic hyperactivation of the mTORC1 pathway, the transcription factor NRF1 mediates an increase in proteasome and intracellular amino acid levels (Zhang et al., 2014). Further studies are needed to clarify the role of mTORC1 in regulating the proteasome.

4. Regulation of mTORC1 activity

Of the multiple environmental inputs that regulate mTORC1 activity, nutrients and growth factors are absolutely required for mTORC1 activity, even in the presence of other activating stimuli. Here, we describe the mechanisms by which nutrients regulate the mTORC1 pathway.

4.1 Discovery of nutrient regulation of mTORC1 activity

The connection between TORC1 and the response to the nutritional state emerged from observations in S. cerevisiae, D. melanogaster, and mammalian cells where TOR inhibition leads to phenotypes akin to those observed under starvation (Barbet et al., 1996; Oldham, 2000; Peng et al., 2002; Zhang et al., 2000). Environmental amino acid levels were found to activate the mTORC1 pathway as measured by the phosphorylation of S6K1 and 4EBP1, two well-known mTORC1 substrates (Hara, 1998; Wang et al., 1998), and to signal independently of the growth factor input to mTORC1 (Hara, 1998; Nobukuni et al., 2005; Roccio et al., 2005; Smith et al., 2005; Svanberg and Moller-Loswick, 1996; Wang et al., 1998).

More recent work showing that the Rag GTPases are necessary and sufficient
for mTORC1 to sense amino acids (Kim et al., 2008; Sancak et al., 2008) is beginning to reveal the logic of how the pathway integrates inputs from nutrients and growth factors. What has emerged is a bipartite mechanism of mTORC1 activation involving two distinct small GTPases: first, the control of mTORC1 subcellular localization by nutrients through the Rag GTPases, and, second, the control of mTORC1 kinase activity by growth factors and energy levels through the Rheb GTPase (Zoncu et al., 2011). Both inputs are needed for full activation of mTORC1 as in the absence of either the pathway is off.

4.2 Growth factors regulate the nucleotide state of Rheb, an essential mTORC1 kinase activator

Growth factors and energy levels regulate the Rheb input to mTORC1 (Inoki et al., 2003; Long et al., 2005) through a heterotrimeric complex comprised of the tuberous sclerosis complex (TSC) proteins, TSC1, TSC2, and TBC1D7, which together function as a GTPase activating protein (GAP) for Rheb, which functions as an essential kinase activator for mTORC1 that resides on the lysosomal surface (Brugarolas et al., 2004; Dibble et al., 2012; Garami et al., 2003; Inoki et al., 2003; Long et al., 2005; Sancak et al., 2008; Saucedo et al., 2003; Stocker et al., 2003; Tee et al., 2002). Growth factors control the localization of TSC on the lysosome (Menon et al., 2014). When present, the TSC complex dissociates from the lysosomal surface and enables Rheb to remain GTP-loaded and capable of activating mTORC1 kinase activity. Conversely, upon growth factor deprivation, TSC is recruited to the lysosome to inhibit the mTORC1 pathway (Menon et al., 2014).

4.2 Amino acids control mTORC1 localization to the lysosome

In order for Rheb to activate mTORC1, mTORC1 must interact with Rheb on the lysosomal surface. The function of nutrients is to control the translocation of mTORC1 to the lysosome. Central to this regulation are the Rag GTPases, which exist as heterodimers of the related and functionally redundant RagA or RagB bound to RagC or
Figure 1

A

B

Figure 1
RagD, which are also very similar (Hirose et al., 1998; Schürmann et al., 1995; Sekiguchi et al., 2001). Under amino acid starvation, RagA/B is GDP-bound while RagC/D is GTP-bound. Under nutrient replete conditions the nucleotide state of the Rag GTPases switch, and the Rags are competent to bind to mTORC1 and promote its recruitment to the lysosomal surface where its activator Rheb also resides (Buerger et al., 2006; Saito et al., 2005; Sancak et al., 2008) (Figure 1). The function of each Rag within the heterodimer is poorly understood and their regulation is undoubtedly complex as many distinct factors play key roles.

4.2.1 Regulation of the Rag GTPases

A lysosome-associated molecular machine consisting of the Ragulator and vacuolar ATPase (v-ATPase) complexes regulates the Rag GTPases and is necessary for the sensing of amino acids by mTORC1 (Sancak et al., 2010; Zoncu et al., 2011). Ragulator is comprised of five proteins: p18, p14, MP1, HBXIP, and c7orf59. It is necessary to localize the Rags to the lysosome, and it is required for the activation of mTORC1 by amino acids. Forced targeting of mTORC1 to the lysosomal surface renders the pathway insensitive to amino acid levels independent of Rags and Ragulator, but not of Rheb function. Ragulator does not simply function as a lysosomal

Figure 1: Nutrient Sensing by the mTORC1 Pathway

(A) In the absence of amino acids and growth factors, mTORC1 is inactive. This is controlled by two separate signaling pathways. First, in the absence of amino acids, Sestrin1/2 and CASTOR1 bind and inhibit GATOR2. GATOR1 is an active GAP toward RagA, causing it to become GDP bound. In this state, mTORC1 does not localize to the lysosomal surface. Second, in the absence of insulin or growth factors, TSC is an active GAP toward Rheb, localizes to the lysosomal surface, and stimulates Rheb to be GDP bound.

(B) In the presence of amino acids and growth factors, mTORC1 is active. Lysosomal amino acids signal through SLC38A9 to activate the amino acid sensing branch. Ragulator is active, causing RagA to be GTP bound. This binding state is reinforced by the fact that GATOR1 is inactive in the presence of amino acids, as GATOR2 inhibits it following the dissociation of Sestrin1/2 and CASTOR1 upon binding to leucine and arginine, respectively. The Rag heterodimer in this nucleotide conformation state recruits mTORC1 to the lysosomal surface. In addition, the presence of growth factors activates a pathway that inhibits TSC, causing it to dissociate from the lysosomal surface, and leaving Rheb GTP bound. In this state, Rheb activates mTORC1 when it translocates to the lysosomal surface.
scaffold for the Rags, but also modulates the nucleotide state of RagA/B, potentially as a guanine exchange factor (GEF) (Bar-Peled et al., 2012). Upon amino acid stimulation, Ragulator promotes the exchange of GDP for GTP on RagA/B (Figure 1), putting it in a state to bind and recruit mTORC1 to the lysosomal surface. Reflecting the importance of Ragulator in controlling the mTORC1 pathway, severe growth defects are seen in individuals with mutations that lead to reduced p14 levels (Bohn et al., 2007). While the role of Ragulator in the pathway is well defined, the 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. GATOR1 is a trimeric complex of DEPDC5, Nprl2, and Nprl3 and acts on RagA/B (Bar-Peled et al., 2013) while Folliculin-FNIP2 acts on RagC/D (Tsun et al., 2013). When amino acids are present, GATOR1 is inactive while Folliculin-FNIP2 is active (Figure 1). Altogether, this enables RagA/B to be GTP-loaded and RagC/D to be GDP-loaded, and in a state that is competent to bind and recruit mTORC1 to the lysosomal surface.

Finally, a distinct complex called GATOR2 negatively regulates GATOR1 through mechanisms that remain to be defined (Bar-Peled et al., 2013). GATOR2 is comprised of five proteins (mios, WDR24, WDR59, sec13, and seh1L) initially identified as an interacting partner of GATOR1. The identification and characterization of both GATOR complexes in mammalian cells will be described in detail in Chapters 2 and 7.

4.3 Sensing of amino acids

While many components of the nutrient-sensing pathway upstream of mTORC1 have been identified, the identity of the amino acid sensor(s) remained elusive until recently. Amino acid sensing could initiate from the extracellular, cytosolic, or lysosomal compartments. The presence of many mTORC1 pathway components on the lysosome suggests that this organelle is more than simply a scaffold surface for mTORC1 activation. Rather, there is the intriguing possibility that lysosomes act as storage sites.
for amino acids, and that amino acid availability within this compartment is sensed by mTORC1. The storage of nutrients in vacuoles, which is established in yeast, may also occur in mammalian cells as some studies suggest that certain amino acids, like arginine, are highly enriched in lysosomes relative to the cytosol (Harms et al., 1981). A cell free assay revealed that the lysosome itself contains the minimal machinery needed for the amino acid-mediated recruitment of mTORC1 to the lysosomal surface (Zoncu et al., 2011).

In an “inside-out” model of sensing, a lysosome-based transmembrane protein would be an alluring candidate amino acid sensor. One such protein is SLC38A9, a newly identified Ragulator-interacting amino acid transporter that resides in the lysosomal membrane and is required for arginine sensing by mTORC1 (Rebsamen et al., 2015; Wang et al., 2015). SLC38A9 contains a N-terminal extension that appears necessary for the downstream signaling event (Bernard and André, 2001; Wang et al., 2015). In cells lacking SLC38A9 the mTORC1 pathway has a selective defect in sensing arginine, suggesting that SLC38A9 is an attractive candidate to be an arginine sensor (Wang et al., 2015). While the mechanism through which SLC38A9 regulates the mTORC1 pathway remains unknown, this transporter is the best candidate so far identified for reporting the contents of lysosomes to mTORC1 in the cytosol.

It is very likely that in addition to sensing lysosomal amino acids, mTORC1 will be found to also sense cytosolic amino acids and integrate information from both amino acid pools. Indeed, Sestrin1/2 and CASTOR1 have been identified as the cytosolic leucine and arginine sensors, respectively, for the mTORC1 pathway. Their identification and characterization will be discussed in detail in Chapters 3-6.

4.4 Nutrient regulation of TORC1 in yeast

More recent evidence indicates that TORC1 is involved in amino acid signaling in yeast (Binda et al., 2009; De Virgilio and Loewith, 2006). TORC1 resides on the vacuole, the equivalent of the metazoan lysosome, although it does not shuttle on and
off its surface in response to nutrient levels as it does in mammals (Binda et al., 2009). Homologs of the Rag GTPases, Gtr1 and Gtr2, exist in yeast and they associate with a vacuolar docking complex consisting of Ego1 and Ego3, which has some structural similarity to Ragulator (Bun-Ya et al., 1992; Dubouloz et al., 2005; Gao and Kaiser, 2006; Kogan et al., 2010). Yeast also have GATOR1 and GATOR2 equivalents, called SEACIT and SEACAT (Panchaud et al., 2013a, b). SEACIT has been proposed to inhibit TORC1 in response to deprivation of sulfur-containing amino acids, such as methionine and cysteine, and controls glutamine synthesis and consumption (Laxman et al., 2014; Laxman et al., 2013; Sutter et al., 2013).

Although TORC1 has been posited to respond to amino acids, constitutively active Gtr1 does not make the TORC1 pathway completely resistant to leucine deprivation, unlike constitutively active RagA/B, which in mammals makes mTORC1 signaling resistant to total amino acid deprivation (Binda et al., 2009; Sancak et al., 2008(Efeyan et al., 2012). Furthermore, the Gtr GTPases are dispensable for growth on glutamine or ammonium (Stracka et al., 2014) and constitutively active Gtr1 fails to rescue the TORC1 signaling defect under ammonium deprivation (Binda et al., 2009). If amino acids signal to TORC1, the mechanisms of its activation are likely to be distinct from those through which amino acids activate mTORC1. For instance, orthologs of Sestrins do not exist in yeast, suggesting divergence in the regulation of the upstream components of the nutrient-sensing pathway.

While in mammals, growth is intimately linked to amino acid availability, yeast are more concerned with the quality and abundance of nitrogen and can uptake and metabolize a host of nitrogen sources, including amino acids which are deaminated to yield ammonia which will rapidly become ammonium in the cell. In yeast, the actual intracellular signal for TORC1 remains less clear (Broach, 2012). Early studies showed that TORC1 is a major regulator of the nitrogen catabolite repression program (Hardwick et al., 1999; Shamji et al., 2000), although later work emphasizes that TORC1 is likely not the sole player regulating this pathway (Broach, 2012). Further studies are needed to ascertain whether TOR is involved in the sensing of an as yet
unidentified nitrogen source in yeast.

5. GCN2: a parallel amino acid sensing pathway

In addition to the TORC1/mTORC1 pathway, eukaryotes evolved a parallel pathway to detect intracellular amino acid levels: the general amino acid control non-derepressible 2 (GCN2) pathway. GCN2 senses the uncharged tRNAs that accumulate upon amino acid deprivation. GCN2 attenuates translation, which not only consumes amino acids but is also one of the most energy demanding processes in the cell (Lane and Martin, 2010). Like the TORC1 pathway, the GCN2 pathway is conserved from yeast to mammals.

In yeast, GCN2 is dedicated to sensing uncharged tRNAs (Hinnebusch, 1984). Under conditions of amino acid limitation or a defect in an amino acyl tRNA synthetase, S. cerevisiae upregulate the transcription of genes involved in amino acid biosynthesis, a process termed general amino acid control (Hinnebusch, 1988; Hinnebusch, 2005; Wek et al., 1995). When present, uncharged tRNAs bind to the histidyl tRNA synthetase-like domain of GCN2, which lacks residues critical for synthetase activity and histidine specific binding, thus enabling GCN2 to respond to a variety of uncharged tRNAs (Wek et al., 1989; Wek et al., 1995). The binding triggers GCN2 homodimerization (Narasimhan et al., 2004) and autophosphorylation (Diallinas and Thireos, 1994), allowing it to phosphorylate and inhibit its only known substrate, eukaryotic initiation factor 2a (eIF2a) (Dever et al., 1992). This phosphorylation prevents efficient translation initiation of most mRNAs by limiting the pool of ternary complex, which consists of eIF2, GTP, and methionyl initiator tRNA and is required for translation initiation (Abastado et al., 1991; Dever et al., 1992; Hinnebusch, 1993).

While most mRNAs are translationally repressed upon amino acid deprivation, the mRNA encoding the GCN4 transcription factor is derepressed so that GCN4 can accumulate and activate the expression of genes that promote amino acid biosynthesis (Abastado et al., 1991; Dever et al., 1992; Hinnebusch, 1993). A cluster of four
upstream open reading frames (uORFs) in the 5' untranslated region of the GCN2 mRNA permits this unique regulation (Hinnebusch, 2005). Under nutrient replete conditions, a ternary complex forms at the first uORF. It then dissociates and another forms at the subsequent uORFs, thus preventing translation of the main ORF. However, upon starvation, ternary complex formation is delayed, and rebinding at latter uORFs reduced. Larger proportions of preinitiation complexes bypass the uORFs and form ternary complexes before reaching and translating the main ORF (Abastado et al., 1991; Hinnebusch, 1984; Mueller and Hinnebusch, 1986).

In mammals, GCN2 pathway architecture is reminiscent of that in yeast (Berlanga et al., 1999; Sood et al., 2000) as it is activated by a limitation in an essential amino acid or inhibition in the synthesis of a nonessential amino acid. uORFs also regulate the translation of the mammalian GCN4 orthologue, ATF4, a basic leucine zipper transcription factor (Vattem and Wek, 2004). ATF4 induces a cascade of transcriptional regulators that contribute to a gene expression program that modulates apoptosis, autophagy, and amino acid metabolism, including upregulation of select amino acyl tRNA synthetases and amino acid transporters (B'chir et al., 2013; Bunpo et al., 2009; Harding et al., 2000; Harding et al., 2003); (Krokowski et al., 2013). Deletion of GCN2 in mice decreases their viability during prenatal and postnatal development under conditions of nutritional stress, most notably leucine deprivation (Zhang et al., 2002). When challenged with a leucine-free diet for several days, GCN2-null mice lose more body weight than wild type counterparts and a significant proportion perish (Anthony et al., 2004).

GCN2 has acquired a critical role in controlling feeding behavior in animals. When rodents encounter a food source that lacks a single essential amino acid, they recognize this deficiency and reduce the intake of the imbalanced food (Koehnle et al., 2003). GCN2 activity in the anterior piriform cortex (APC) mediates this behavior. Injection of amino acid alcohol derivatives such as threoninol into the APC increases the levels of uncharged tRNAs and promotes the rejection of diets low in the corresponding amino acid (Hao et al., 2005). Furthermore, mice with full body or brain specific GCN2
deletions fail to reject food depleted of leucine or threonine, unlike wild type counterparts (Hao et al., 2005; Maurin et al., 2005). At the signaling level, ingestion of a meal imbalanced in amino acid composition rapidly elevates phosphorylated elf2a in APC neurons of wild type, but not GCN2-null mice (Hao et al., 2005; Maurin et al., 2005(Gietzen et al., 2004). The need to adapt feeding behavior to changes in nutrient levels is by no means restricted to animals. Drosophila also sense changes in dietary amino acids and reduce their intake of foods deficient in essential amino acids (Bjordal et al., 2014; Ribeiro and Dickson, 2010; Toshima and Tanimura, 2012; Vargas et al., 2010). As in animals, GCN2 plays a critical role within neuronal circuits to mediate this rejection by repressing GABA signaling within dopaminergic neurons of the brain (Bjordal et al., 2014). Together, these findings point to a role for the detection of uncharged tRNAs by GCN2 in controlling circuits in flies and animals that protect against the consumption of imbalanced food sources.

TORC1/mTORC1 evolved in parallel to the GCN2 pathway to sense the availability of intracellular amino acids. The mechanisms for crosstalk between the TORC1/mTORC1 and GCN2 pathways were acquired at least twice during evolution, albeit in opposing directions. While in yeast GCN2 lies downstream of TORC1, it functions upstream of mTORC1 in mammals (Anthony et al., 2004; (Cherkasova and Hinnebusch, 2003; Kubota et al., 2003; Staschke et al., 2010).

7. Conclusions

TORC1/mTORC1 is a master regulator of growth that is evolutionarily conserved from yeast to mammals. Nutrients are a key input into this pathway, and regulate the activity of mTORC1 by controlling its localization to the lysosomal surface. Central to this regulation are the Rag GTPases and multiple distinct protein complexes that control the nucleotide state of the Rags in response to nutrient levels. Although we have gained an increased understanding of the mechanisms by which amino acids activate mTORC1, much remains to be discovered.
In this thesis, I discuss how we identified the first major negative regulator of the amino-acid sensing pathway of mTORC1, GATOR1, a protein complex that contains DEPDC5, Nprl2, and Nprl3. GATOR1 functions as a GAP for RagA/B and is required for mTORC1 to sense the absence of amino acids. GATOR1 interacts with another distinct protein complex called GATOR2, which contains mios, WDR24, WDR59, sec13 and seh1L. GATOR2 functions upstream or parallel to GATOR1 to positively regulate the mTORC1 pathway through unknown mechanisms. Finally, a separate four-membered protein complex, which we call KICSTOR, is necessary to anchor GATOR1 to the lysosome so that it can interact with GATOR2.

To elucidate the molecular function of GATOR2, we searched for GATOR2-interacting proteins using immunoprecipitation and mass spectrometry (IP/MS). We identified Sestrin1/2 and CASTOR1 as two proteins that bind to GATOR2 at distinct sites to inhibit mTORC1 signaling. We determined that Sestrin1/2 and CASTOR1 function as leucine and arginine sensors, respectively, for the mTORC1 pathway. Both bind directly to their respective amino acids at affinities consistent with those necessary for sensing. Importantly, the amino acid binding capacity of both proteins are required for amino acids to activate the mTORC1 pathway. While these findings advance our understanding of how amino acids activate mTORC1, many exciting questions remain to be addressed and will be discussed in Chapter 7.
References


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CHAPTER 2

A tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1

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This work has been published in:


Experiments in Figures 2, 4, 5, S1, S2, S3, S4, and S5 were performed by LC. Experiments in Figures 1, 2, 3, 4, 5, S1, S2, S3, S4, and S5 were performed by LBP. Analysis in Figures 5 and S5 were performed by ADC. Experiments in Figure S5 were performed by WWC.
Summary

The mTOR Complex 1 (mTORC1) pathway promotes cell growth in response to many cues, including amino acids, which act through the Rag GTPases to promote mTORC1 translocation to the lysosomal surface, its site of activation. Although progress has been made in identifying positive regulators of the Rags, it is unknown if negative factors also exist. Here, we identify GATOR as an octomeric complex that interacts with the Rags and is composed of two subcomplexes we call GATOR1 and 2. Inhibition of GATOR1 subunits (DEPDC5, Nprl2, and Nprl3) makes mTORC1 signaling resistant to amino acid deprivation. In contrast, inhibition of GATOR2 subunits (Mios, WDR24, WDR59, Seh1L, Sec13) suppresses mTORC1 signaling and epistasis analysis shows that GATOR2 negatively regulates DEPDC5. GATOR1 has GTPase activating protein (GAP) activity for RagA and RagB and its components are mutated in human cancer. In cancer cells with inactivating mutations in GATOR1, mTORC1 is hyperactive and insensitive to amino acid starvation and such cells are hypersensitive to rapamycin, an mTORC1 inhibitor. Thus, we identify a key negative regulator of the Rag GTPases and reveal that, like other mTORC1 regulators, the Rags can be deregulated in cancer.
Introduction

The mTOR complex 1 (mTORC1) kinase is a master regulator of growth and its deregulation is common in human disease, including cancer and diabetes (1). In response to a diverse set of environmental inputs, including amino acid levels, mTORC1 regulates many anabolic and catabolic processes, such as protein synthesis and autophagy (1, 2). The sensing of amino acids by mTORC1 initiates from within the lysosomal lumen (3) and requires a signaling machine associated with the lysosomal membrane that consists of the Rag GTPases (4, 5), the Ragulator complex (6, 7), and the vacuolar ATPase (V-ATPase) (3). The Rag GTPases exist as obligate heterodimers of RagA or RagB, which are highly homologous, with either RagC or RagD, which are also very similar to each other (4, 5, 8). Through a poorly understood mechanism requiring the V-ATPase, luminal amino acids activate the guanine nucleotide exchange factor (GEF) activity of Ragulator towards RagA/B that, when GTP-loaded, recruits mTORC1 to the lysosomal surface (7). There, mTORC1 interacts with its activator Rheb, which is regulated by many upstream signals, including growth factors (1). Upon amino acid withdrawal RagA/B become GDP-bound (4) and mTORC1 leaves the lysosomal surface, leading to its inhibition. The negative regulators that inactivate the Rag GTPases are unknown.
Results and Discussion

We suspected that important regulators of the Rags might have escaped prior identification because their interactions with the Rags are too weak to persist under standard purification conditions. Thus, to preserve unstable protein complexes (9), we treated human embryonic kidney (HEK)-293T cells expressing FLAG-tagged RagB with a chemical cross-linker, and identified via mass spectrometry proteins that co-immunoprecipitate with FLAG-RagB. This analysis revealed the presence in the immunoprecipitates of known Rag interacting proteins as well as Mios, a 100 kDa WD40-repeat protein not previously studied (Fig. S1A). Consistent with this finding, endogenous RagA and RagC co-immunoprecipitated with recombinant Mios expressed in HEK-293T cells and isolated under similar purification conditions (Fig. 1A). Suppression of Mios, by RNA interference (RNAi) in human cells, strongly inhibited the amino acid-induced activation of mTORC1, as detected by the phosphorylation state of its substrate S6K1 (Fig. 1B, S2B). Moreover, in Drosophila S2 cells, dsRNAs targeting

Figure. 1. GATOR is a Rag-interacting complex, whose Mios component is necessary for the activation of mTORC1 by amino acids.
(A) Mios interacts with endogenous RagA and RagC. HEK-293T cells were transfected with the indicated cDNAs in expression vectors. Cells were treated with a cell permeable chemical cross-linker, lysates were prepared and subjected to Flag immunoprecipitation (IP) followed by immunoblotting for indicated proteins.
(B) Mios is necessary for the activation of the mTORC1 pathway by amino acids. HEK-293T cells expressing shRNAs targeting GFP or Mios were starved of amino acids for 50 min or starved and then re-stimulated with amino acids for 10 min. Cell lysates were analyzed for the phosphorylation state of S6K1.
(C) S2 cells treated with dsRNAs targeting Mio or GFP were starved of amino acids for 90 min or starved and re-stimulated with amino acids. The indicated proteins were detected by immunoblotting.
(D) Cell size histogram of S2 cells after dsRNA-mediated depletion of Mio.
(E)-(F) GATOR is an octomeric complex defined by two distinct subcomplexes and interacts with the Rag GTPases. HEK-293T cells were transfected and processed as in (A) with the exclusion of the cross-linking reagent, and cell lysates and FLAG-immunoprecipitates were subjected to immunoblotting.
(G) HEK-293T cells stably expressing FLAG-tagged DEPDC5 or WDR24 were lysed and cell lysates and FLAG immunoprecipitates were analyzed by immunoblotting for endogenous RagA, RagC, Mios and Nprl3.
(H) Schematic summarizing GATOR-Rag interactions. GATOR2 (Mios, Seh1L, WDR24, WDR59 and Sec13) interacts with GATOR1 (DEPDC5, Nprl2 and Nprl3), which likely then binds the Rags.
Figure 1

a, cells expressing:

RagC
RagA
Mios
AG
Nprl3

b, amino acids:

P-T389-S6K1
S6K1
Mios

c, amino acids:

P-T398-dS6K
dS6K

d, lysate:

E, cells expressing:

HA-DEPDC5, HA-WDR59, HA-Mios, HA-Npr13, HA-SEH1L, HA-Sec13

F, cells expressing:

HA-GST-RagC, HA-RagB

G, cells expressing:

HA-GATOR

H, GATOR1

RegA, RegC
DEPDC5
Npr12, Npr13
Mios, SEH1L, WDR24, WDR59, Sec13

GATOR2

lysate
Proteins in nuc-FlagB IP

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**Supp Figure 1**

**A**

Proteins in nuc-FlagB IP

- p18: 217
- p14: 88
- HBXIP: 68
- C7orf59: 16
- V-type proton ATPase subunit G1: 9
- V-type proton ATPase subunit E1: 18
- Mios: 16

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**B**

**C**

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**D**

**Supp Figure 1**

- IP: FLAG-Mios
- IP: FLAG-DEPDC5
- IP: FLAG-Nprt2
Mio (10), the fly ortholog of Mios, ablated dTORC1 signaling and also reduced cell size (Fig. 1, C and D). Thus, in human and fly cells, Mios is necessary for amino acid signaling to TORC1.

In vitro we failed to detect a strong interaction between purified Mios and the Rag heterodimers, suggesting that within cells other components exist that are needed for complex formation. Indeed, in FLAG-Mios immunoprecipitates prepared from HEK-293T cells, we detected 7 additional proteins (WDR24, WDR59, Seh1L, Sec13, DEPDC5, Nprl2, and Nprl3) by mass spectrometry. The proteins varied in abundance, however, with much greater amounts of WDR24, WDR59, Seh1L, and Sec13 immunoprecipitating with Mios than DEPDC5, Nprl2, and Nprl3 (Fig. S1B). In contrast, in FLAG-DEPDC5 immunoprecipitates, Nprl2 and Nprl3 were more abundant than Mios, WDR24, WDR59, Seh1L, and Sec13 and experiments with FLAG-Nprl2 gave analogous results (Fig. S1B). These findings suggest that two subcomplexes exist, one consisting of Mios, WDR24, WDR59, Seh1L, and Sec13, and the other of DEPDC5, Nprl2, and Nprl3. To test this notion, we co-expressed FLAG-WDR24 or FLAG-Nprl2 together with HA-tagged versions of the other seven proteins. As expected, DEPDC5 and Nprl3 co-immunoprecipitated with Nprl2 much more strongly than with WDR24, while the opposite was true for Mios, WDR59, Seh1L, and Sec13 (Fig. 1E). For reasons

**Figure S1. The octomeric GATOR complex is composed of two distinct subcomplexes.**

(A) Table summarizing peptide counts of proteins that co-immunoprecipitate with FLAG-RagB. HEK-293T cells stably expressing FLAG-RagB were treated with a chemical cross-linker and cell lysates were subject to FLAG-immunoprecipitation followed by mass spectrometry analysis of co-immunoprecipitated proteins.

(B) Cartoon summarizing peptide counts from mass spectrometric analyses of anti-FLAG immunoprecipitates from HEK-293T cells expressing FLAG-Mios (left), FLAG-DEPDC5 (center) and FLAG-Nprl2 (right). GATOR subunits are color-coded according to their peptide counts.

(C) The GATOR-Rag interaction is primarily mediated by the GATOR1 subcomplex. HEK-293T cells were transfected with the indicated cDNAs in expression vectors. Cell lysates were prepared and subjected to FLAG immunoprecipitation (IP) followed by immunoblotting for indicated proteins.

(D) Table of peptide counts for the RagA and RagC proteins that co-immunoprecipitate with FLAG-DEPDC5 or FLAG-WDR24 in HEK-293T cells stably expressing FLAG-tagged proteins were processed as described in (A).
Figure 2

A

shRNA: GFP

amino acids: 

T389-S6K1

SGK1

B

shRNA: GFP

amino acids: 

T389-S6K1

SGK1

C

dsRNA: GFP

amino acids:

T389-dS6K

dS6K

D

dsRNA: GFP

amino acids:

T389-dS6K

dS6K

E

dsRNA: GFP

amino acids:

T389-dS6K

dS6K

F

gFP

cell number vs. cell diameter (µm)

G

dsRNA: GFP

amino acids:

T389-dS6K

dS6K

H

mTORC1

GATOR1

GATOR2

DEPDC5

mTORC1

Nprl2 Nprl3 GATOR2

Sci Was OI

DEPODS
described later, we call the 8-protein complex GATOR for GAP Activity Towards Rags and the two subcomplexes GATOR1 (DEPDC5, Nprl2, and Nprl3) and GATOR2 (Mios, WDR24, WDR59, Seh1L, and Sec13) (Fig. 1H).

When the eight proteins were co-expressed with RagA and RagC, GATOR interacted strongly with the Rag heterodimer (Fig. 1F). Similar experiments in which single GATOR proteins were omitted, revealed complex relationships between the components, but suggested that GATOR1 mediates the GATOR-Rag interaction (Fig. S1C). Consistent with this conclusion, when stably expressed in HEK-293T cells, FLAG-DEPDC5 co-immunoprecipitated much more endogenous RagA and RagC than FLAG-WDR24, as detected by immunoblotting (Fig. 1G) and mass spectrometric analysis (Fig. S1D).

The finding that GATOR components interact with the Rag GTPases was intriguing because their likely budding yeast orthologs (IML1, NPR2, NPR3) positively regulate autophagosome formation, a TORC1-dependent process (11), and, at least in certain yeast strains, also inhibit TORC1 signaling upon nitrogen starvation (12-14). Moreover, recently the likely yeast orthologs of GATOR2 (Sea2, Sea3, Sea4, Seh1L,

**Figure 2. The GATOR complex is required for the regulation of the TORC1 pathway by amino acids.**

(A) shRNA-mediated depletion of the GATOR2 components Seh1L, WDR24, or WDR59 in HEK-293T cells inhibits amino acid-induced S6K1 phosphorylation.

(B) In HEK-293Ts expressing shRNAs targeting the GATOR2 components DEPDC5, Nprl2, and Nprl3, S6K1 phosphorylation is insensitive to amino acid withdrawal. In (A) and (B) cells were starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min. Cell lysates were immunoblotted for the phosphorylation state of S6K1.

dsRNA-mediated depletion in S2 cells of (C) dSeh1L; (D) dWDR59 and dWDR24; and (E) dDEPDC5, dNprl2, and dNprl3. S2 cells were treated with the indicated dsRNAs and were starved of amino acids for 90 min or starved and restimulated with amino acids for 30 min. Immunoblotting was used to detect the phosphorylation state of dS6K.

(F) S2 cell sizes after dsRNA-mediated depletion of dSeh1L and dDEPDC5.

(G) GATOR2 functions upstream of GATOR1. S2 cells were treated with the indicated combinations of dsRNAs and starved and restimulated with amino acids and analyzed as in (C)-(E).

(H) Schematic depicting the relationship between GATOR1 and GATOR2 in their regulation of mTORC1.
Supplemental Figure 2

A

shRNA: GFP WDR4_1 WDR4_2 WDR4_3
FLAG-WDR24

shRNA: GFP Sah1L_1 Sah1L_2
FLAG-Sah1

shRNA: GFP WDR59_1 WDR59_2
FLAG-WDR59

B

siRNA: Screened Mios WDR4 Sah1L WDR59
Amino acids: |
| - | + | - | + | - | + | - | + |

P-T389-S6K1
S6K1
Mios
and Sec13) were shown to interact with IML1, NPR2, and NPR3 to form a complex that has been called SEA (15). However, unlike GATOR, the SEA complex does not appear to consist of two distinct subcomplexes as its components are found in stoichiometric amounts.

We used RNAi in HEK-293T and Drosophila S2 cells to examine the function of each GATOR component in amino acid sensing by mTORC1 and dTORC1, respectively. We excluded Sec13 from further analysis as it functions in several protein complexes (16) and so its inhibition might have effects that are difficult to interpret. Consistent with Mios being required for amino acids to activate mTORC1 (Fig.1B), depletion of other GATOR2 components or their Drosophila orthologs strongly blunted amino acid-induced activation of mTORC1 and dTORC1, respectively (Fig. 2, A and C, S2A). In contrast, loss of GATOR1 proteins had the opposite effect and prevented the inactivation of mTORC1 and dTORC1 normally caused by amino acid deprivation (Fig. 2, B and D, S2B). Consistent with the opposite roles of GATOR1 and GATOR2 on dTORC1 signaling, dsRNAs targeting dSeh1L or dDEPDC5 decreased and increased, respectively, S2 cell size (Fig. 2E). To clarify the relationship between GATOR1 and GATOR2, we used RNAi to inhibit dDEPDC5 at the same time as Mio or dSeh1L in S2 cells. Interestingly, in the background of GATOR1 inhibition, loss of GATOR2 had no effect on dTORC1 activity, indicating that GATOR2 functions upstream of GATOR1 (Fig. 2, F and G). Thus, GATOR2 is an inhibitor of an inhibitor (GATOR1) of the amino acid sensing branch of the TORC1 pathway.

Figure S2. Validation of shRNAs targeting GATOR components. (A) HEK-293T cells were transfected with indicated cDNAs and corresponding shRNAs in expression vectors. Cell lysates were analyzed by immunoblotting to determine the extent of recombinant protein depletion by the indicated shRNAs. (B) siRNA mediated depletion of Mios, WDR24, Seh1L and WDR59 demonstrates that these proteins are necessary for amino-acid induced activation of mTORC1. HEK-293T cells treated with the indicated siRNAs were starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min. Cell lysates were prepared followed by immunoblotting to detect the levels of the indicated proteins.
Figure 3

A shRNA: antibody
- a.a. for 50 min
- a.a. for 50 min
+ a.a. for 10 min

B shRNA: antibody
- a.a. for 50 min
- a.a. for 50 min
+ a.a. for 10 min

C cells expressing: antibody
- a.a. for 50 min
- a.a. for 50 min
+ a.a. for 10 min

D transfected cDNAs:

- FLAG-S6K1 &
- Mtrp2
- RagB
- RagC
- RagBE
- RagC75N

- a.a. for 50 min
- a.a. for 50 min
+ a.a. for 10 min

IP-FLAG

lysate

- HA-DEPDC5
- HA-Npr1
- HA-Npr2

- a.a. for 50 min
+ a.a. for 10 min
A key step in the amino acid-induced activation of mTORC1 is its recruitment to the lysosomal surface, an event that requires known positive components of the amino acid sensing pathway, like Ragulator (7) and the v-ATPase (3). Consistent with a positive role for GATOR2, in HEK-293T cells expressing shRNAs targeting Mios or Seh1L, mTOR did not translocate to LAMP2-positive lysosomal membranes upon amino acid stimulation (Fig. 3A). In contrast, in cells expressing an shRNA targeting DEPDC5, mTOR localized constitutively to the lysosomal surface regardless of amino acid availability (Fig. 3B). Moreover, just overexpression of DEPDC5 was sufficient to block the amino acid-induced translocation of mTOR to the lysosomal surface (Fig. 3C). Unlike Ragulator, which tethers the Rags to the lysosomal surface (6-7), GATOR2 is not needed for the proper Rag localization (Fig. S3A). Thus, GATOR1 and GATOR2 have opposite effects on the activity and subcellular localization of mTORC1.

Consistent with GATOR1 being an inhibitor of the mTORC1 pathway, concomitant overexpression of its three components blocked the amino acid-induced activation of mTORC1 (Fig. 3D) to a similar extent as RagB\textsuperscript{T54N}-RagC\textsuperscript{Q120L}, a Rag heterodimer that is dominant negative because the RagB\textsuperscript{T54N} mutant cannot bind GTP (7). In contrast, expression of the dominant active RagB\textsuperscript{Q99L}-RagC\textsuperscript{S75N} heterodimer blocked not only amino acid deprivation but also GATOR1 overexpression from inhibiting mTORC1 signaling. Because RagB\textsuperscript{Q99L} is constitutively bound to GTP (17).

**Figure 3. GATOR regulates mTORC1 localization to the lysosomal surface and functions upstream of the Rag GTPases.**

(A) RNAi-mediated depletion of the GATOR2 components Mios and Seh1L prevents amino acid-induced mTOR lysosomal translocation. HEK-293T cells expressing the indicated shRNAs were starved or starved and restimulated with amino acids for the specified times prior to co-immunostaining for mTOR (red) and Lamp2 (green).

(B) Reduced expression of DEPDC5 in HEK-293T cells results in constitutive mTOR localization to the lysosomal surface. HEK-293T cells treated with the indicated lentiviral shRNAs were processed as described in (A).

(C) Images of HEK-293T cells stably expressing FLAG-DEPDC5 starved for or starved and restimulated with amino acids. Cells were processed as described in (A).

(D) GATOR1 functions upstream of the nucleotide binding state of the Rags. HEK-293T cells transfected with the indicated cDNAs in expression vectors were starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min. The indicated proteins were detected by immunoblotting. In all images, insets show selected fields that were magnified five times and their overlays. Scale bar equals 10 μM.
Supplemental Figure 3

A

<table>
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<tr>
<th>shRNA:</th>
<th>GFP</th>
<th>Mios_2</th>
<th>Seh1L_2</th>
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<tr>
<td>antibody:</td>
<td>RagA</td>
<td>Lamp2</td>
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</table>

- Merge
- LAMP2
- Merge
Figure 4

A. **RagA-RagC**

B. **RagB-RagC**

C. **RagB*-RagC*

D. **Rap2A**

E. Graph showing time (min) vs. % GDP.

F. Diagram showing in vitro binding assay results.
and RagC$^{S75N}$ cannot bind GTP (7), this result suggests that GATOR1 functions upstream of the regulation of the nucleotide binding state of the Rags.

To test the possibility that GATOR1 is a guanine nucleotide exchange factor (GEF) or a GTPase activating protein (GAP) for the Rags we prepared Rag heterodimers consisting of a wild-type Rag and a Rag$^\times$ mutant (see methods) (7). The Rag$^\times$ mutants are selective for xanthosine rather than guanine nucleotides, allowing us to prepare heterodimers in vitro in which the wild-type Rag is loaded with radiolabeled GTP or GDP while the Rag$^\times$ partner is bound to XDP or XTP (7). In vitro, purified GATOR1 did not stimulate the dissociation of GDP from RagB or RagC when each was bound to its appropriate Rag$^\times$ partner (Fig. S4A-B), ruling out its function as a GEF. In contrast, GATOR1 strongly increased, in a time and dose-dependent manner, GTP hydrolysis by RagA or RagB within RagC$^\times$-containing heterodimers, irrespective of what nucleotide RagC$^\times$ was loaded with (Fig. 4, A, B and E; S4, C and D). GATOR1 also slightly boosted GTP hydrolysis by RagC within a RagB$^\times$-RagC heterodimer (Fig. 4C), but had no effect on the GTPase activity of Rap2a (Fig. 4D). Consistent with the binding
preference of many GAPs for the GTP-loaded state of target GTPases, in vitro
GATOR1 preferentially interacted with the RagB^{G99L}-containing heterodimer (Fig. 4F).
Thus, GATOR1 is a GAP complex for RagA/B, providing a mechanism for its inhibitory role in mTORC1 signaling.

Because the pathways that convey upstream signals to mTORC1 are frequently
deregulated by mutations in cancer (reviewed in (18)), we thought it possible that
GATOR1 components might be mutated in human tumors. Indeed previous studies
identified in lung and breast cancers deletions of a 630 kb region of 3p21.3 that includes
NPRL2 (19, 20), and one study reported two cases of glioblastoma with deletions in a
three-gene region of 22q12.2 that contains DEPDC5 (21). Our analyses of publically
available data from the cancer genome atlas (TCGA) identified a subset of
glioblastomas and ovarian cancers with homozygous deletions or LOH of DEPDC5 or
NPRL2 (Fig. 5A), but copy number data for NPRL3 was too sparse to accurately assess
deletions in it. In most glioblastomas with LOH for DEPDC5 or NPRL2 the remaining
alleles had nonsense or frame shift mutations that are likely to inactivate the gene
products (Fig. 5B, S5A). In ovarian cancers with LOH for DEPDC5 the remaining
DEPDC5 alleles had a mix of missense and nonsense or frameshift mutations as well
as internal exon deletions (Fig. 5, B and C), whereas NPRL2 was exclusively focally

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**Figure S4.** GATOR1 does not promote nucleotide exchange by RagB or RagC.
(A) GATOR1 does not alter GDP dissociation by RagC. GDP dissociation assay, in which
RagC-RagB^{X} was loaded with [^{3}H]GDP and incubated with GATOR1, Ragulator, or a control.
Dissociation was monitored by a filter-binding assay. Each value represents the normalized
mean for n=2.
(B) GATOR1 does not alter GDP dissociation by RagB. RagB-RagC^{X} was loaded with
[^{3}H]GDP, incubated with GATOR1, Ragulator, or a control protein and analyzed as in (A). Each
value represents the normalized mean for n=2.
(C) The nucleotide-bound state of RagC does not alter GATOR1-GAP activity for RagB. RagB-
RagC^{X} was loaded with [a-^{32}P] GTP and XDP or XTP and incubated with GATOR1 or a control
protein. GTP hydrolysis was determined by thin layer chromatography.
(D) GATOR1 stimulates GTP hydrolysis of RagB in a dose dependent manner. RagB-RagC^{X}
was loaded with [a-^{32}P]GTP and incubated with the indicated molar amount of GATOR1 or a
control protein. GTP hydrolysis was determined as described in (C).
Tumor: Glioblastoma
Sample size: (283)
Homozygous Hemizygous
LOH focal deletion focal deletion

OEPDC5 5 (1.77%) 1 (0.35%)
NPRL2 0 (0.3%) 1 (0.35%)

Tumor: Ovarian cancer
Sample size: (441)
Homozygous Hemizygous
LOH focal deletion focal deletion

DEPDC5 5 (1.13%) 1 (0.23%)
NPRL2 0 0

Figure 5

Table:

<table>
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<tr>
<th>Cancer</th>
<th>GATOR1 Deletion</th>
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<tr>
<td>HeLa</td>
<td>-</td>
<td>&gt;4000</td>
</tr>
<tr>
<td>HT29</td>
<td>-</td>
<td>&gt;4000</td>
</tr>
<tr>
<td>Jurkat</td>
<td>-</td>
<td>16 - 194</td>
</tr>
<tr>
<td>PC3</td>
<td>-</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>HCC1500</td>
<td>N4PR2-</td>
<td>0.19 ± 0.02</td>
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<tr>
<td>SW780</td>
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<td>Li7</td>
<td>N4PR2-</td>
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<tr>
<td>MRKNU1</td>
<td>DEPDC5*</td>
<td>0.14 ± 0.04</td>
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<tr>
<td>HA7RCC</td>
<td>DEPDC5*</td>
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Diagram:

- mTOR
- Lamp2
- merge
- GATOR1
- Rapamycin
- GATOR2
- DEPDC5
- N4PR2
- N4PR2
- GAP activity
- lysosome
deleted (Fig. 5A). In aggregate, inactivating mutations in GATOR1 components are present in low single digit percentages of glioblastomas and ovarian cancers, numbers that may change upon better assessment of NPRL3.

In order to study the effects of GATOR1 loss on cancer cells, we used the Cosmic and CCLE resources (see methods) to identify human cancer cell lines with homozygous deletions in DEPDC5, NPRL2, or NPRL3, which we confirmed via immunoblotting or PCR of genomic DNA (Fig. S5C). In seven such lines, but not in Jurkat or HeLa cells, mTORC1 signaling was hyperactive and completely insensitive to amino acid deprivation, irrespective of which GATOR1 component was lacking (Fig. 5D). Furthermore, in GATOR1-null cells mTOR localized to the lysosomal surface even in the absence of amino acids (Fig. 5E). The proliferation of the GATOR1-null cancer cells was very sensitive to the mTORC1 inhibitor rapamycin, with IC50s in the 0.1-0.4 nM range (Fig. 5F). This value is many orders of magnitude less than for cell lines that are not considered rapamycin sensitive, like HeLa and HT29 cells, and at the low end of cancer cells, like PC3 and Jurkat cells, which have lost PTEN function (22-24), an established negative regulator of the mTORC1 pathway. Thus, loss of GATOR1 confers hypersensitivity to pharmacological mTORC1 inhibition.

Figure 5. GATOR1 components are mutated in cancer and GATOR1-null cancer cells are hypersensitive to the mTORC1 inhibitor rapamycin.

(A) Table summarizing mutation type and frequency for DEPDC5 and NPRL2 in glioblastomas and ovarian tumors.
(B)-(C) Mutations identified in DEPDC5 in glioblastomas and ovarian cancers.
(D) In GATOR1-null cancer cells the mTORC1 pathway is resistant to amino acid starvation. Cells were starved of amino acids for 50 min and starved and restimulated with amino acids for 10 min. Cell lysates were analyzed by immunoblotting for levels of the indicated proteins.
(E) Cancer cells were starved or starved and restimulated with amino acids for the specified times prior to co-immunostaining for mTOR (red) and Lamp2 (green). In all images, insets show selected fields that were magnified five times and their overlays. Scale bar equals 10 μM.
(F) GATOR1-null cancer cells are hypersensitive to Rapamycin. Rapamycin IC50 values for indicated cancer cell lines. Values are presented as mean ± SD (n = 3). (G) Model for the role of the GATOR complex in amino acid sensing branch of the mTORC1 pathway. GATOR2 is a negative regulator of GATOR1, which inhibits the mTORC1 pathway by functioning as a GAP for RagA.
Frameshift
Tumor classification
Glioblastoma

Cell line: Glioblastoma
gene: NPRL2

Amino acid positions: 1 50 100 150 200 250 300 350 381

Cell lines: Jurkat MRKNU1 HA7RCC
Genes: Depdc5 Runx2
Intron positioning:
- Jurkat: Intron 7, 12, 24, 29
- MRKNU1: Intron 7, 12, 24, 29
- HA7RCC: Intron 7, 12, 24, 29

Cell line: Nprl3: 2/-f
Gene: Nprl2 Runx2

Intron positioning:
- Nprl3: Intron 7, 12, 24, 29
- Nprl2: Intron 1, 8, 11
- Runx2: Intron 1, 8, 11

Cell lines: 293T HCC1500 SW780 NCI-H740
Genes: Nprl2 Runx2

Intron positioning:
- 293T: Intron 1, Exon 8, Intron 11
- HCC1500: Intron 1, Exon 8, Intron 11
- SW780: Intron 1, Exon 8, Intron 11
- NCI-H740: Intron 1, Exon 8, Intron 11

Supplemental Figure 5

Supplementary Materials
In conclusion, we identify the octomeric GATOR complex as a critical regulator of the pathway that signals amino acid sufficiency to mTORC1. The GATOR1 subcomplex has GAP activity for RagA and RagB and its loss makes mTORC1 signaling insensitive to amino acid deprivation. Inactivating mutations in GATOR1 are present in cancer and may help identify patients likely to respond to clinically-approved pharmacological inhibitors of mTORC1.

Figure S5. Validation of loss of genes encoding GATOR1 components in human cancer cell lines.
(A) Mutations found in NPRL2 in glioblastoma tumors.
(B) Immunoblot for the levels of Nprl3 in indicated cancer cell. NS= non-specific band.
(C) Genomic PCR for indicated DEPDC5 and NPRL2 regions from various cancer cell lines. Primers were designed to amplify the indicated intronic or exonic regions of the specified genes and PCR products were resolved by gel electrophoresis.
Materials and Methods

Materials

Reagents were obtained from the following sources: antibody to Nprl3 from Atlas Antibodies; HRP-labeled anti-mouse and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, S6K1, RagA and RagC, phospho-T398 dS6K, mTOR, and FLAG epitope from Cell Signaling Technology; the antibody for Mios was produced in collaboration with Cell Signaling Technology; antibodies to the HA epitope from Bethyl laboratories. RPMI, FLAG M2 affinity gel, ATP, GDP, and amino acids from Sigma Aldrich; DMEM from SAFC Biosciences; FuGENE 6 and Complete Protease Cocktail from Roche; Alexa 488 and 568-conjugated secondary antibodies; Schneider’s media, Express Five-SFM, and Inactivated Fetal Calf Serum (IFS) from Invitrogen; amino acid-free RPMI, and amino acid free Schneider’s media from US Biological; Cellulose PEI TLC plates from Sorbent Technologies; [\(^{3}H\)]GDP, [\(^{a-32P}\)]GTP, and [\(^{g-32P}\)]yGTP from Perkin Elmer; GTP, XTP and XDP from Jena Biosciences; siRNAs targeting indicated genes and siRNA transfection reagent from Dhharmacon; nitrocellulose membrane filters from Advantec; DSP from Pierce. The dS6K antibody was a generous gift from Mary Stewart (North Dakota State University).

Cell lines and tissue culture

HEK-293T, HeLa, and HT-29 cells were cultured in DMEM 10% IFS; Jurkat, HCC1500, NCI-H740 and Li7 were cultured in RPMI supplemented with 10% FBS; MRKNU1 were cultured in DMEM supplemented with 10% FBS and 2 mM glutamine; SW780 and HA7RCC were cultured in IMDM supplemented with 10% FBS; MKN45 were cultured in DMEM supplemented with 20% FBS; S2 cells were cultured in Express-Five SFM. All cell lines were maintained at 37°C, 5% CO2, with the exception of S2 cells which were grown at 25°C. HCC1500, NCI-H740 and SW780s were obtained from the American Type Culture Collection (ATCC), Li7 from the Riken Bio Resource Center, MRKNU1 from the Health Science Research Resources Bank (HSRRB), MKN45 from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and HA7RCC were a generous gift from Benoît Vandeneynde (de Duve Institute and Université catholique de Louvain and Ludwig Institute for Cancer
Cell lysis and immunoprecipitation

HEK-293T cells transiently transfected with cDNA expression vectors (see below) were rinsed once with ice-cold PBS and lysed with Triton lysis buffer (1% Triton X-100, 10 mM β-glycerol phosphate, 10 mM pyrophosphate, 40 mM Hepes pH 7.4, 2.5 mM MgCl₂ and 1 tablet of EDTA-free protease inhibitor [Roche] (per 25 ml buffer)). Lysis of HEK-293T cells stably expressing FLAG-tagged DEPDC5 or Metap2, was identical to the procedure described above with the exception that 1% Triton in the lysis buffer was substituted with 0.3% CHAPS. The soluble fractions of cell lysates were isolated by centrifugation at 13,000 rpm in a microcentrifuge for 10 minutes. For anti-FLAG-immunoprecipitations, the FLAG-M2 affinity gel was washed with lysis buffer 3 times. 20 μl of a 50% slurry of the affinity gel was then added to cleared cell lysates and incubated with rotation for 2 hours at 4°C. The beads were washed 3 times with lysis buffer containing 150 mM NaCl. Immunoprecipitated proteins were denatured by the addition of 50 μl of sample buffer and boiling for 5 minutes as described (25), resolved by 8%-16% SDS-PAGE, and analyzed by immunoblotting.

For co-transfection experiments, 2,000,000 HEK-293T cells were plated in 10 cm culture dishes. Twenty-four hours later, cells were transfected using Fugene 6 transfection reagent with the pRK5-based cDNA expression plasmids indicated in the figures in the following amounts: 100 ng HA-RagB; 100 ng HA- or HA-GST-RagC; 300 ng HA-GST-RagB^{Q69L} or 300 ng HA-GST-RagB^{T54N}; 300 ng HA-GST-RagC^{S75N} or 300 ng HA-GST-RagC^{Q120L}; 500 ng HA- or 100-1000 ng Flag-Metap2; 2000 ng Flag- or 100 ng HA-Mios; 300 ng Flag- or 100 ng HA-WDR24; 200 ng Flag- or 100-300 ng HA-Nprl2; 100 ng HA-WDR59; 100-300 ng HA-Nprl3; 100 ng HA-Seh1L; 100 ng HA-Sec13, 3 ng Flag-S6K. The total amount of plasmid DNA in each transfection was normalized to 2 μg with empty pRK5. Thirty-six hours after transfection, cells were lysed as described above.

Identification of GATOR

HEK-293T cells stably expressing FLAG-tagged Metap2, RagB, Mios, DEPDC5
or Nprl2 were chemically crosslinked with DSP prior to cell lysis with Triton lysis buffer as described in (6). Cell lysates and FLAG-immunoprecipitations were preformed as described above. Proteins were eluted with the FLAG peptide (sequence DYKDDDDK) from the FLAG-M2 affinity gel, resolved on 4-12% NuPage gels (Invitrogen), and stained with simply blue stain (Invitrogen). Each gel lane was sliced into 10-12 pieces and the proteins in each gel slice digested overnight with trypsin. The resulting digests were analyzed by mass spectrometry as described (4). Peptides corresponding to GATOR members, Ragulator, v-ATPase or Rags were detected in the FLAG-RagB, FLAG-Mios, FLAG-DEPDC5, FLAG-Nprl2 and FLAG-WDR24 immunoprecipitates, while no peptides were detected in negative control immunoprecipitates of FLAG-Metap2.

Amino acid starvation of cell lines

HEK-293T cells in culture dishes or coated glass cover slips were rinsed with and incubated in amino acid-free RPMI for either 50 minutes and stimulated with a 10X mixture of amino acids for 10-15 minutes. After stimulation, the final concentration of amino acids in the media was the same as in RPMI. The 10X mixture was prepared from individual powders of amino acids. Amino acid starvation for other cancer cell lines cells was identical to the procedure described above, with the addition of 10% dialyzed IFS to amino acid-free RPMI.

RNAi in Drosophila S2 cells

dsRNAs against Drosophila GATOR genes were designed as described in (4). Primer sequences used to amplify DNA templates for dsRNA synthesis for Mio, dSeh1L, dWDR24, dWDR59, dDEPDC5, dNprl2, and dNprl3 including underlined 5' and 3' T7 promoter sequences, are as follows:

Mio (CG7074)
Forward primer CG7074_1F:
GAATTAATACGACTCTATAGGGAGATGCTTATATATCCTATCCGTGAACCT
Reverse primer CG7047_1R:
GAATTAATACGACTCTATAGGGAGACTCAATGTCCCAGATGGTGAT
Forward primer CG7074_2F:
GAATTAATACGACTCATACTATAGGGAGAAGATGATGAAAGCTGTGCTCGATCTCGAG
Reverse primer CG7047_2R:
GAATTAATACGACTCATACTATAGGGAGAATCGCTTTATAGACGATGTGATG
Forward primer CG7074_3F:
GAATTAATACGACTCATACTATAGGGAGAAGCTTGGTCTCCGATAGATATTTG
dSehl L (CG8722)
Forward primer CG8722_1F:
GAATTAATACGACTCATACTATAGGGAGAAGCTGCTTAAACTTTCCAC
Reverse primer CG8722_1R:
GAATTAATACGACTCATACTATAGGGAGAAGCTTGGTCTCCGATAGATATTTG
dWDR59 (CG4705)
Forward primer CG4705_1F:
GAATTAATACGACTCATACTATAGGGAGAAGCTTGGTCTCCGATAGATATTTG
Reverse primer CG4705_1R:
GAATTAATACGACTCATACTATAGGGAGAAGCTTGGTCTCCGATAGATATTTG
dWDR24 (CG7609)
Forward primer CG7609_1F:
GAATTAATACGACTCATACTATAGGGAGAAGCTTGGTCTCCGATAGATATTTG
Reverse primer CG7609_1R:
GAATTAATACGACTCATACTATAGGGAGAAGCTTGGTCTCCGATAGATATTTG
dDEPDC5 (CG12090)
dsRNAs targeting GFP and dRagC were used as positive and negative controls, respectively. On day one, 4,000,000 S2 cells were plated in 6-cm culture dishes in 5 ml of Express Five SFM media. Cells were transfected with 1 μg of dsRNA per million cells using Fugene (Roche). Two days later, a second round of dsRNA transfection was
performed. On day five, cells were rinsed once with amino acid-free Schneider's medium, and starved for amino acids by replacing the media with amino acid-free Schneider's medium for 1.5 hours. To stimulate with amino acids, the amino acid-free medium was replaced with complete Schneider's medium for 30 minutes. Cells were then washed with ice cold PBS, lysed, and subjected to immunoblotting for phospho-T398 dS6K and total dS6K.

Mammalian RNAi

On day one, 200,000 HEK-293T cells were plated in a 6 well plate. Twenty-four hours later, the cells were transfected with 250 nM of a pool of siRNAs [Dharmacon] targeting Mios, WDR24, WDR59 or Seh1L or a non-targeting pool. On day four, the cells were transfected again but this time with double the amount of siRNAs. On day five, the cells were either split onto coated glass cover slips or rinsed with ice-cold PBS, lysed and subjected to immunoblotting as described above.

Lentiviral shRNAs targeting Mios, WDR24, WDR59, Seh1L, DEPDC5, Nprl2, Nprl3 and GFP were obtained from the TRC. The TRC number for each shRNA is as follows:

Human Mios shRNA_1: TRCN0000303645
Human Mios shRNA_2: TRCN0000370186
Human WDR24 shRNA_1: TRCN0000130142
Human WDR24 shRNA_2: TRCN0000416122
Human WDR24 shRNA_3: TRCN0000445462
Human WDR59 shRNA_1: TRCN0000156940
Human WDR59 shRNA_2: TRCN0000156940
Human Seh1L shRNA_1: TRCN0000156869
Human Seh1L shRNA_2: TRCN0000330510
Human Seh1L shRNA_3: TRCN0000330507
Human Nprl2 shRNA_1: TRCN0000234677
Human Nprl2 shRNA_2: TRCN0000234673
Human Nprl3 shRNA_2: TRCN0000135594
Human DEPDC5 shRNA_1: TRCN0000137523
The following shRNAs targeting Nprl3 and DEPDC5 were made in the lab and cloned into pLKO.1 vector as described (26).

The target sequence for the Nprl3 shRNA:
Human Nprl3 shRNA_1: GATGTTATTCTGGCAACAATT

The target sequence for the DEPDC5 shRNA:
Human DEPDC5 shRNA_2: CAGGTATTTGAAGAGTTTATT

shRNA-encoding plasmids were co-transfected with the Delta VPR envelope and CMV VSV-G packaging plasmids into actively growing HEK-293T cells using FuGENE 6 transfection reagent as previously described (26). Virus-containing supernatants were collected 48 hours after transfection, filtered to eliminate cells and target cells were infected in the presence of 8 μg/ml polybrene. 24 hours later, cells were selected with puromycin and analyzed on the 2nd or 3rd day after infection.

Immunofluorescence assays

Immunofluorescence assays were performed as described in (6). Briefly, 200,000 HEK-293T (infected with lentiviral shRNAs) cells or 75,000 cells for other cell lines used (HeLa, SW780, HCC1500) were plated on fibronectin-coated glass coverslips in 12-well tissue culture plates. Twenty-four hours later, the slides were rinsed with PBS once and fixed for 15 min with 4% paraformaldehyde in PBS at room temperature. The slides were rinsed twice with PBS and cells were permeabilized with 0.05% Triton X-100 in PBS for 5 min. After rinsing twice with PBS, the slides were incubated with primary antibody in 5% normal donkey serum for 1 hr at room temperature, rinsed four times with PBS, and incubated with secondary antibodies produced in donkey (diluted 1:1000 in 5% normal donkey serum) for 45 min at room temperature in the dark and washed four times with PBS. Slides were mounted on glass coverslips using Vectashield (Vector Laboratories) and imaged on a spinning disk confocal system (Perkin Elmer) or a Zeiss Laser Scanning Microscope (LSM) 710.

S2 cell size determinations

For measurements of cell size, S2 cells treated with dsRNAs as described above
were harvested in a 4 ml volume and diluted 1:20 with counting solution (Isoton II Diluent, Beckman Coulter). Cell diameters were determined with a particle size counter (Coulter Z2, Beckman Coulter) running Coulter Z2 AccuComp software.

Purification of recombinant Rag heterodimers, GATOR1, and Ragulator for GAP/GEF/In-vitro binding assays

To produce protein complexes used for GAP or GEF assays, 4,000,000 HEK-293T cells were plated in 15 cm culture dishes. Forty-eight hours later, cells were transfected with the following combination of constructs (all cDNAs were expressed from pRK5 expression plasmid). For Ragulator: 4 µg Flag-p14, 8 µg HA-MP1, 8 µg HA-p18G2A (a lipidation defective mutant), 8 µg HA-HBXIP, and 8 µg HA-C7orf59. For FLAG-GATOR1: 4 µg FLAG-DEPDC5, 8 µg HA-Nprl2 and 8 µg HA-Nprl3. For RagA/B-RagC: 16 µg HA-RagB or HA-RagA and 8 µg Flag-RagC_D181N, for RagB^X-RagC: 8 µg FLAG-RagB_D163N and 16 µg HA-RagB. For Rags used in in-vitro binding: 8 µg HA-GST-RagB_T54N and 16 µg HA-RagC_Q120L, 4 µg HA-GST-RagB_Q99L and 8 µg HA-RagC_S75N. For individual proteins: 10 µg Flag- or HA-GST-Rap2a, 15 µg of FLAG-Leucyl tRNA synthetase (LRS), or 10 µg Flag-Metap2.

Thirty-six hours post transfection cell lysates were prepared as described above, with the exception that for all GATOR1 purifications, 1 mM ATP was added to the lysis buffer. 200 µl of a 50% slurry of FLAG-M2 affinity gel or immobilized glutathione beads were added to lysates from cells expressing FLAG-tagged proteins or HA-GST tagged proteins, respectively. Recombinant proteins were immunoprecipitated for 3 hours at 4°C. Each sample was washed once with Triton lysis buffer, followed by 3 washes with Triton lysis buffer supplemented with 500 mM NaCl and finally, 4 washes with CHAPS buffer. FLAG-tagged proteins were eluted from the FLAG-M2 affinity gel with a competing FLAG peptide for 1 hour as described above. GATOR1 and Ragulator proteins were stored in CHAPS buffer supplemented with 10% glycerol and stored at -80°C.

To remove the FLAG peptide, GTPases were subsequently purified on a HiLoad 16/60 Superdex 200 FPLC column (GE) pre-equilibrated with CHAPS buffer supplemented with 150 mM salt. The peak corresponding to the desired complex was
concentrated in 10,000 MW CO columns (Amicon), snap frozen in CHAPS buffer supplemented with 10% glycerol and stored at -80°C.

**In Vitro Binding Assays**

For the binding reactions, 20 µl of a 50% slurry containing immobilized HA-GST-tagged proteins were incubated in binding buffer (1% Triton X-100, 2.5 mM MgCl₂, 40 mM HEPES [pH 7.4], 2 mM DTT, and 1 mg/ml BSA) with 2 µg of FLAG-GATOR1 in a total volume of 50 µl for 1 hr and 30 min at 4°C. To terminate binding assays, samples were washed three times with 1 ml of ice-cold binding buffer supplemented with 300 mM NaCl followed by the addition of 50 µl of sample buffer.

**Rag GTP hydrolysis assays**

14 µg of the indicated Rag heterodimers or Rap2a were incubated for 2 hours at 4°C with 20 µl of FLAG-M2 affinity gel prewashed in CHAPS loading buffer (4 mM HEPES pH 7.4, 30 mM NaCl, 0.3% CHAPS). The resin was then washed 3 times with CHAPS loading buffer to remove unbound protein. The GTPases were loaded in 100 µl CHAPS loading buffer containing 0.1 µM XDP or 0.1 µM XTPgS, 70 pmoles of the specified radioactive GTP species ([α-³²P]GTP for TLC assay or [γ-³²P]GTP for phosphate capture assay), 2 mM DTT, 0.01 µg/µl BSA, and 5 mM EDTA at 25°C for 10 minutes. Following this nucleotide loading, MgCl₂ was added to a final concentration of 10 mM and the GTPases were incubated for an additional 5 minutes at 25°C. The GTPases were placed back on ice and washed 6 times with GTPase wash buffer (4 mM HEPES pH 7.4, 5 mM MgCl₂, 20 mM NaCl, 0.3% Chaps, 2 mM DTT, 0.01 µg/µl BSA) to remove unbound nucleotide. 30 µl of competing FLAG-peptide was then added and the GTPases were eluted from the affinity gel for 2 hours. Protein concentrations were determined prior to use.

For the TLC-based GTP hydrolysis assay, 5 pmoles of the indicated Rag heterodimer or Rap2a loaded with xanthosine nucleotides and [α-³²P]GTP were added to 20 pmoles Flag-LRS or Flag-GATOR1 in 45 µl of GTPase wash buffer. The reaction was incubated at 25°C for the indicated times. The assay was terminated upon addition of 5 µl of 6X Elution Buffer (6.7 mM GTP, 6.7 mM GDP, 100 mM EDTA, 2% SDS)
followed by further incubation for 5 minutes at 65°C at 1400 rpm. Chloroform was added to separate the nucleotides from denatured proteins and the sample was spun at 13200 rpm in a microcentrifuge for 1 min to separate the aqueous and organic phases. 30 μl of the aqueous layer was removed. Samples were spotted on a PEI Cellulose TLC plates and developed for 2.5 hours in 0.5 M KH₂PO₄ pH 3.4. Plates were exposed to film and spot densities were quantified with Multi Gauge V2.2 (Fujifilm).

For the phosphate capture GTP hydrolysis assay, Flag-RagC^{D181N}-HA-RagB was loaded with [g-^{32}P]GTP and XDP as described above. A total of 48 pmoles of loaded Flag-RagC^{D181N}-HA-RagB was added to GTPase wash buffer containing Flag-LRS or FLAG-GATOR1 in a total volume of 140 μl and incubated at 25°C. At the indicated time points three aliquots of 10 μl were taken and quenched by addition of 500 μl of activated charcoal mixture (5% activated charcoal, Norit® (Sigma) in 50 mM NaH₂PO₄). This mixture was then vortexed and spun at 13200 rpm in a microcentrifuge for 10 minutes at 4°C. 375 μl of the supernatant was added to 3.5 ml of Optifluor scintillation fluid and free ^{32}P was measured using a TriCarb scintillation counter (Perkin Elmer).

Nucleotide Exchange Assays

These assays were essentially performed as described in (7). Briefly, 40 pmols of FLAG-RagB^{X}-HA-RagC or FLAG-RagC^{X}-HA-RagB were loaded with 2 μM of [^{3}H]GDP (25-50 Ci/mmol). The GTPase-[^{3}H]GDP were stabilized by addition of 20 mM MgCl₂ followed by a further incubation at 4°C for 12 hours. To initiate the GEF assay, 40 pmols of the indicated proteins were added along with 200 μM GTPγS and incubated at 25°C. Samples were taken every 2 minutes and spotted on nitrocellulose filters, which were washed with 2 ml of wash buffer (40 mM Hepes pH 7.4, 150 mM NaCl and 5 mM MgCl₂). Filter-associated radioactivity was measured using a TriCarb scintillation counter (Perkin Elmer).

Identification of GATOR1-null cancer cell lines

To identify cancer cell lines null for GATOR1 components we searched the following publically available databases: Cancer Cell Line Encyclopedia (CCLE) (http://www.broadinstitute.org/ccle/home) and Cancer Genome Project (CPG)
GATOR1 null cell lines were identified in CCLE based on a value <-4 when sorted by deletion of the indicated GATOR1 genes. GATOR1-null cells were identified at CGP based on copy number analysis using CONAN. NPRL3-null cells were verified by immunoblotting for the Npr3 protein. DEPDC5- and NPRL2-null cells were verified by genomic PCR as follows. Genomic DNA from cancer cells was extracted using the QiAmp DNA Mini kit Blood and Tissue (Qiagen) and used in PCR reactions with the gene-specific primers listed below. Runx2 is a positive control.

Runx2_exon6_fwd: CGCATTCCCTCATCCAGATG
Runx2_exon6_rev: AAAGGACTTGACAGGTTACAG
DEPDC5_intron7_fwd: CCAAGCAACTAAAGCACAACCCAA
DEPDC5_intron7_rev: CAGGCTTCCTGACCTGATAC
DEPDC5_intron12_fwd: TGGGACATCTGCTGTACTGAC
DEPDC5_intron12_rev: CAGAAGAGCTCTCATGGTTCCTGG
DEPDC5_intron24_fwd: AGTGAATATCTCAAGCCATCCT
DEPDC5_intron24_rev: CCTTAGACAGTGCCTAGTTCA
DEPDC5_intron29_fwd: TGAAGCTCAGGGATGACGTGC
DEPDC5_intron29_rev: AATCAGGGCAGCAGACGTGC
NRPL2_intron1_fwd: GCTCCCAATGTGGCAGGGAA
NRPL2_intron1_rev: TCACCTTCTGTGACCTTGA
NRPL2_exon8_fwd: CTCATCCCTGACCCACACAG
NRPL2_exon8_rev: CCAATGACGCTCTGCACAGT
NRPL2_intron11_fwd: GAGCTGATGACGCGCTTGA
NRPL2_intron11_rev: AGGAGGACTACCCACAGCA

Cell proliferation assays
The indicated cancer cell lines were seeded in 96 well plates (Corning). Rapamycin, (0.4 pM – 4 μM), was added 24 hours post-seeding, and DMSO was used as the control. Cell viability was measured 4 days after drug addition with CellTiter Glo luminescent viability assay (Promega). Readings were normalized to the untreated cells.
and IC₅₀ calculated with Prism 5 (Graphpad). All assays were repeated in triplicate.
Acknowledgements

We thank all members of the Sabatini lab for helpful suggestions, E. Spooner for the mass spectrometric analysis of samples, and N. Kory for technical assistance. This work was supported by grants from the NIH (CA103866 and AI47389) and Department of Defense (W81XWH-07-0448) to D.M.S. and the National Cancer Institute (NIH) (U24CA143867) to M.M. and awards from the David H. Koch Graduate Fellowship Fund to L.B.-P.; the NSF Graduate Research Fellowship Program to L.C.; the Harvard-MIT Health, Sciences, and Technology IDEA² program to W.W.C.; and the American Cancer Society to B.C.G. D.M.S. is an investigator of the Howard Hughes Medical Institute.
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CHAPTER 3

The Sestrins interact with GATOR2 to negatively regulate the amino acid sensing pathway upstream of mTORC1

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This work has been published in:


Experiments in Figures 1, 2, S1, S2, and S3 were performed by LC. Experiments in Figures 1, 4, S1, S2, S3, and S4 were performed by RLW. Experiments in Figure 1 were performed by SMS. Experiments in Figures 2 and 3 were performed by JMO. Experiments in S1 were performed by RAS.
Summary

The mTORC1 kinase is a major regulator of cell growth that responds to numerous environmental cues. A key input is amino acids, which act through the heterodimeric Rag GTPases (RagA/B bound to RagC/D) to promote the translocation of mTORC1 to the lysosomal surface, its site of activation. GATOR2 is a complex of unknown function that positively regulates mTORC1 signaling by acting upstream of or in parallel to GATOR1, which is a GTPase activating protein (GAP) for RagA/B and an inhibitor of the amino acid sensing pathway. Here, we find that the Sestrins, a family of poorly understood growth regulators (Sestrin1-3), interact with GATOR2 in an amino acid-sensitive fashion. Sestrin2-mediated inhibition of mTORC1 signaling requires GATOR1 and the Rag GTPases, and the Sestrins regulate the localization of mTORC1 in response to amino acids. Thus, we identify the Sestrins as GATOR2-interacting proteins that regulate the amino acid sensing branch of the mTORC1 pathway.
Introduction

The mechanistic target of rapamycin complex 1 (mTORC1) protein kinase is a master growth regulator that senses diverse environmental cues, such as growth factors, cellular stresses, and nutrient and energy levels. When activated, mTORC1 phosphorylates substrates that potentiate anabolic processes, such as mRNA translation and lipid synthesis, and that limit catabolic ones, such as autophagy. mTORC1 deregulation occurs in a broad spectrum of diseases, including diabetes, epilepsy, and cancer (Howell et al., 2013; Kim et al., 2013; Laplante and Sabatini, 2012).

Many upstream inputs, including growth factors and energy levels, signal to mTORC1 through the TSC complex, which regulates Rheb, a small GTPase that is an essential activator of mTORC1 (Brugarolas et al., 2004; Garami et al., 2003; Inoki et al., 2003; Long et al., 2005; Sancak et al., 2008; Saucedo et al., 2003; Stocker et al., 2003; Tee et al., 2002). Amino acids do not appear to signal to mTORC1 through the TSC-Rheb axis and instead act through the heterodimeric Rag GTPases, which consist of RagA or RagB bound to RagC or RagD (Hirose et al., 1998; Kim et al., 2008; Nobukuni et al., 2005; Roccio et al., 2005; Sancak et al., 2008; Schürmann et al., 1995; Sekiguchi et al., 2001; Smith et al., 2005).

The Rags control the subcellular localization of mTORC1 and amino acids promote its recruitment to the lysosomal surface, where Rheb also resides (Buerger et al., 2006; Dibble et al., 2012; Menon et al., 2014; Saito et al., 2005; Sancak et al., 2008). Several positive components of the pathway upstream of the Rag GTPases have been identified. The Ragulator complex localizes the Rags to the lysosomal surface and, along with the vacuolar-ATPase, promotes the exchange of GDP for GTP on RagA/B (Bar-Peled et al., 2012; Sancak et al., 2010; Zoncu et al., 2011). The distinct FLCN-FNIP complex acts on RagC/D and stimulates its hydrolysis of GTP into GDP (Tsun et al., 2013). When RagA/B is loaded with GTP and RagC/D with GDP, the heterodimers bind and recruit mTORC1 to the lysosomal surface, where it can come in...
contact with its activator Rheb.

Recent work has identified the GATOR1 complex as a major negative regulator of the amino acid sensing pathway and its loss causes mTORC1 signaling to be completely insensitive to amino acid starvation (Bar-Peled et al., 2013; Panchaud et al., 2013). GATOR1 consists of DEPDC5, Nprl2, and Nprl3, and is a GTPase activating protein (GAP) for RagA/B. The GATOR2 complex, which has five known subunits (WDR24, WDR59, Mios, Sec13, and Seh1L), is a positive component of the pathway and upstream of or parallel to GATOR1, but its molecular function is unknown (Bar-Peled et al., 2013).

Here, we identify the Sestrins as interacting partners of GATOR2. The Sestrins are three related proteins (Sestrin1-3) of poorly characterized molecular functions (Buckbinder et al., 1994; Budanov et al., 2002; Peeters et al., 2003). Sestrin2 inhibits mTORC1 signaling and has been proposed to activate AMPK upstream of TSC as well as to interact with TSC (Budanov and Karin, 2008). We find that the Sestrins interact with GATOR2 in an amino acid sensitive fashion, regulate the subcellular localization of mTORC1, and require GATOR1 and the Rag GTPases to inhibit mTORC1 signaling. Thus, we conclude that the Sestrins are components of the amino acid sensing pathway upstream of mTORC1.
Results and Discussion

The Sestrins Interact with GATOR2 in an Amino Acid-Sensitive Fashion

To begin to probe how the GATOR complexes might be regulated, we sought to identify GATOR2-interacting proteins. In mass spectrometric analyses of anti-FLAG immunoprecipitates prepared from HEK-293T cells stably expressing FLAG-tagged GATOR2 components (WDR24, Mios, or WDR59), we consistently detected peptides derived from Sestrin2, at levels comparable to those from the bona fide GATOR2 component Sec13 (Figure 1A). Sestrin1 and Sestrin3 were also present, albeit at lower amounts than Sestrin2 (Figure 1A).

Consistent with the Sestrins being GATOR2-interacting proteins, recombinant FLAG-tagged Sestrin1, Sestrin2, or Sestrin3 when transiently co-expressed in HEK-

Figure 1: The Sestrins interact with GATOR2, but not GATOR1, in an amino acid-sensitive fashion
(A) GATOR2 interacts with the Sestrins. Mass spectrometric analyses identify Sestrin-derived peptides in immunoprecipitates from HEK-293T cells stably expressing FLAG-tagged GATOR2 components.
(B) Recombinant Sestrin 1, 2, and 3 interact with recombinant GATOR2 but not GATOR1. Anti-FLAG immunoprecipitates were collected from HEK-293T cells expressing the indicated cDNAs in expression vectors and were analyzed, along with cell lysates, by immunoblotting for the relevant epitope tags.
(C) Stably expressed Sestrin2 co-immunoprecipitates endogenous GATOR2 components. Immunoprecipitates were prepared from HEK-293T cells stably expressing the indicated FLAG-tagged proteins, and were analyzed along with cell lysates by immunoblotting for the indicated proteins.
(D) Stably expressed GATOR2 and endogenous Sestrin2 interact in an amino acid dependent fashion. HEK-293T cells stably expressing the indicated FLAG-tagged proteins were starved of amino acids for 50 minutes, or starved and then stimulated with amino acids for 10 minutes. Anti-FLAG immunoprecipitates were analyzed as in (C).
(E) Stably expressed Sestrin2 interacts with endogenous GATOR2 in an amino acid sensitive fashion. HEK-293T cells expressing the indicated epitope tagged proteins were amino acid starved or starved and restimulated with amino acids as in (D), and anti-FLAG immunoprecipitates were analyzed as in (C).
(F) The GATOR2-Sestrin2 interaction is sensitive to both amino acid and glucose availability, but is not affected by growth factors. HEK-293T cells stably expressing the indicated FLAG-tagged proteins were starved of either amino acids, glucose, or growth factors for 50 minutes, or starved and restimulated with amino acids, glucose, or insulin, respectively, for 10 minutes. Anti-FLAG immunoprecipitates were analyzed as in (C).
Figure 1

A

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<th>Unique Peptide Counts</th>
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B

transfected cDNAs:

- hA-GATOR1: + + + + +
- hA-GATOR2: + + + + +
- hA-WDR59: + + + + +
- hA-WDR24: + + + + +
- hA-Mios: + + + + +
- hA-Nprl3: + + + + +

IP:FLAG

- FLA-metp2: + + + + +
- FLA-WDR24: + + + + +
- FLA-WDR59: + + + + +
- FLA-Mios: + + + + +
- FLA-Nprl3: + + + + +
- FLA-WDR24 & hA-Mios: + + + + +
- FLA-WDR24 & hA-Nprl3: + + + + +

C

cells expressing:

- Mios: FLA-WDR24
- Nprl2: FLA-WDR24

D

cells expressing:

- amino acids: + + +
- Mios: FLA-WDR24
- Nprl2: FLA-WDR24

E

cells expressing:

- amino acids: + + +
- Mios: FLA-WDR24
- Nprl2: FLA-WDR24

F

cells expressing:

- amino acids: + + +
- Mios: FLA-WDR24
- Nprl2: FLA-WDR24
Figure S1
Figure S1, related to Figure 1: Regulation of the GATOR2-Sestrin2 interaction by nutrients and not insulin and GATOR2 is important for Sestrin2 stability.

(A) Stably expressed components of GATOR2, but not GATOR1, interact with endogenous Sestrin2. Anti-FLAG immunoprecipitates were analyzed as in Figure 1C. The asterisk indicates a nonspecific band that appears in all lanes at a molecular weight just below that of endogenous Sestrin2.

(B) Sestrin2 does not disrupt the interaction between GATOR1 and GATOR2. Anti-FLAG immunoprecipitates were collected and analyzed as in Figure 1B from HEK-293T cells transiently overexpressing the indicated cDNAs.

(C) The amino acid-sensitive interaction between GATOR2 and Sestrin2 is not dependent on mTOR activity. HEK-293T cells stably expressing the indicated FLAG-tagged proteins were starved or starved and restimulated with amino acids as in Figure 1D, and concurrently treated with either DMSO, 250 nM rapamycin, or 250 nM Torin1 for 60 minutes.

(D) Sestrin2 levels are decreased in cells with reduced expression of GATOR2. Cell lysates from HEK-293T cells genetically modified with the indicated guide RNAs using the CRISPR-Cas9 system were immunoblotted for levels of the indicated endogenous proteins.

(E) Recombinant TSC1 does not co-immunoprecipitate endogenous Sestrin2. Anti-FLAG immunoprecipitates were collected from HEK-293T cells expressing the indicated cDNAs and were analyzed by immunoblotting for endogenous proteins.

293T cells co-immunoprecipitated GATOR2, but not GATOR1 or the metap2 control protein (Figure 1B). When stably expressed in HEK-293T cells, FLAG-Sestrin2 co-immunoprecipitated endogenous GATOR2 as detected through its Mios component (Figure 1C). The reciprocal was also true because stably expressed FLAG-WDR24 co-immunoprecipitated abundant amounts of endogenous Sestrin2 alongside the established components of GATOR2 (Figure S1A). In contrast, FLAG-DEPDC5, a GATOR1 component, did not co-immunoprecipitate endogenous Sestrin2, suggesting that GATOR1 and Sestrin2 do not make a readily detectable interaction (Figure S1A). Given that GATOR1 is known to interact with GATOR2 (Bar-Peled et al., 2013), we tested the effect of expressing increasing amounts of FLAG-Sestrin2 on this interaction and found that Sestrin2 did not perturb the ability of GATOR1 to co-immunoprecipitate GATOR2 (Figure S1B).

Amino acids regulate the interaction between multiple critical components of the amino acid pathway (Bar-Peled et al., 2012; Sancak et al., 2010; Sancak et al., 2008; Tsun et al., 2013; Zoncu et al., 2011). Likewise, amino acid deprivation strongly increased the GATOR2-Sestrin2 interaction, whether monitored by immunoprecipitating GATOR2 or Sestrin2 and probing for endogenous Sestrin2 or GATOR2, respectively.
(Figure 1D, 1E). Pretreatment of cells with rapamycin, an allosteric mTORC1 inhibitor, or Torin1, an ATP-competitive mTOR inhibitor, did not prevent the amino acid-induced decrease in the GATOR2-Sestrin2 interaction, indicating that mTORC1 activity does not control the interaction (Figure S1C). Consistent with the notion that the pathways upstream of mTORC1 that sense amino acids and growth factors are largely independent, insulin treatment of cells did not regulate the Sestrin2-GATOR2 interaction (Figure 1E). Interestingly, however, glucose deprivation led to a modest increase in the amount of Sestrin2 bound to GATOR2, albeit to a much lesser extent than that caused by amino acid starvation (Figure 1E). Glucose levels have been previously described as an upstream input to the Ragulator-v-ATPase input to Rag GTPases (Efeyan et al., 2012a), and these results are consistent with glucose also affecting the GATOR2 input to the Rags.

Given the robust interaction between Sestrin2 and GATOR2, we reasoned that within cells the levels of GATOR2 might affect those of Sestrin2, in an analogous fashion to the components of other complexes, like Ragulator or GATOR1 (Bar-Peled et al., 2013; Sancak et al., 2008). Indeed, endogenous Sestrin2 expression was severely depressed in cells in which strongly suppressed either the Mios or WDR24 components of GATOR2 via CRISPR/Cas9-mediated genome editing (Figure S1D).

Together, these results identify the Sestrins as GATOR2 interacting proteins and establish that Sestrin2 and GATOR2 interact in an amino-acid sensitive fashion, suggesting a regulatory role for the Sestrins in signaling amino acid sufficiency to mTORC1.

The Sestrins inhibit the amino acid sensing pathway upstream of mTORC1

The Sestrins have previously been reported to be negative regulators of mTORC1 signaling and to function by activating AMPK, which in turn stimulates TSC to inhibit Rheb, and by binding TSC (Budanov and Karin, 2008). In our experimental system, under conditions where GATOR2 and Sestrin2 interact, we were unable to
detect an interaction between recombinant TSC1 and endogenous Sestrin2 (Figure S1E). Given the strong interaction of Sestrin2 with GATOR2, we reasoned that Sestrin2 might regulate the capacity of the mTORC1 pathway to sense amino acids. Indeed, stable over-expression of Sestrin2 dose-dependently inhibited mTORC1 activation by amino acids, as determined by the phosphorylation of S6K1, confirming its role as a negative regulator (Figure 2A and S2A). In addition, consistent with previous reports (Budanov and Karin, 2008), stable over-expression of FLAG-Sestrin2 caused a dramatic reduction in cell size (Figure 2B), a well-known consequence of mTORC1 inhibition (Fingar et al., 2002).

In HEK-293T cells, inhibition of just Sestrin1 or Sestrin2, caused by either short-hairpin RNA (shRNA)-mediated knockdown or CRISPR/Cas9-mediated knockout, caused only a slight defect in mTORC1 inhibition upon amino acid withdrawal (Figures 2C and S2B-E). The double knockdown of Sestrin1 and Sestrin3 had a similarly weak effect (Figure 2C) while that of Sestrin1 and Sestrin2 more robustly rescued mTORC1 activation.

Figure 2: The Sestrins are negative regulators of the amino acid sensing pathway upstream of mTORC1.
(A) Stable overexpression of Sestrin2 inhibits mTORC1 signaling, but does not affect the phosphorylation of Akt. HEK-293T cells stably expressing the indicated proteins were starved of amino acids for 50 minutes, or starved and restimulated with amino acids for 10 minutes. Immunoblotting of cell lysates allowed for the analysis of levels and the phosphorylation states of the indicated proteins.
(B) Stable overexpression of Sestrin2 severely decreased cell size. HEK-293T cells stably expressing the indicated proteins and wild-type HEK-293T cells were analyzed for cell size.
(C) A decrease in the levels of the Sestrins leads to an inability to fully inhibit mTORC1 signaling under amino acid deprivation. HEK-293T cells which were genetically modified with the indicated guide RNAs using the CRISPR/Cas9 system were subsequently treated with the indicated shRNAs, then starved of amino acids for 50 minutes, or starved and restimulated with amino acids for 10 minutes, and analyzed as in (A).
(D) The indicated shRNAs reduced the mRNA levels of Sestrin1 and 3. Quantitative polymerase chain reaction (qPCR) was performed on the samples described in (C) to assess the efficacy of shRNA-mediated knockdown of Sestrin1 and 3. Errors depicted are standard error of the mean calculated based on samples from a single qPCR run.
(E) Double-knockdown of Sestrin1 and 2 exaggerates the observed phenotype. Cells were treated and cell lysates were analyzed as in (A).
Figure S2

A

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B

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D

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Figure S2, related to Figure 2: The Sestrins are necessary for full inhibition of mTORC1 signaling in the absence of amino acids.

(A) Sestrin1 and 2 dose-dependently inhibit mTORC1 signaling. Recombinant Sestrin1 or 2 were transiently overexpressed in HEK-293T cells, which were amino acid starved or starved and restimulated with amino acids as in Figure 1D. Cell lysates were immunoblotted for levels and phosphorylation states of the indicated proteins.

(B) Sestrin2-null cells have a slight defect in their ability to inhibit mTORC1 signaling in the absence of amino acids. Sestrin2-null HEK-293T cells, or HEK-293T cells which were treated with a guide RNA targeting GFP, were amino acid starved or starved and restimulated as in Figure 1D and 1E, and levels and phosphorylation states of the indicated proteins were analyzed by immunoblotting. Three distinct guide RNAs targeting Sestrin2 were used to produce three different Sestrin2-null clones.

(C) shRNA-mediated knockdown of Sestrin1 or Sestrin2 leads to a slight increase in mTORC1 signaling under amino acid deprivation conditions. HEK-293T cells were treated with the indicated shRNAs, amino acid starved or starved and restimulated as described in Figure 1D, and cell lysates were analyzed by immunoblotting.

(D) Knockdown of Sestrin1 leads to a slight defect in the ability to inhibit mTORC1 signaling in the absence of amino acids. Cells were treated and cell lysates were analyzed as in (C).

(E) shRNA-mediated depletion of Sestrin3 leads to a phenotype that is consistent with that of knockdown of Sestrin 1 or 2. Cells were treated and cell lysates were analyzed as in (C).

(F) shRNA-mediated knockdown of all three Sestrins in HEK-293E cells renders the cells completely insensitive to glucose deprivation. Cells were starved of glucose for 50 minutes and restimulated with 500mM glucose for 10 minutes. Cell lysates were analyzed as in (C).

signaling in the absence of amino acids (Figure 2E). Finally, when we inhibited all three Sestrins by expressing shRNAs targeting Sestrin1 and Sestrin3 in Sestrin2-null cells created with the CRISPR/Cas9 system, we obtained a strong but still partial rescue of mTORC1 signaling upon amino acid deprivation (Figure 2C). In addition, triple knockdown of all three Sestrins using shRNAs in HEK-293E cells rendered the cells insensitive to glucose deprivation (Figure S2F). These data indicate that the Sestrins play redundant roles within the mTORC1 pathway and collectively are necessary for the full inhibition of mTORC1 signaling that occurs in the absence of amino acids or glucose.

The Sestrins function upstream of GATOR1 and the Rag GTPases

To further understand how the Sestrins play a regulatory role in the amino acid sensing pathway, we investigated whether they require other components of the pathway to inhibit mTORC1 signaling. The nucleotide loading state of the Rag GTPase
Figure 3
heterodimer is critical for the proper sensing of amino acids by mTORC1 (Sancak et al., 2008). Amino acids promote GTP loading of RagA/B and GDP loading of RagC/D, enabling them to recruit mTORC1 to the lysosomal surface (Sancak et al., 2008). The GAP activity of GATOR1 leads to GTP hydrolysis of RagA/B and inhibition of the pathway (Bar-Peled et al., 2013).

Several lines of evidence support the notion that the Sestrins lie upstream of the Rags and depend on GATOR1 to function as negative regulators of mTORC1. First, concomitant overexpression of recombinant Sestrin2 and the dominant active RagB^{Q99L}-RagC^{S75N} pair prevented Sestrin2-mediated inhibition of the pathway, thus placing the Sestrins upstream of the Rag GTPases (Figure 3A). Second, while Sestrin2 overexpression strongly abrogated signaling in cells expressing GATOR1, in Nprl3-null HEK-293E cells produced via the CRISPR/Cas9-system, Sestrin2 failed to inhibit the constitutive mTORC1 signaling observed in the absence of GATOR1. Thus, GATOR1 is epistatic to Sestrin2 (Figure 3B).

Given that Sestrin2 functions upstream of GATOR1, we tested the possibility that it might inhibit the pathway by enhancing the GAP activity of GATOR1, however, GATOR1 GAP activity is unaltered when isolated from cells overexpressing Sestrin2 (Figure S3A).

Previous work has shown that lysosome-associated machinery, which includes the v-ATPase, is necessary for the amino acid-induced activation of mTORC1 (Zoncu et al., 2011). Interestingly, inhibition of the v-ATPase with concanamycin A (ConA), which

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**Figure 3: The Sestrins function upstream of the Rag GTPases and GATOR1**

(A) The Sestrins function upstream of the Rag GTPases. Rag heterodimers containing constitutively active RagB^{S65L}-RagC^{S75N} or the dominant negative RagB^{S4N}-RagC^{121L} mutants were cotransfected alongside the indicated cDNAs in HEK-293T cells. Anti-FLAG immunoprecipitates were prepared and protein lysates were analyzed by immunoblotting for the indicated proteins.

(B) GATOR1 is necessary for the Sestrins to inhibit mTORC1 signaling. The indicated constructs were stably overexpressed in control HEK-293E cells or in cells lacking the indicated GATOR1 components generated via the CRISPR/Cas9 method. Lysates were probed via immunoblotting for the indicated proteins.
Figure S3
decreased mTORC1 signaling, also reduced the interaction between Sestrin2 and GATOR2 in the absence of amino acids (Figure S3B).

Taken together, these results demonstrate that Sestrin2 requires GATOR1 and the Rags in order to inhibit mTORC1 signaling and are consistent with it having a modulatory role in the amino acid sensing pathway upstream of mTORC1.

**The Sestrins are necessary for the amino acid-regulated subcellular localization of mTORC1**

Given that Sestrin2 is upstream of GATOR1 and the Rags, we reasoned that the Sestrins might inhibit the pathway by controlling the subcellular localization of mTORC1, analogous to previously characterized regulators of the amino acid sensing pathway (Bar-Peled et al., 2013; Petit et al., 2013; Sancak et al., 2010; Sancak et al., 2008; Tsun et al., 2013; Zoncu et al., 2011). Consistent with this notion, in HEK-293T cells stably overexpressing FLAG-Sestrin2, mTORC1 failed to translocate to LAMP2-positive lysosomes despite the presence of amino acids (Figure 4A and S4A). Conversely, shRNA-mediated knockdown of Sestrin1 and Sestrin2 led to increased levels of lysosome-associated mTORC1 even in the absence of amino acids (Figure 4B). The shRNA-mediated knockdown of Sestrin1 and Sestrin3 in Sestrin2-null cells further increased the localization of mTORC1 to lysosomes under amino acid deprivation conditions (Figure S4B). In combination, these results indicate that the Sestrins are

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**Figure S3, related to Figure 3: The Sestrins do not affect the GAP activity of GATOR1 and inhibition of the v-ATPase affects the Sestrin2-GATOR2 interaction.**

(A) GATOR1 purified from HEK-293T cells overexpressing Sestrin2 is equally active towards RagB as GATOR1 purified from cells without Sestrin2 overexpression. Five picomoles of Flag RagB HA RagC 181N were loaded with [γ32P]GTP and incubated with the indicated proteins. LRS is leucyl tRNA synthetase. GTP hydrolysis was determined by thin layer chromatography. 
(B) Inhibition of the v-ATPase with concanamycin A (ConA) reduces the interaction between GATOR2 and Sestrin2. HEK-293T cells were treated with either 11.5 uM ConA or DMSO for 1 hour, during which time the cells were starved of amino acids for 1 hour or starved for 50 minutes and restimulated with amino acids for 10 minutes. Immunoprecipitates were collected using either an antibody targeting WDR24 or a control protein, GSK3β, and were analyzed, along with cell lysates, by immunoblotting for the indicated proteins.
Figure 4

A

Cells expressing antibody

- a.a. for 50 min
- a.a. for 50 min
+ a.a. for 10 min

FLAG-metap2
FLAG-Seatin2

mTOR LAMP2
mTOR LAMP2

merge
merge

B

shRNA: antibody:

- a.a. for 50 min
- a.a. for 50 min
+ a.a. for 10 min

GFP Sesn1 & Sesn2

mTOR LAMP2
mTOR LAMP2

merge
merge

C

amino acids

Sesnin 1/2

mTORC1

GATOR1

GATOR2

mTORC1

lysosome

GTP

GAP activity

translocation
negative regulators of mTORC1 signaling and are necessary for the amino acid-dependent localization of mTORC1 to the lysosomal surface (Figure 4C).

Amino acids must be present in the cellular environment for mTORC1 to be active and are sensed through a signaling pathway that culminates with the nucleotide loading of the Rag GTPases (reviewed in (Bar-Peled and Sabatini, 2014; Efeyan et al., 2012b; Kim et al., 2013; Yuan et al., 2013)). Many regulators impact the nucleotide loading state of the Rags in response to amino acid availability, most notably Ragulator and GATOR1, which are a GEF and GAP, respectively, for RagA/B. GATOR2 is a poorly studied complex that acts upstream of or in parallel with GATOR1 and is a positive component of the mTORC1 pathway. Here, we identify the Sestrins as GATOR2-interacting proteins that require GATOR1 and the Rags to function as negative regulators of the amino acid pathway upstream of mTORC1. In addition, we show that amino acids levels regulate the strength of the interaction between Sestrin2 and GATOR2, and that the Sestrins are necessary for mTORC1 recruitment to the lysosomal surface in response to amino acids.

Interestingly, inhibition of the v-ATPase, a known regulator of mTORC1 activity which engages in amino acid-regulated interactions with Ragulator on the lysosomal surface, disrupts the Sestrin2-GATOR2 interaction in the absence of amino acids (Figure S3B). As of yet, the relationship between the branch involving the GATOR complexes and the branch involving the Ragulator/v-ATPase complexes upstream of mTORC1 has not been thoroughly investigated, and these data imply that there may be

Figure 4: The Sestrins control mTORC1 localization in response to amino acids
(A) Sestrin2 overexpression prevents proper mTORC1 recruitment to lysosomes. HEK-293T cells stably expressing the indicated recombinant proteins were starved or starved and restimulated with amino acids for the indicated times prior to processing for immunofluorescence. Insets depict selected fields that were magnified 3.24 times and their overlays.
(B) Sestrin1 and Sestrin2 loss results in constitutive mTORC1 localization to the lysosome. HEK-293T cells stably expressing the indicated shRNA constructs were processed as described above in (A).
Figure S4

A

Cells expressing: FLAG-Sestrin2

- a.a. for 50 min

- a.a. for 50 min
  + a.a. for 10 min

B

HEK-293T clone: shRNA: antibody:

- a.a. for 50 min

- a.a. for 50 min
  + a.a. for 10 min

FLuG-Sestrin2

mTOR

LAMP2

merge

*gGFP

GFP

merge

Iii

&gSean2

Sesni-2

and Seen3-2

mTOR

LAMP2

merge

mTOR

LAMP2

merge
Figure S4, related to Figure 4: The Sestrins control amino acid-dependent mTORC1 localization to the lysosome.

(A) Overexpression of FLAG-Sestrin2 impairs proper mTOR localization to the lysosome in response to amino acids. HEK-293T cells stably overexpressing the indicated recombinant proteins were processed for immunofluorescence as indicated in Figure 4A.

(B) Knockdown of Sestrin1 and Sestrin3 leads to an increase in mTOR localization to the lysosomes under amino acid deprivation. shRNA-mediated knockdown of the indicated genes was performed in Sestrin2-null cells generated by the CRISPR/Cas9 system and processed for immunofluorescence as indicated in Figure 4A. All scale bars represent 10 μm.

some crosstalk between the two branches. However, further work must be performed to determine if the effect of concanamycin A on the Sestrin2-GATOR2 interaction is a direct or an indirect effect of inhibiting the v-ATPase.

Our work raises several interesting questions. First, the mechanism through which the Sestrins act as negative regulators of mTORC1 signaling remains unknown. Although initially our most appealing hypotheses, the Sestrins do not appear to inhibit the pathway by disrupting the interaction between GATOR1 and GATOR2 (Figure S1B), nor do they affect the GAP activity of GATOR1 towards RagA/B (Figure S3A). Another possibility is that the Sestrins inhibit GATOR2 function, which is in turn necessary to signal amino acid sufficiency to the Rags. The function of GATOR2 is unknown, and therefore, while this is a tempting mechanism through which the Sestrins may affect the mTORC1 pathway, it is currently impossible to test.

Although the Sestrins have weak homology to a family of alkyl hydroxyperoxidase enzymes in Mycobacterium tuberculosis, they do not appear to possess any reductase activity (Budanov et al., 2004; Woo et al., 2009). An intriguing possibility is that the Sestrins possess an enzymatic function that is linked to their role as negative regulators of the amino acid sensing branch of mTORC1, but further studies are needed to understand if the Sestrins retain any enzymatic activity.

Another question is what role, if any, the Sestrins play in tumorigenesis. Here, we demonstrate that loss of the Sestrins renders cells unable to fully inhibit mTORC1 in the absence of amino acids. Similarly, GATOR1-null cells retain constitutive mTORC1

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signaling in the absence of amino acids. DEPDC5, Nprl2, and Nprl3, which together encode GATOR1, are thought to act as tumor suppressor genes (Bar-Peled et al., 2013). It has previously been posited that the Sestrins may act as tumor suppressor genes (Budanov et al., 2010), and mutations in all three Sestrin genes have been detected by cancer genome sequencing efforts (Bamford et al., 2004). However, we show here that the three Sestrins have a large degree of redundancy, and thus a cancer cell may need to lose two or all Sestrins to significantly affect mTORC1 signaling, which may be unlikely.

Finally, the Sestrins have previously been reported to be negative regulators of mTORC1 signaling through AMPK and TSC, which act in the growth factor sensing branch upstream of mTORC1, distinct from the amino acid sensing branch (Budanov and Karin, 2008). Although it is clear from our work that the Sestrins affect the amino acid sensing pathway, it remains to be clarified whether they modulate both of these branches upstream of mTORC1, and what the relative importance of each of these potential effects is. While we were unable to detect any interaction between recombinant Sestrin2 and endogenous TSC (Figure S1E), further work is needed to fully understand the effect of the Sestrins on these two separate signaling pathways.
Materials and Methods

Materials

Reagents were obtained from the following sources: LAMP2 H4B4 and HRP-labeled anti-mouse and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, S6K1, Sestrin2, mTOR, Mios and FLAG epitope from Cell Signaling Technology; antibodies to the HA epitope from Bethyl laboratories. RPMI, FLAG M2 affinity gel, ATP, GDP, and amino acids from Sigma Aldrich; DMEM from SAFC Biosciences; XtremeGene9 and Complete Protease Cocktail from Roche; Alexa 488 and 568-conjugated secondary antibodies; Inactivated Fetal Calf Serum (IFS) from Invitrogen; amino acid-free RPMI from US Biologicals. The WDR24 and WDR59 antibodies were generously provided by Jianxin Xie (Cell Signaling Technology).

Cell lysis and immunoprecipitation

Cells were rinsed once with ice-cold PBS and lysed immediately with Triton lysis buffer (1% Triton, 10 mM β-glycerol phosphate, 10 mM pyrophosphate, 40 mM Hapes pH 7.4, 2.5 mM MgCl2 and 1 tablet of EDTA-free protease inhibitor [Roche] (per 25 ml buffer). The cell lysates were clarified by centrifugation at 13,000 rpm at 4°C in a microcentrifuge for 10 minutes. For anti-FLAG-immunoprecipitations, the FLAG-M2 affinity gel was washed with lysis buffer 3 times. 30 μl of a 50% slurry of the affinity gel was then added to cleared cell lysates and incubated with rotation for 2 hours at 4°C. The beads were washed 3 times with lysis buffer containing 500 mM NaCl. In the case of transient cotransfection assays to explore the interaction of the Sestrins with GATOR2, beads were incubated in the final salt wash for 30 minutes to reduce non-specific binding. Immunoprecipitated proteins were denatured by the addition of 50 μl of sample buffer and boiling for 5 minutes as described (Kim et al., 2002), resolved by 8%–16% SDS-PAGE, and analyzed by immunoblotting.

For co-transfection experiments in HEK-293T cells, 2 million cells were plated in 10 cm culture dishes. Twenty-four hours later, cells were transfected via the
polyethylenimine method (Boussif et al., 1995) with the pRK5-based cDNA expression plasmids indicated in the figures in the following amounts: 300 ng Flag-Metap2, 100 ng Flag-WDR24, 50 ng Flag-Sestrin1, 25 ng Flag-Sestrin2, or 200 ng Flag-Sestrin3; 2 ng of Flag-S6K1, or 200 ng each of HA-Mios, HA-WDR59, HA-WDR24, HA-Sec13, HA- Seh1L, HA-Depdc5, HA-Nprl3, or HA-Nprl2. The total amount of plasmid DNA in each transfection was normalized to 5 μg with empty pRK5. Thirty-six hours after transfection, cells were lysed as described above.

For experiments which required amino acid starvation or restimulation, cells were treated as previously described (Tsun et al., 2013). Briefly, cells were incubated in amino acid free RPMI for 50 minutes and then stimulated with amino acids for 10 minutes. For glucose starvation, cells were incubated in RPMI media lacking glucose but containing amino acids and dialyzed serum for 50 minutes, followed by a 10 minute restimulation with 5 mM D-Glucose. For insulin deprivation, cells were incubated in RPMI without serum for 50 minutes and restimulated with 1 ug/ml insulin for 10 minutes. Finally, when Torin1 or Rapamycin was used, cells were incubated with 250 nM of each throughout the starvation and restimulation period.

**Generation of CRISPR/Cas9 genetically modified cells**

To generate HEK-293T cells with loss of GATOR2 components or Sesn2, the following sense (S) and antisense (AS) oligonucleotides encoding the guide RNAs were cloned into the pX330 vector (Petit et al., 2013).

sgMios_1S: caccgATCACATCAGTAAACATGAG  
sgMios_1AS: aaacCTCATGTTTACTGATGTGATc

sgWDR24_1S: caccgACCCAGGGCTGTGGTCACAC  
sgWDR24_1AS: aaacGTGTGACCACAGCCCTGGGTc

sgWDR59_1S: caccgCGGGGGAGATGGCGGCGAGA  
sgWDR59_1AS: aaacTCGCGCCGCATCTCCCCCGc
On day one, 200,000 cells were seeded into 6 wells of a 6-well plate. Twenty-four hours post seeding, each well was transfected with 250 ng shGFP pLKO, 1 ug of the pX330 guide construct, 0.5 ug of empty pRK5 using XtremeGene9. The following day, cells were trypsinized, pooled in a 10 cm dish, and selected with puromycin to eliminate untransfected cells. Forty-eight hours after selection, the media was aspirated and replenished with fresh media lacking puromycin. The following day, cells were single cell sorted with a flow cytometer into the wells of a 96-well plate containing 150 ul of DMEM supplemented with 30% IFS. Cells were grown for two weeks and the resultant colonies were trypsinized and expanded. Clones were validated for loss of the relevant protein via immunoblotting.

Extended Experimental Procedures

Cell lines and tissue culture

HEK-293T and HEK-293E cells were cultured in DMEM 10% IFS supplemented with 2 mM glutamine. All cell lines were maintained at 37°C and 5% CO2.
Identification of Sestrin by mass spectrometry

Immunoprecipitates from HEK-293T cells stably expressing FLAG-Metap2, FLAG-Mios, FLAG-WDR24 or FLAG-WDR59 were prepared using Triton or Chaps lysis buffer without crosslinking as described earlier. Proteins were eluted with the FLAG peptide (sequence DYKDDDDK) from the FLAG-M2 affinity gel, resolved on 4-12% NuPage gels (Invitrogen), and stained with simply blue stain (Invitrogen). Each gel lane was sliced into 10-12 pieces and the proteins in each gel slice digested overnight with trypsin. The resulting digests were analyzed by mass spectrometry as described (Sancak et al., 2008). Peptides corresponding to Sestrin family members were detected in the FLAG-Mios, FLAG-WDR24 and FLAG-WDR59 immunoprecipitates, while no peptides were detected in negative control immunoprecipitates of FLAG-Metap2.

Mammalian lentiviral shRNAs

On day one, 750,000 HEK-293T cells were seeded in a 6 well plate in DMEM supplemented with 20% inactivated fetal bovine serum (IFS). Twenty-four hours later, the cells were transfected with shRNA-encoding plasmids indicated below alongside the Delta VPR envelope and CMV VSV-G packaging plasmids using XtremeGene9 transfection reagent. Lentiviral shRNAs targeting Sestrin1, Sestrin2, and Sestrin3 were obtained from the TRC. The TRC number for each shRNA is as follows:

Human Sestrin1 shRNA_1: TRCN0000143187
Human Sestrin1 shRNA_2: TRCN0000435014
Human Sestrin2 shRNA_1: TRCN0000143630
Human Sestrin2 shRNA_2: TRCN0000122802
Human Sestrin3 shRNA_1: TRCN0000412760
Human Sestrin3 shRNA_2: TRCN0000088252

Twelve hours post transfection, the old media was aspirated and replaced with 2 ml fresh media. Virus-containing supernatants were collected 36 hours after replacing media and passed through a 0.45 micron filter to eliminate cells. Four million cells in the presence of 8 μg/ml polybrene (Millipore) were infected with 1 ml of virus for each construct in the case of single knockdown or with 500 ul of virus in the case of double or triple knockdown in 2 ml total volume of media and then spun at 2,200 rpm for 45 minutes at 37°C. Forty-eight hours after selection, cells were trypsinized and selected with puromycin and seeded on the 3rd day for signaling experiments, as described.
To validate knockdown of Sesn1 and Sesn3, the following primer pairs were used in an
RT-PCR reaction due to the lack of antibodies to these proteins. The data were analyzed via the
delta-delta Ct method (Schmittgen and Livak, 2008).

Sesn1 Forward: TGGCAATGCACAAAGATGTTG
Sesn1 Reverse: GCTACGATCCAATAGCCTGGTT

Sesn3 Forward: TGCGTTTGTGATCTTGCTAATG
Sesn3 Reverse: CGCCTCTTCATCTTCCCTTTCC

**Immunofluorescence assays**

Immunofluorescence assays were performed as described in (Sancak et al., 2010). Briefly, 300,000 HEK-293T cells were plated on fibronectin-coated glass coverslips in 6-well tissue culture plates. Twenty-four hours later, the slides were rinsed with PBS once and fixed for 15 min with 4% paraformaldehyde in PBS at room temperature. The slides were rinsed three times with PBS and cells were permeabilized with 0.05% Triton X-100 in PBS for 5 min. After rinsing three times with PBS, the slides were blocked for 1 hour in Odyssey blocking buffer, and then incubated with primary antibody in Odyssey blocking buffer for 1 hr at room temperature, rinsed three times with PBS, and incubated with secondary antibodies produced in donkey (diluted 1:1000 in Odyssey blocking buffer) for 45 minutes at room temperature in the dark and washed three times with PBS. Slides were mounted on glass coverslips using Vectashield (Vector Laboratories) and imaged on a spinning disk confocal system (Perkin Elmer).

**Rag GTP hydrolysis assay**

GAP assays were performed essentially as previously described (Bar-Peled et al., 2013). In brief, the indicated GTPases were bound to FLAG-M2 affinity gel and loaded with XDP and [γ-32P]GTP at room temperature followed by an incubation with MgCl2 to stabilize the nucleotide. The GTPases were subsequently washed to remove unbound nucleotide and eluted from the affinity gel with competing FLAG peptide. Protein concentrations were determined prior to use.

For the TLC-based GTP hydrolysis assay, 5 pmoles of the indicated Rag heterodimer was loaded with xanthine nucleotides and [γ32P]GTP were added to 20 pmoles of purified LRS, GATOR1, or GATOR1 purified from HA-Sesn2 overexpressing cells in 45 μl of GTPase wash
buffer. The reaction was incubated at 25°C for the indicated times and eluted samples were spotted on PEI Cellulose plates and developed for 2.5 hr in 0.5 M KH2PO4 pH 3.4. Plates were exposed to film and spot densities were quantified with ImageJ.

References for extended experimental procedures


Acknowledgements

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CHAPTER 4

Sestrin2 is a leucine sensor for the mTORC1 pathway

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This work has been published in:


Experiments in Figures 1, 3, 4, S2, and S3 were performed by LC. Experiments in Figures 2, 3, 4, S1, S2, and S4 were performed by RLW. Experiments in Figures 1 were performed by RAS.
Summary

Leucine is a proteogenic amino acid that also regulates many aspects of mammalian physiology, in large part by activating the mTOR complex 1 (mTORC1) protein kinase, a master growth controller. Amino acids signal to mTORC1 through the Rag guanine triphosphatases (GTPases). Several factors regulate the Rags, including GATOR1, a GTPase activating protein (GAP); GATOR2, a positive regulator of unknown function; and Sestrin2, a GATOR2-interacting protein that inhibits mTORC1 signaling. We find that leucine, but not arginine, disrupts the Sestrin2-GATOR2 interaction by binding to Sestrin2 with a $K_d$ of 20 μM, which is the leucine concentration that half-maximally activates mTORC1. The leucine-binding capacity of Sestrin2 is required for leucine to activate mTORC1 in cells. These results indicate that Sestrin2 is a leucine sensor for the mTORC1 pathway.
Introduction

It has long been appreciated that in addition to being a proteogenic amino acid, leucine is also a signaling molecule that directly regulates animal physiology, including satiety (1), insulin secretion (2), and skeletal muscle anabolism (3, 4). Because the liver has a low capacity to metabolize leucine, its blood concentrations fluctuate in accord with its consumption so that dietary leucine can directly impact physiology (5-7). A key mediator of the effects of leucine is the mTORC1 protein kinase (8, 9), which regulates growth by controlling processes like protein and lipid synthesis as well as autophagy.

Many environmental signals besides leucine regulate the mTORC1 pathway, including other amino acids like arginine, as well as glucose and various growth factors and forms of stress (10, 11). How mTORC1 senses and integrates these diverse inputs is not well understood, but it is clear that the Rheb and Rag guanine triphosphatases (GTPases) have necessary but distinct roles. Rheb is a monomeric GTP binding protein and the Rags function as obligate heterodimers of RagA or RagB bound to RagC or RagD (12-14). Both the Rheb and Rag GTPases localize, at least in part, to the lysosomal surface (15-18), which is an important site of mTORC1 regulation (19). In a Rag-dependent manner amino acids promote the translocation of mTORC1 to the lysosome where Rheb, if bound to GTP, stimulates its kinase activity. Growth factors trigger the GTP-loading of Rheb by driving its GTPase activating protein (GAP), the tuberous sclerosis (TSC) complex, off the lysosomal surface (18).

Regulation of the Rag GTPases by amino acids is complex, and many distinct factors have important roles (20). A lysosome-associated super-complex containing Ragulator, SLC38A9, and the vacuolar adenosine triphosphase (v-ATPase) interacts with the Rag GTPases and is necessary for the activation of mTORC1 by amino acids (21-24). Ragulator anchors the Rag heterodimers to the lysosome and has nucleotide exchange activity for RagA and RagB (21, 25). SLC38A9 is an amino acid transporter and a potential lysosomal arginine sensor (23), but the function of the v-ATPase in mTORC1 activation is unclear. Two GAP complexes stimulate GTP hydrolysis by the
Rag GTPases, with GATOR1 acting on RagA and RagB (26) and Folliculin (FLCN)-Folliculin interacting protein 2 (FNIP2) on RagC and RagD (27). The separate GATOR2 complex negatively regulates GATOR1 through an unknown mechanism and is necessary for mTORC1 activation (26). Lastly, the Sestrins are GATOR2-interacting proteins that inhibit mTORC1 signaling but whose molecular function is not known (28, 29).

The amino acids sensors upstream of mTORC1 have been elusive for many years. While SLC38A9 is a strong candidate for sensing arginine at lysosomes (23), the long-sought sensor of leucine was unknown. We demonstrate that Sestrin2 is a leucine sensor for the mTORC1 pathway.
Results and Discussion

Leucine directly regulates the Sestrin2-GATOR2 interaction

Activation of mTORC1 by amino acids requires the pentameric GATOR2 complex (26). Although its molecular function is unknown, epistasis-like experiments suggest that GATOR2 suppresses GATOR1, the GAP for and inhibitor of RagA and RagB (26). Within cells Sestrin2 binds to GATOR2 in an amino acid-sensitive manner (28, 29), with removal and re-addition of all amino acids from the culture media inducing and reversing the interaction, respectively (28). Although several amino acids can regulate mTORC1 signaling, arginine and leucine are the best established and deprivation of either strongly inhibits mTORC1 in various cell types (8, 30, 31). In human embryonic kidney-293T (HEK-293T) cells removal of either leucine or arginine from the cell medium inhibited mTORC1 signaling, as read out by ribosomal protein S6 kinase 1 (S6K1) phosphorylation, to similar extents. Strikingly, however, only leucine depletion caused Sestrin2 to bind to GATOR2, inducing the interaction as effectively as complete amino acid starvation (Fig. 1A). Leucine re-addition rapidly reversed the binding and amino acids did not affect the interaction between WDR24 and Mios, two core components of GATOR2 (Fig. 1A, and S1A).

Sestrin2 is homologous to two other proteins, Sestrin1 and Sestrin3 (32-34), and when overexpressed all three can interact with GATOR2 (28). As with Sestrin2, leucine starvation and stimulation strongly regulated the interaction of endogenous Sestrin1 with GATOR2 (Fig. S1B). In contrast, endogenous Sestrin3 bound to GATOR2 irrespective of leucine concentrations (Fig. S1B), suggesting that this interaction is constitutive or regulated by signals that remain to be defined.

While enzymatic events triggered by leucine might mediate the effects of leucine on the Sestrin2-GATOR2 interaction, it was tempting to consider that leucine might act directly on the complex. Consistent with this possibility, the addition of leucine, but not arginine, to ice-cold detergent lysates of cells deprived of all amino acids abrogated the
Figure 1

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IP: FLA-WDR24
When added to the purified complexes, leucine dose-dependently disrupted the Sestrin2-GATOR2 complex, with the half maximal effect at about 1 μM (Fig. 1D). Methionine and isoleucine were considerably less potent, acting at concentrations approximately 10- and 25-fold greater than leucine, respectively (Fig. 1E). These values reflect only the relative potencies of these amino acids as equilibrium conditions were not attained because the large assay volume precluded Sestrin2 from rebinding to GATOR2 once dissociated.

**Sestrin2 binds leucine with a $K_d$ of 20 μM**

Given that leucine disrupts the purified complex, we reasoned that leucine might directly bind to Sestrin2 or GATOR2. To test this, we developed an equilibrium binding...
Sestrin2 binds leucine with a $K_d$ of 20 μM

Given that leucine disrupts the purified complex, we reasoned that leucine might directly bind to Sestrin2 or GATOR2. To test this, we developed an equilibrium binding assay in which purified proteins immobilized on agarose beads were incubated with radioactive amino acids, and the bound amino acids were quantified after washing. Radiolabeled leucine bound to Sestrin2, but not WDR24, the GATOR2 complex, or the control protein Rap2A, in a manner that was fully competed by excess non-radiolabeled leucine (Fig. 2A). In contrast, arginine did not bind to either Sestrin2 or Rap2A (Fig. S2A). Consistent with the differential sensitivities of the Sestrin1- and Sestrin3-GATOR2 complexes to leucine, Sestrin1 bound leucine to a similar extent as did Sestrin2, whereas Sestrin3 bound very weakly (Fig. 2B and S2A). Drosophila dSestrin (CG11299-PD) also bound leucine, albeit at lower amounts than the human protein (Fig. S2B and C).
whereas Sestrin3 bound very weakly (Fig. 2B and S2A). *Drosophila dSestrin* (CG11299-PD) also bound leucine, albeit at lower amounts than the human protein (Fig. S2B and C).

As all of these proteins were expressed in and purified from human HEK-293T cells, it remained formally possible that an unidentified protein that co-purifies with Sestrin2 (and Sestrin1) is the actual receptor for leucine. To address this possibility, we prepared human Sestrin2 in bacteria, a heterologous system that does not encode a Sestrin homologue or even a TOR pathway. Consistent with the results obtained with Sestrin2 prepared in human cells, radiolabelled leucine bound to bacterially-produced

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**Figure 2: Sestrin2 binds leucine with a $K_d$ of 20 μM**

(A) Binding of radiolabeled leucine to Sestrin2, but not WDR24, GATOR2, or the control protein Rap2A. FLAG immunoprecipitates prepared from HEK-293T cells transiently expressing indicated proteins or complexes were used in binding assays with $[^3H]$Leucine as described in the methods. Unlabeled leucine was added where indicated. Values are Mean ± SD for 3 technical replicates from one representative experiment. SDS-PAGE followed by Coomasie blue staining was used to analyze immunoprecipitates prepared in parallel to those included in the binding assays. Asterisks indicate breakdown products in the WDR24 and GATOR2 purifications.

(B) Leucine-binding capacities of Sestrin1 (two isoforms), Sestrin2, and Sestrin3. FLAG immunoprecipitates were prepared and binding assays performed and analyzed as in (A).

(C) Leucine binds to bacterially-produced Sestrin2, but not the RagA/RagC heterodimer. Leucine binding assays were performed as described in the methods and analyzed as in (A) with His-MBP-Sestrin2 or His-RagA/RagC bound to the Ni-NTA resin.

(D) Effects of leucine and arginine on the melting temperature of bacterially-produced Sestrin2 in a thermal shift assay. His-MBP-Sestrin2 was incubated with the Sypro orange dye with or without leucine or arginine. Upon heating the sample the change in fluorescence was captured and used to calculate melting temperatures (Tm) under the indicated conditions. Values are Mean ± SD from 3 replicates.

(E) Sestrin2 binds leucine with a $K_d$ of 20 μM. FLAG-Sestrin2 immunoprecipitates prepared as in (A) were used in binding assays with 10 μM or 20 μM $[^3H]$Leucine and indicated concentrations of unlabeled leucine. In the representative graph shown each point represents the normalized mean ± SD for n = 3 in an assay with 10 μM $[^3H]$Leucine. The $K_d$ was calculated from the results of six experiments (three with 10 μM and three with 20 μM $[^3H]$Leucine).

(F) Methionine can compete the binding of leucine to Sestrin2. FLAG-Sestrin2 immunoprecipitates prepared as in (A) were used in binding assays with 10 μM $[^3H]$Leucine and indicated concentrations of unlabeled methionine. In the graph shown each point represents the normalized mean ± SD for n = 3. The $K_i$ was calculated using data from the three experiments.

(G) Isoleucine can compete the binding of leucine to Sestrin2. FLAG-Sestrin2 immunoprecipitates prepared as in (A) were used in binding assays with 10 μM $[^3H]$Leucine and indicated concentrations of unlabeled isoleucine. In the graph shown each point represents the normalized mean ± SD for n = 3. The $K_i$ was calculated using data from the three experiments.
Sestrin2, but not the RagA-RagC heterodimer, which was used as a control (Fig. 2C). Furthermore, in a thermal shift assay, leucine, but not arginine, shifted the melting temperature by up to 8.5°C of bacterially-produced Sestrin2, but not of two control proteins (Fig. 2D and S2E-F). Collectively, these data strongly argue that leucine binds directly to Sestrin2.

While the thermal shift assay is valuable for assessing the capacity of a protein to bind a ligand, this method is not suitable for obtaining an accurate $K_d$ (35). Therefore, we used a competition binding assay with increasing amounts of unlabeled leucine to determine that leucine has a $K_d$ for Sestrin2 of 20 ± 5 μM (Fig. 2E). In comparison, methionine and isoleucine competed leucine binding with inhibitory constants ($K_i$) of 354 ± 118 μM and 616 ± 273 μM, respectively (Fig. 2F and G). These values are approximately one eighteenth and one thirtieth the affinity of leucine for Sestrin2, and correlate well with the relative potencies of leucine, methionine, and isoleucine in disrupting the Sestrin2-GATOR2 interaction in vitro (Fig. 1D and E).

**Sestrin2 regulates mTORC1 through GATOR2**
Consistent with leucine regulating mTORC1 by modulating the binding of Sestrin2 to GATOR2, 20-40 μM leucine had half-maximal effects on both the Sestrin2-

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**Figure S2: Sestrin2 does not bind arginine, and the capacity of Sestrin2 to bind leucine is conserved in Drosophila Sestrin**

(A) Sestrin2 does not bind arginine. FLAG-Sestrin2 immunoprecipitates prepared as in Figure 2A were used in binding assays with [³H]Arginine with or without unlabeled arginine.

(B) Effects of leucine or arginine on the melting temperature of human choline acetyltransferase (ChAT) in a thermal shift assay. ChAT prepared in bacteria was subjected to a thermal shift assay as in Figure 2C.

(C) Effects of leucine or arginine on the melting temperature of another control protein, Physconitrella patens hydroxycinnamoyl transferase (PpHCT) in a thermal shift assay. (D) SDS-PAGE and Coomasie blue staining analyses of the bacterially prepared proteins (His-MBP-Sestrin2, ChAT, and PpHCT) used in the thermal shift assays.

(E) dSestrin (CG11299-PD) binds to leucine. Binding assays were performed and immunoprecipitates analyzed as in Figure 2A. F) dSestrin (CG11299-PD) interacts with human WDR24, a component of GATOR2. FLAG immunoprecipitates were prepared from HEK-293T cells expressing the indicated proteins, and immunoprecipitates and cell lysates analyzed via immunoblotting.
A. Leucine added to media (pM): 0 1 10 20 40 60 100 200 400

B. Cells expressing: metap2 WDR24

C. Transfected cDNAs:

D. %SAE bound (cpm)

E. HEK-293T cells: Wild-type Sestrin-3 triple null

Figure 3
GATOR2 interaction and mTORC1 activity in HEK-293T cells (Fig. 3A and B). This concentration range encompasses the $K_d$ of leucine for Sestrin2, indicating that the affinity of Sestrin2 for leucine is physiologically relevant.

To formally test whether Sestrin2 regulates mTORC1 by interacting with GATOR2, we identified a Sestrin2 mutant (S190W) that still binds leucine but has a severely decreased capacity to bind GATOR2 (Fig. 3C and D). In Sestrin1-3 triple null HEK-293T cells, mTORC1 signaling was active and unaffected by leucine deprivation (Fig. 3E). In these cells expression of wild-type Sestrin2 restored the leucine sensitivity of the mTORC1 pathway, but that of Sestrin2 S190W had no effect (Fig. 3E). Thus, Sestrin2 must be able to interact with GATOR2 for the mTORC1 pathway to sense the absence of leucine.

For leucine to activate mTORC1, Sestrin2 must be able to bind leucine

To test whether the leucine-binding capacity of Sestrin2 is necessary for mTORC1 to sense the presence of leucine, we identified two Sestrin2 mutants, L261A and E451A, which do not bind leucine to an appreciable degree (Fig. 4A). Leucine did not affect mTORC1 signaling in these cells, indicating that the leucine-binding capacity of Sestrin2 is necessary for it to sense the presence of leucine.

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Figure 3: Sestrin2 regulates mTORC1 through GATOR2

(A) Effects of varying leucine concentrations on mTORC1 activity, as measured by the phosphorylation of S6K1. HEK-293T cells were deprived of leucine for 50 minutes and restimulated with leucine at the indicated concentrations for 10 minutes. Cell lysates were analyzed via immunoblotting for the indicated proteins and phosphorylation states.

(B) Effects of varying leucine concentrations on the Sestrin2-GATOR2 interaction. HEK-293T cells stably expressing the indicated proteins were starved and restimulated as in (A). FLAG immunoprecipitates were prepared and analyzed by immunoblotting for the indicated proteins.

(C) Decreased GATOR2-binding capacity of the Sestrin2 S190W mutant. FLAG immunoprecipitates were prepared from cells transiently expressing the indicated proteins. The immunopurified complexes were treated with the indicated concentrations of leucine and then analyzed as in Figure 1C.

(D) Determination of leucine-binding capacity of Sestrin2 S190W. Assays were performed and immunoprecipitates analyzed as in Figure 2A.

(E) In Sestrin1-3 triple null cells expressing Sestrin2 S190W the mTORC1 pathway cannot sense the absence of leucine. Wild-type HEK-293T cells and Sestrin1-3 triple null HEK-293T cells generated with the CRISPR/Cas9 system were used to express the indicated FLAG-tagged proteins. Cells were starved for leucine for 50 minutes and, where indicated, stimulated with leucine for 10 minutes and lysates analyzed via immunoblotting.
### HEK-293T cells: Wild-type Sestrin1-3 triple null

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**Supp. Figure 3**
not significantly affect the interaction of the mutants with GATOR2 in vitro, consistent with Sestrin2 mediating the effects of leucine on the Sestrin2-GATOR2 complex (Fig. 4B). Expression of wild-type Sestrin2 in the Sestrin1-3 triple null cells restored the leucine sensitivity of the mTORC1 pathway in these cells, but that of either mutant inhibited signaling and rendered it insensitive to leucine (Fig. 4C and S3A). Furthermore, in Sestrin1-3 triple null cells expressing the mutants, the localization of mTOR to lysosomes in the presence of leucine was decreased, while that of RagC was not affected (Fig. S4A-D). Thus, activation of mTORC1 by leucine requires the binding of leucine to Sestrin2.

Figure S3: Expression in Sestrin1-3 triple null cells of Sestrin2 L261A or E451A at levels much lower than wild-type Sestrin2 still renders mTORC1 signaling insensitive to leucine stimulation

(A) Expression in Sestrin1-3 triple null cells of Sestrin2 L261A or E451A does not restore the capacity of the mTORC1 pathway to sense the presence of leucine, even when mutants are expressed at levels much lower than wild-type Sestrin2. Cells were generated and analyzed as in Figure 3E. Note that wild-type recombinant Sestrin2 is overexpressed relative to endogenous levels, explaining why it partially suppresses mTORC1 signalling. The Sestrin2 E451A mutant is expressed at levels similar to the endogenous protein, and both mutants are expressed at much lower levels than wild-type Sestrin2. All forms of Sestrin2 in this experiment were expressed from the pLJC5 plasmid.
Figure 4

A

B

C

D
Figure 4: The capacity of Sestrin2 to bind leucine is required for the mTORC1 pathway to sense leucine

(A) The Sestrin2 L261A and E451A mutants do not bind leucine. Binding assays were performed and immunoprecipitates analyzed as in Figure 2A.

(B) Leucine-insensitivity of the interactions of Sestrin2 L261A or E451A with GATOR2. FLAG immunoprecipitates were prepared from cells transiently expressing the indicated proteins. The immunoprecipitates were treated with the indicated concentrations of leucine and analyzed as in Figure 1C.

(C) In Sestrin1-3 triple null cells expressing Sestrin2 L261A or E451A the mTORC1 pathway cannot sense the presence of leucine. Cells were generated and analyzed as in Figure 3E.

(D) Model showing how amino acid inputs arising from multiple sensors in distinct compartments impinge on the Rag GTPases to control mTORC1 activity.
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Figure S4: Expression of Sestrin2 L261A or E451A in Sestrin1-3 triple null cells decreases the localization of mTOR to lysosomes in the presence of leucine

(A) mTOR localization upon leucine deprivation and restimulation in wild-type and Sestrin1-3 triple null cells. HEK-293T cells (wild-type or Sestrin1-3 triple null created via the CRISPR/Cas9 system) stably expressing the indicated proteins were deprived of or deprived of and restimulated with leucine for the indicated times prior to processing for immunofluorescence. Insets depict selected fields that were magnified 3.24X and their overlays. All scale bars represent 10 μm.

(B) Effects of wild-type Sestrin2, or Sestrin2 L261A or E451A, on mTOR localization. Sestrin1-3 triple null HEK-293T cells stably expressing the indicated proteins were treated and analyzed as in (A).

(C) RagC localizes to the lysosome in wild-type and Sestrin1-3 triple null cells. HEK-293T cells (wild-type and Sestrin1-3 triple null) were treated and processed as in (A).

(D) RagC localization in Sestrin1-3 triple null cells reconstituted with wild-type Sestrin2 or either Sestrin2 mutant. Cells were processed for immunofluorescence as in (A).
Conclusions

Sestrin2 has several properties consistent with it being a leucine sensor for the mTORC1 pathway: (1) it binds leucine at affinities consistent with the concentrations at which leucine is sensed; (2) Sestrin2 mutants that do not bind leucine cannot signal the presence of leucine to mTORC1; and (3) loss of Sestrin2 and its homologs renders the mTORC1 pathway insensitive to the absence of leucine. Although we have not investigated Sestrin1 as thoroughly, it appears to behave similarly to Sestrin2, so we propose that Sestrin1 and Sestrin2 are leucine sensors upstream of mTORC1.

Given that Sestrin2 has appreciable affinity for methionine, it would not be surprising if in contexts where leucine concentrations are low and those of methionine are high, Sestrin2 may serve as a methionine sensor for the mTORC1 pathway.

Sestrin2 binds to and likely inhibits GATOR2, but how this leads to suppression of mTORC1 awaits elucidation of the molecular function of GATOR2. In addition, structural studies are needed to understand how the binding of leucine to Sestrin2 disrupts its interaction with GATOR2 and why leucine binds very poorly to Sestrin3.

As Sestrin1 and Sestrin2 are soluble proteins, it is likely that they sense free leucine in the cytosol. Although these concentrations are unknown, the $K_m$ of the human leucyl-tRNA synthetase (LRS) for leucine has been reported to be 45 μM (36), which is similar to the affinity of Sestrin2 for leucine, suggesting that cytosolic free leucine concentrations are within this range. Like Sestrin2, LRS can bind isoleucine and methionine at lower affinities than leucine (about 30 fold less in the case of LRS) (36). The similarities between the amino acid binding characteristics of Sestrin2 and LRS support the notion that the affinity and specificity of Sestrin2 for leucine are sufficient for it to serve as a leucine sensor.

Our work suggests a model in which signals emerging from distinct amino acid sensors in different cellular compartments converge on the Rag GTPases at the
lysosomal surface to regulate mTORC1 activity (Fig. 4D). The putative arginine sensor SLC38A9 likely monitors lysosomal contents and Sestrin2 is almost certainly a cytosolic sensor. There must also be an amino acid sensor upstream of the GAP for RagC and RagD, the FLCN-FNIP2 complex, but its identity and cellular localization are unknown. A future challenge is to elucidate how the Rag GTPases integrate the inputs coming from the different sensors, which will likely require a much better understanding of the function of each Rag in the heterodimer. Moreover, in vivo characterization of the different sensors will be needed to comprehend how specific tissues adapt the amino acid sensing pathway to their particular needs.

Given that Sestrin2 (and Sestrin1) are likely to have leucine-binding pockets, these proteins may be targets for developing small molecule modulators of the mTORC1 pathway. Leucine attenuates the decrease in skeletal muscle protein synthesis that occurs in the elderly and stimulates satiety (1, 37). Thus, small molecules that potently mimic the effects of leucine on Sestrin2 could have therapeutic value. Furthermore, caloric restriction (CR) inhibits mTORC1 signaling (38, 39) and is associated with increases in healthspan and lifespan in multiple organisms (40, 41). Thus, small molecules that antagonize the effects of leucine on Sestrin2 might have CR-mimicking properties.
Materials and Methods

Materials

Reagents were obtained from the following sources: HRP-labeled anti-mouse and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, S6K1, Sestrin2, Mios and the FLAG epitope from Cell Signaling Technology; antibodies to the HA epitope from Bethyl laboratories; antibody to raptor from Millipore. FLAG M2 affinity gel, ATP, and amino acids from Sigma Aldrich; RPMI without leucine, arginine, or lysine from Pierce; DMEM from SAFC Biosciences; XtremeGene9 and Complete Protease Cocktail from Roche; Inactivated Fetal Calf Serum (IFS) and SimplyBlue SafeStain from Invitrogen; amino acid-free RPMI from US Biologicals; [3H]-labelled amino acids from American Radiolabeled Chemicals. The WDR24, Mios, Sestrin1, and Sestrin3 antibodies were generously provided by Jianxin Xie of Cell Signaling Technology.

Cell lines and tissue culture

HEK-293T cells were cultured in DMEM 10% IFS supplemented with 2 mM glutamine. All cell lines were maintained at 37°C and 5% CO2.

Cell lysis and immunoprecipitation

Cells were rinsed one time with ice-cold PBS and immediately lysed with Triton lysis buffer (1% Triton, 10 mM β-glycerol phosphate, 10 mM pyrophosphate, 40 mM Hapes pH 7.4, 2.5 mM MgCl2 and 1 tablet of EDTA-free protease inhibitor [Roche] (per 25 ml buffer). The cell lysates were cleared by centrifugation at 13,000 rpm at 4°C in a microcentrifuge for 10 minutes. For anti-FLAG-immunoprecipitations, the FLAG-M2 affinity gel was washed 3 times with lysis buffer. 30 μl of a 50/50 slurry of the affinity gel was then added to clarified cell lysates and incubated with rotation for 2 hours at 4°C. Where indicated, leucine or arginine (500 μM final) were added to lysates immediately prior to addition of the Flag affinity gel. Following immunoprecipitation, the beads were washed one time with lysis buffer and 3 times with lysis buffer containing 500 mM NaCl. Immunoprecipitated proteins were denatured by the addition of 50 μl of sample buffer
and boiling for 5 minutes as described (42), resolved by 8%–16% SDS-PAGE, and analyzed by immunoblotting.

For co-transfection experiments in HEK-293T cells, 2 million cells were plated in 10 cm culture dishes. Twenty-four hours later, cells were transfected using the polyethylenimine method (43) with the pRK5-based cDNA expression plasmids indicated in the figures in the following amounts: 100 ng HA-WDR24 and 300 ng FLAG-Metap2, 25 ng FLAG-Sestrin2, or 50 ng FLAG-dSestrin (CG11299-PD); 100 ng FLAG-WDR24 and 15 ng each of HA-Sestrin2, HA-Sestrin2 S190W, HA-Sestrin2 L261A, or HA-Sestrin2 E451A. The total amount of plasmid DNA in each transfection was normalized to 5 µg with empty pRK5. Thirty-six hours after transfection, cells were lysed as described above.

For experiments which required amino acid starvation or restimulation, cells were treated as previously described (27). Briefly, cells were incubated in amino acid free RPMI for 50 minutes and then restimulated with amino acids for 10 minutes. The same protocol was followed for both leucine and arginine single starvation and restimulations.

**Purification of proteins expressed in bacteria**

Recombinant Sestrin 2 was expressed in *Escherichia coli* (strain BL21 DE3 star) from the His-MBP-TEV-Sestrin2 in pMAL6H-C5XT plasmid. The bacterial cultures were grown at 30°C to an optical density of 0.4 at which point the temperature was lowered to 18°C. After 30 minutes at 18°C, the cultures were induced overnight at 18°C with 0.5 mM IPTG. The cells were subsequently resuspended in lysis buffer with TCEP (50 mM Tris pH 7.4, 200 mM NaCl, 5 mM MgCl₂, 0.1% CHAPS, 1 mM TCEP, 200 µM leucine, and protease inhibitor tablets), which was then supplemented with lysozyme and crude DNAs. The cells underwent mechanical homogenization and the lysates were cleared by centrifugation and then loaded onto the Ni-NTA resin. After incubation, the resin was washed once with lysis buffer with TCEP, once with lysis buffer with TCEP + 300 mM NaCl, and once with lysis buffer with TCEP + 25 mM imidazole. The proteins were eluted with lysis buffer with TCEP + 300 mM imidazole. The eluted proteins were concentrated and purified using size exclusion chromatography on a HiLoad 16/60
Superdex 200 column (GE Healthcare), which was equilibrated with the following buffer: 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, and 200 μM leucine. The collected protein was concentrated and immediately used in binding assays or frozen at -80°C. Before use in any binding assays, the protein was diluted sufficiently to significantly decrease the leucine that may have remained bound through the purification steps. The control His-RagA/RagC heterodimer was purified through a similar protocol, using the Ni-NTA resin and subsequent size exclusion chromatography.

**Purification of proteins expressed in human cells and the leucine binding assay**

4 million HEK-293T cells were plated in a 15 cm plate four days prior to the experiment. Each plate would yield the protein for one sample. Forty-eight hours after plating, the cells were transfected via the polyethylenimine method (43) with the pRK5- based cDNA expression plasmids indicated in the figures in the following amounts: 5 or 3 μg FLAG-Sestrin2; 12 μg FLAG-Rap2A; 5 μg WDR24-FLAG; 1 μg WDR24-FLAG with 4.75 μg each of Seh1L, Sec13, Mios, and WDR59; 12 μg FLAG-dSestrin (CG11299- PD); 12 μg FLAG-Sestrin1.1; 12 μg FLAG-Sestrin1.2; 12 μg FLAG-Sestrin3; 12 μg FLAG-Sestrin2 mutants (L261A, E451A, S190W) and up to 20 μg total DNA with empty-PRK5. Forty-eight hours after transfection cells were lysed as previously described. If multiple samples of the same type were represented in the experiment, the cell lysates were combined, mixed, and evenly distributed amongst the relevant tubes, to ensure equal protein amounts across samples of the same type.

Anti-FLAG immunoprecipitates were prepared as previously described, with the exception that prior to incubation with lysates, the beads were blocked by rotating in 1 μg/μl bovine serum albumin (BSA) for 20 minutes at 4°C and subsequently washed twice in lysis buffer. 30 μl of the 50/50 slurry of beads in lysis buffer was added to each of the clarified cell lysates and incubated as previously described.

For the binding assays, two tubes at a time were washed as previously indicated for immunoprecipitations. All the liquid was subsequently aspirated and a 15 μl aliquot of proteins bound to the beads was incubated for one hour on ice in cytosolic buffer (0.1%
Triton, 40 mM HEPES pH 7.4, 10 mM NaCl, 150 mM KCl, 2.5 mM MgCl2) with the appropriate amount of [3H]-labelled amino acids and cold amino acids. Tubes were flicked every five minutes. At the end of one hour, the beads were briefly spun down, aspirated dry, and rapidly washed three times with binding wash buffer (0.1% Triton, 40 mM HEPES pH 7.4, 150 mM NaCl). The beads were aspirated dry again and resuspended in 85 µl of binding wash buffer. With a cut tip, each sample was mixed well and three 10 µl aliquots were separately quantified using a TriCarb scintillation counter (PerkinElmer). This process was repeated in pairs for each sample, to ensure similar incubation and wash times for all samples analyzed across different experiments.

For each sample, an immunoprecipitation was performed in parallel. After washing four times as previously described and once with CHAPS buffer (0.3% CHAPS, 40 mM HEPES pH 7.4), the protein was eluted in 250 µl of CHAPS buffer with 300 mM NaCl and 1 mg/ml FLAG peptide for 1 hour at 4°C. The eluent was subsequently concentrated, quantified for protein amount using Bradford reagent, and resuspended in sample buffer. The proteins were resolved by 4-12% SDS-PAGE, and stained with SimplyBlue SafeStain.

For binding assays performed with bacterially-produced proteins, 23.6 µg His-RagA/RagC, 23.6 µg His-MBP-TEV-Sestrin2, or 73.6 µg His-MBP-TEV-Sestrin2 were diluted into 500 µl lysis buffer (50 mM Tris pH 7.4, 200 mM NaCl, 5 mM MgCl2, 0.1% CHAPS) and incubated with 15 µl compact Ni-NTA resin as previously described. For the binding assays, two tubes were washed at a time. The Ni-NTA resin with proteins bound to it was washed one time with lysis buffer and three times with lysis buffer supplemented with 300 mM NaCl. After washing, the liquid was aspirated and the protein bound to the resin was incubated for one hour on ice with the appropriate amount of [3H]-labelled amino acids and, where indicated, cold amino acids. The tubes were flicked every five minutes. The samples were subsequently washed three times after binding with wash buffer (lysis buffer with 300 mM additional NaCl). The resin was aspirated dry and resuspended in 85 µl of wash buffer. The samples were then well mixed with a cut tip and 10 µl of each was loaded into scintillation fluid in triplicate and
quantified with a TriCarb Scintillation Counter. Samples performed in parallel were eluted with lysis buffer + 300 mM imidazole and analyzed by SDS-PAGE as described above.

**Kd/Ki calculations**

Amino acid affinities to Sestrin2 were determined by first normalizing the bound [³H]-labeled amino acid concentrations across three separate binding assays performed with varying amounts of cold amino acid competition. These values were plotted and fit to a hyperbolic equation (Cheng-Prusoff equation) to estimate the IC50 value. Kd or Ki values were derived from the IC50 value using the equation: Kd or Ki = IC50 / (1 + ([³H]Leucine)/Kd).

**In vitro Sestrin2-GATOR2 dissociation assay**

HEK-293T cells stably expressing FLAG-WDR24 were starved for all amino acids for 50 minutes, lysed and subjected to anti-FLAG immunoprecipitation as described previously. The GATOR2-Sestrin2 complexes immobilized on the agarose beads were washed twice in lysis buffer with 500 mM NaCl, as previously described, and then incubated for 10 minutes in 1 mL of cytosolic buffer with the indicated concentrations of individual amino acids. The amount of GATOR2 and Sestrin2 that remained bound was assayed by SDS-PAGE and immunoblotting as described previously.

**Thermal shift assay**

The thermal shift (protein melting) assays were performed according to the LightCycler 480 instruction manual. Briefly, for Sestrin2, 5X Sypro orange dye and Sestrin2 at 4 µM were combined with or without leucine or arginine (at the indicated concentrations) in thermal shift buffer (100 mM Tris pH 7.4, 100 mM NaCl, and 1 mM DTT) in a volume of up to 10 µl in one well of a LightCycler Multiwell 384-well plate. 20X Sypro orange dye was used for the two control proteins, human choline acetyltransferase (ChAT) (at 4 µM) or *Physcomitrella patens* hydroxycinnamoyl transferase (PpHCT) (at 2.5 µM). Each condition was tested in triplicate. The plate was subjected to a protocol in which the
temperature increased from 20° to 85°C at 0.06°C/second. Fluorescence was recorded and plotted over time, and melting temperatures were calculated as described in the LightCycler 480 instruction manual. Briefly, the negative first derivative of the curve shown (change in fluorescence/change in temperature) was plotted against the temperature. The peak (i.e., lowest point on this curve) reflects the melting temperature. Each reported melting temperature is the mean ± SD for three replicates from one experiment.

**Generation of CRISPR/Cas9 genetically modified cells**

To generate HEK-293T cells with loss of all three Sestrins, the following sense (S) and antisense (AS) oligonucleotides encoding the guide RNAs were cloned into the pX330 vector.

- sgSesn2_S: caccgGACTACCTGCCTCGGCC
- sgSesn2_AS: aaacGGGCGAACCGCAGGTAGTCc
- sgSesn3_1S: caccgCAGCCACGATGAACCGGGG
- sgSesn3_1AS: aaacCCCCGGTTCATCGTGGCTGc
- sgSesn1_1S: caccgTGCATGTACCAATTCCGCAA
- sgSesn1_1AS: aaacTTGCGGAATTGGTACATGCAc

On day one, 200,000 cells were seeded into 6 wells of a 6-well plate. Twenty-four hours post seeding, each well was transfected with 250 ng shGFP pLKO, 1 ug of the pX330 guide construct, and 500 ng of empty pRK5 using XtremeGene9. The following day, cells were trypsinized, pooled in a 10 cm dish, and selected with puromycin to eliminate untransfected cells. Forty-eight hours after selection, the media was aspirated and replenished with fresh media lacking puromycin. The following day, cells were single cell sorted with a flow cytometer into the wells of a 96-well plate containing 150 µl of DMEM supplemented with 30% IFS. Cells were grown for two weeks and the resultant colonies were trypsinized and expanded. Clones were validated for loss of the relevant protein via immunoblotting.

To create the Sestrin1-3 triple null cells, Sestrin1 null cells were generated first. The
same method was repeated in the Sestrin1 null cells with Sestrin2 guides and Sestrin1-2 double null cells were produced. The method was repeated for a third time with the Sestrin1-2 double null cells and a guide RNA targeting Sestrin3 to created the Sestrin1-3 triple null cells.

**pS2 plasmid**

To produce the pS2 plasmid the 1500 base pairs upstream of the human Sestrin2 gene start site was amplified using the following primers and subsequently subcloned into the pLJC5 lentiviral vector in place of the UbC promoter.

S2_promoter_F: CCACCGGT TAGTGAATGTGATA CATGTGAAAAG
S2_promoter_R: GCGT GTCGAC GCACCACCACCACCACCCTTGT CATCGTCA TCCTTGTA GTCCATGGTGCGTGCGTGCGCCAGCAGCGGT CGCGG

**Lentivirus production and lentiviral transduction**

Lentiviruses were produced by transfection of HEK-293T cells with either pLJC5-FLAG-metap2, pLJC5-FLAG-Sestrin2 (wild-type or mutant), or pS2-FLAG-Sestrin2 (wild-type or mutant) plasmids in combination with the VSV-G envelope and CMV ΔVPR packaging plasmids. Twenty-four hours after transfection, the media was changed to DMEM with 20% IFS. Forty-eight hours after transfection, the virus-containing supernatant was collected from the cells and passed through a 0.45 μm filter. Target cells were plated in 6-well plates containing DMEM 10% IFS with 8 μg/mL polybrene and infected with virus containing media. Twenty-four hours later, the media was changed to fresh media containing puromycin for selection. To obtain the equal expression levels shown in Figure 4C the pS2 plasmid was used to express wild-type Sestrin2 and Sestrin2 L261A while the pLJC5 plasmid was used to express Sestrin2 E451A and metap2.

**Immunofluorescence assays**

Immunofluorescence assays were performed as described in (21). Briefly, 400,000 HEK-293T cells were plated on fibronectin-coated glass coverslips in 6-well tissue
culture plates. Twenty-four hours later, the slides were rinsed once with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The slides were subsequently rinsed three times with PBS and cells were permeabilized with 0.05% Triton X-100 in PBS for 5 min. After rinsing three times with PBS, cells were incubated with primary antibody in Odyssey blocking buffer for 1 hr at room temperature, rinsed three times with PBS, and incubated with secondary antibodies produced in donkey (diluted 1:400 in Odyssey blocking buffer) for 45 minutes at room temperature in the dark and washed three times with PBS. Slides were mounted on glass coverslips using Vectashield (Vector Laboratories) and cells imaged on a spinning disk confocal system (Perkin Elmer).

**Statistical analyses**

Two-tailed t tests were used for comparison between two groups. All comparisons were two-sided, and P values of less than 0.001 were considered to indicate statistical significance.
Acknowledgements

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CHAPTER 5

The CASTOR proteins are arginine sensors for the mTORC1 pathway

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Experiments in Figures 1-6, S1-S6 were performed by LC, with assistance from SMS. Experiments in Figure 1 were performed by JWH and SPG. Experiments in Figure 4 were performed by RAS.
Summary

Amino acids signal to the mTOR complex I (mTORC1) growth pathway through the Rag GTPases. Multiple distinct complexes regulate the Rags, including GATOR1, a GTPase activating protein (GAP), and GATOR2, a positive regulator of unknown molecular function. Arginine stimulation of cells activates mTORC1, but how it is sensed is not well understood. Recently, SLC38A9 was identified as a putative lysosomal arginine sensor required for arginine to activate mTORC1 but how arginine deprivation represses mTORC1 is unknown. Here, we show that CASTOR1, a previously uncharacterized protein, interacts with GATOR2 and is required for arginine deprivation to inhibit mTORC1. CASTOR1 homodimerizes and can also heterodimerize with the related protein, CASTOR2. Arginine disrupts the CASTOR1-GATOR2 complex by binding to CASTOR1 with a dissociation constant of ~30 μM, and its arginine-binding capacity is required for arginine to activate mTORC1 in cells. Collectively, these results establish CASTOR1 as an arginine sensor for the mTORC1 pathway.
Introduction

Arginine is a conditionally essential amino acid with many metabolic and regulatory roles, serving as a proteogenic amino acid as well as a precursor for critical molecules such as nitric oxide, creatine, and glutamate (Wu and Morris, 1998). Arginine regulates key aspects of mammalian physiology, including insulin release, intestinal stem cell migration, and neonatal growth (Ban et al., 2004; Floyd et al., 1966; Rhoads et al., 2006; Yao et al., 2008). These effects stem at least in part from the ability of arginine to activate mTORC1, a master growth controller that integrates diverse environmental inputs to coordinate many anabolic and catabolic processes in cells (Ban et al., 2004; Dibble and Manning, 2013; Efeyan et al., 2012; Hara, 1998).

The lysosome is a critical organelle for mTORC1 activation, and amino acids promote the translocation of mTORC1 to its surface where its kinase activator Rheb, a small GTPase, resides (Buerger et al., 2006; Dibble et al., 2012; Menon et al., 2014; Saito et al., 2005; Sancak et al., 2008). Necessary for this recruitment are the Rag GTPases, which form heterodimeric complexes comprised of RagA or RagB bound to RagC or RagD (Hirose et al., 1998; Sancak et al., 2008; Schürmann et al., 1995; Sekiguchi et al., 2001). Amino acid availability controls the nucleotide state of the Rags, and this regulation depends on a complex interplay between multiple distinct factors, including Ragulator, which serves as a lysosomal scaffold for RagA/B (Bar-Peled et al., 2012; Sancak et al., 2010); FLCN/FNIP2, a GAP for RagC/D (Petit et al., 2013; Tsun et al., 2013); and GATOR1, a GAP for RagA/B and a critical negative regulator of the mTORC1 pathway (Bar-Peled et al., 2013). The GATOR2 complex, which has five subunits (mios, WDR24, WDR59, sec13, seh1L), acts upstream or parallel to GATOR1 and is a key positive regulator of the mTORC1 pathway, although its molecular function is unknown (Bar-Peled et al., 2013).

The proteins that sense amino acids and signal to the Rag GTPases were elusive until recently. We identified Sestrin2 as a cytosolic leucine sensor and SLC38A9 as a putative lysosomal arginine sensor for the mTORC1 pathway (Rebsamen et al.,...
While Sestrin2 interacts with GATOR2 to inhibit mTORC1 signaling in the absence of leucine, SLC38A9 forms a supercomplex with Ragulator and is necessary for transmitting arginine, but not leucine, sufficiency to mTORC1 (Chantranupong et al., 2014; Jung et al., 2015; Lynch et al., 2000; Rebsamen et al., 2015; Saxton et al., 2015; Wang et al., 2015; Wolfson et al., 2015; Zoncu et al., 2011). Despite these advances, in human cells lacking SLC38A9 arginine starvation still inhibits mTORC1 (Wang et al., 2015), suggesting that our understanding of how arginine is sensed is incomplete.

Here, we demonstrate that CASTOR1, a previously uncharacterized protein, functions in parallel with SLC38A9 to regulate mTORC1 in response to arginine. CASTOR1 forms a homodimer and heterodimerizes with CASTOR2, also a previously unstudied protein, and both complexes interact with GATOR2 to negatively regulate mTORC1 activity. Arginine, but not other amino acids, disrupts this interaction by binding directly to CASTOR1. Importantly, activation of the mTORC1 pathway by arginine requires the arginine-binding capacity of CASTOR1. Thus, CASTOR1 is an arginine sensor for the mTORC1 pathway.
Results and Discussion

CASTOR1 and CASTOR2 are ACT domain-containing proteins that interact with GATOR2

Given its central role as a positive regulator of the mTORC1 pathway, GATOR2 is likely to integrate multiple amino acid inputs to mTORC1, and therefore other sensors in addition to Sestrin2 may interact with it. To identify potential GATOR2-binding partners, we interrogated BioPlex, a database of human protein-protein interactions generated from immunoprecipitation followed by mass spectrometry of 2,594 proteins stably expressed in HEK-293T cells (Huttlin et al., 2015). In this dataset, three core components of GATOR2 – WDR24, WDR59 and mios – were found to interact with the protein encoded by the GATS protein-like 3 (GATSL3) gene (Figure 1A). In addition, proteins encoded by the GATSL2 and FAM164A genes were also present in GATSL3 immunoprecipitates. No prior work exists for GATSL3, GATSL2, or FAM164A. For reasons that are described later, we have renamed GATSL3 as CASTOR1 (Cellular Arginine Sensor for mTORC1) and GATSL2 as CASTOR2.

In humans, CASTOR1 and CASTOR2 reside on chromosome 22 and 7,

Figure 1: CASTOR1 and CASTOR2 are ACT domain-containing proteins that interact with GATOR2
(A) Endogenous GATOR2, FAM164A, and CASTOR2 co-immunoprecipitate with stably expressed CASTOR1. The schematic is adapted from the BioPlex database (Huttlin et al., 2015). Solid blue lines denote proteins that were detected by mass spectrometric analysis of CASTOR1 immunoprecipitates, and dashed purple lines indicate interactions between GATOR2 subunits that were present in Bioplex.
(B) Schematic alignment of human CASTOR1 and CASTOR2 proteins with annotated ACT domains.
(C) The ACT domains of CASTOR1 and CASTOR2 display sequence similarity with the ACT domains of fungal aspartate kinases and putative amino acid binding proteins in bacteria. Amino acid positions are colored from white to blue in order of increasing sequence identity. The red star denotes the position of the I280 residue in CASTOR1.
(D) Recombinant CASTOR1 and CASTOR2 co-immunoprecipitate endogenous GATOR2, as detected by the presence of mios. Anti-HA immunoprecipitates and lysates were prepared from HEK-293T cells cotransfected with the indicated cDNAs in expression vectors. Cell lysates and immunoprecipitates were analyzed by immunoblotting for levels of indicated proteins. HA-metap2 served as a negative control.
Figure 1

A

B

CASTOR1

ACT 1

CASTOR2

ACT 1

CASTOR1

ACT 2

CASTOR2

ACT 2

C

D

transfected

CONAs.

mios

CASTR2

CASTR1

CAStR2

CAStR1

mios

WDR24

IP:

HA

mios

mios

HA

mios

WDR24

cell

lysate
respectively, and are similar, sharing 63% protein sequence identity. Both genes are lowly expressed across most tissues, with higher expression in some, such as the muscle for CASTOR2 (Figure S1A). All human genome assemblies except the most recent (hg38) annotate on chromosome 7 an adjacent duplication of CASTOR2 termed GATSL1. GATSL1 encodes a protein that is nearly identical to CASTOR2, having only an N17K change; however, this change is not conserved across species nor does it lead to functional differences between GATSL1 and CASTOR2 (data not shown). Whether or not GATSL1 exists in the human genome is unclear, but if it does, GATSL1 is unlikely to encode for a protein that is functionally distinct from CASTOR2. Therefore, we do not consider GATSL1 further.

Orthologs of both CASTOR proteins are readily detectable in vertebrates, including zebrafish (Figure S1B and C) but are absent in other established model organisms such as S. cerevisiae, S. pombe, C. elegans, and D. melanogaster. Database searches also revealed the presence of potential CASTOR homologs in invertebrates such as sea urchins and sea anemones (Figure S1D). In contrast to vertebrates that encode two CASTOR proteins, these organisms encode only one CASTOR-like protein, suggesting that the duplication of an ancestral CASTOR gene yielded CASTOR1 and CASTOR2 in vertebrates.

Figure S1, related to Figure 1: CASTOR1 and CASTOR2 homologs are present in vertebrates and invertebrates, and the CASTORs and Sestrins bind to distinct sites on GATOR2. (A) CASTOR1 and CASTOR2 are lowly expressed in most human tissues. mRNA expression data was obtained from GTex. (B) The CASTOR1 and (C) CASTOR2 proteins are highly conserved in vertebrates. Sequence alignments are colored with respect to sequence identity as in Figure 1C. The first and second ACT domains are annotated above the alignment with blue and orange bars, respectively. The red star denotes the position of the I280 residue in CASTOR1. (D) CASTOR1 and CASTOR2 homologs are present in invertebrates. The sequence alignment is annotated as in (B). (E) The GATOR2 components WDR24, mios, and seh1L form a minimal complex that is sufficient to co-immunoprecipitate CASTOR1. HEK-293T cells were cotransfected with the indicated cDNAs, and anti-FLAG immunoprecipitates were analyzed as in Figure 1D. (F) Expression of CASTOR2, but not of Sestrin2, displaces CASTOR1 from GATOR2. HEK-293T cells were cotransfected with the indicated cDNAs, and anti-FLAG immunoprecipitates were analyzed as in Figure 1D.
Intriguingly, both CASTOR1 and CASTOR2 contain two tandem ACT domains of 70 - 80 residues each (Figure 1B). ACTs are evolutionarily ancient domains that function as small molecule binding modules for diverse ligands, including amino acids and nucleotides (Aravind and Koonin, 1999; Chipman, 2001; Grant, 2006; Lang et al., 2014). These domains confer complex allosteric regulation to varied proteins, predominantly bacterial enzymes involved in purine and amino acid metabolism. To date, the aromatic amino acid hydroxylases are the only ACT-containing proteins identified and characterized in mammals (Aravind and Koonin, 1999; Carluccio et al., 2013; Grant, 2006; Kobe et al., 1999; Lang et al., 2014; Siltberg-Liberles and Martinez, 2009). A BLAST search of the NCBI protein database with each individual CASTOR ACT domain revealed that they are most similar to the ACT domains of budding yeast aspartate kinase, which binds lysine (Dumas et al., 2012), as well as several putative amino acid binding proteins in bacteria (Figure 1C).

We first sought to validate the identification of CASTOR1 as a GATOR2-interacting protein. When expressed as an HA-tagged protein in HEK-293T cells, CASTOR1, but not the metap2 control protein, co-immunoprecipitated endogenous mios, an established GATOR2 component (Figure 1D). Given the sequence similarity of CASTOR1 and CASTOR2, we asked whether recombinant CASTOR2 could also interact with GATOR2. Indeed, CASTOR2 co-immunoprecipitated an even greater amount of mios than CASTOR1 (Figure 1D).

To define which GATOR2 components bind the CASTOR proteins, we compared the ability of different GATOR2 subunits to co-immunoprecipitate CASTOR1. Together, WDR24, mios, and seh1L form a minimal unit that was sufficient to interact with CASTOR1, although it did not recapitulate the degree of binding observed with the complete GATOR2 complex (Figure S1E). Given that GATOR2 binds not only to the CASTORs but also to Sestrin1, Sestrin2, and Sestrin3 (Chantranupong et al., 2014; Kim et al., 2015; Parmigiani et al., 2014), we asked whether these proteins occupy unique sites on GATOR2. CASTOR2, but not Sestrin2, effectively displaced CASTOR1 from GATOR2, indicating that the CASTORs bind to the same site on the GATOR2 complex,
Figure 2

A

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D

[Image of gel electrophoresis results]
and that it is distinct from that for the Sestrins (Figure S1F). Collectively, these findings establish CASTOR1 and CASTOR2 as ACT domain-containing proteins that interact with GATOR2.

**CASTOR1 and CASTOR2 form homo- and heterodimeric complexes**

Previous structural studies show that the ACT domains of proteins can oligomerize to form multi-protein complexes (Lang et al., 2014). Consistent with this possibility, we noted a potential interaction between CASTOR1 and CASTOR2 in BioPlex (Figure 1A). Indeed, when overexpressed in cells, CASTOR1 and CASTOR2 robustly interacted with themselves and each other to form homo- and heterooligomers (Figure 2A). Gratifyingly, endogenous CASTOR2 and CASTOR1 can also participate in heterooligomeric complexes as they copurified with recombinant CASTOR1 and CASTOR2, respectively (Figure 2B and C, Figure S2A and B). In addition to CASTOR2, we also identified FAM164A, a zinc finger-containing protein, as a potential interacting partner of CASTOR1 (Figure 1A). However, we do not consider FAM164A further as we could not detect an interaction with CASTOR1 or GATOR2 (Figure S2C and D).

---

**Figure 2: CASTOR1 and CASTOR2 form homo- and heterodimeric complexes**

(A) Recombinant CASTOR1 and CASTOR2 coimmunoprecipitate both themselves and each other. HEK-293T cells were cotransfected with the indicated cDNAs in expression vectors and cell lysates and anti-HA immunoprecipitates were analyzed by immunoblotting for the indicated proteins as in Figure 1D.

(B) Recombinant CASTOR2 coimmunoprecipitates endogenous CASTOR1. HEK-293T cells were cotransfected with the indicated cDNAs in expression vectors and anti-HA immunoprecipitates were collected and analyzed as in Figure 1D. The arrow denotes the band corresponding to CASTOR1.

(C) Recombinant CASTOR1 coimmunoprecipitates endogenous CASTOR2. HEK-293T cells were cotransfected with the indicated cDNAs in expression vectors and anti-HA immunoprecipitates were collected and analyzed as in (A).

(D) Recombinant CASTOR1 and CASTOR2 are present in approximately equal ratios within the heterodimeric complex. SDS-polyacrylamide gel electrophoresis (PAGE), followed by Coomassie blue staining, was used to analyze the indicated protein preparations from HEK-293T cells. The asterisk denotes a common protein contaminant present in these purifications.
Figure S2, related to Figure 2: FAM164A does not interact with CASTOR1, CASTOR2, or GATOR2.
(A) Validation of the anti-serum used to detect endogenous CASTOR2. Lysates were prepared from HEK-293T lines stably expressing the indicated shRNAs and analyzed by immunoblotting for levels of indicated proteins.
(B) Validation of the anti-serum used to detect endogenous CASTOR1. Lysates were prepared from HEK-293T stably lines expressing the indicated shRNAs and analyzed as in (C). The arrow denotes the band corresponding to endogenous CASTOR1.
(C) Recombinant FAM164A does not coimmunoprecipitate CASTOR2 or CASTOR1. HEK-293T cells were cotransfected with the indicated cDNAs in expression vectors, starved of all amino acids for 50 minutes, and anti-HA immunoprecipitates were analyzed as in Figure 1D.
(D) Recombinant FAM164A does not copurify endogenous GATOR2. HEK-293T cells were cotransfected with the indicated cDNAs in expression vectors, starved of all amino acids for 50 minutes, and anti-HA immunoprecipitates were analyzed as in Figure 1D.

SDS-PAGE followed by Coomassie blue staining showed that purifications of recombinant CASTOR1 and CASTOR2 associate in a 1:1 ratio within the heterooligomers (Figure 2D). More definitive methods are needed to determine the exact number of proteins in the complexes, but for simplicity we refer to them as dimers. Altogether, these data support the existence of three CASTOR complexes: the CASTOR1 and CASTOR2 homodimers and the CASTOR1-CASTOR2 heterodimer.

Arginine regulates the interaction of the CASTOR1-homodimer and CASTOR1-CASTOR2 heterodimer with GATOR2

We wondered if the three CASTOR complexes we defined bind differentially to GATOR2. Indeed, when overexpressed in HEK-293T cells, the CASTOR2 homodimer interacted more strongly with endogenous GATOR2 than the CASTOR1 homodimer, while the CASTOR1-CASTOR2 heterodimer bound to GATOR2 at an intermediate level (Figure 3A). Because endogenous CASTOR2 is present in these cells, it is possible that the GATOR2 interaction we observe with the CASTOR1 homodimer is partly due to endogenous CASTOR2 that heterodimerizes with overexpressed CASTOR1. However, this is an unlikely possibility because the RNAi-mediated depletion of CASTOR2 did not alter the level of GATOR2 that copurified with CASTOR1 (Figure S3A).
Figure 3

A

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<th>CASTOR2-FLAG-WDR24</th>
<th>mios</th>
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Because CASTOR1 and CASTOR2 contain ACT domains that have the potential to bind small molecules, we hypothesized that amino acids regulate the CASTOR-GATOR2 interaction in a manner analogous to how leucine controls the Sestrin2-GATOR2 association (Wolfson et al., 2015). Consistent with this prediction, in cells, amino acid withdrawal from the culture medium strengthened the interaction of recombinant CASTOR1-containing dimers with GATOR2 and re-addition of amino acids rapidly disrupted it (Figure 3A). In contrast, amino acids did not affect the interaction between the CASTOR proteins themselves whether in homo- or heterodimeric complexes (Figure 3A). Similar to recombinant CASTOR1, endogenous CASTOR1 associated in a highly amino acid-sensitive manner with endogenous GATOR2 isolated from HEK-293T cells with an antibody directed against WDR24, as well as from a HEK-293T cell line expressing endogenously FLAG-tagged WDR59 (Figure 3B and Figure S3B).

Figure 3: Arginine regulates the interaction of GATOR2 with CASTOR1-homodimers and CASTOR1-CASTOR2 heterodimers in cells and in vitro
(A) Amino acids differentially regulate the interaction of GATOR2 with the three CASTOR complexes. HEK-293T cells cotransfected with the indicated cDNAs were deprived of amino acids for 50 min or starved and restimulated with amino acids for 10 min. Anti-HA immunoprecipitates and cell lysates were analyzed by immunoblotting for levels of the indicated proteins.
(B) Endogenous CASTOR1, but not CASTOR2, associates with GATOR2 in an amino acid-sensitive manner. A HEK-293T cell line expressing endogenously FLAG-tagged WDR59 was treated as in (A) and anti-FLAG immunoprecipitates were analyzed by immunoblotting for the indicated proteins.
(C) Deprivation of arginine, but not leucine, promotes the interaction between the CASTOR1 homodimer and endogenous GATOR2. Cells were deprived of leucine, arginine, or all amino acids for 50 min, and restimulated for 10 min with the respective amino acids where indicated. Anti-HA immunoprecipitates were prepared and analyzed as in (A).
(D) Arginine disrupts the interaction between GATOR2 and CASTOR1-containing dimers in vitro. Anti-HA immunoprecipitates were prepared from HEK-293T cells expressing the indicated cDNAs and deprived of amino acids for 50 min. Indicated amino acids were added directly to the immunoprecipitates, which after re-washing, were analyzed as in (A).
(E) Arginine dose-dependently disrupts the interaction between GATOR2 and CASTOR1-containing dimers in vitro. The experiment was performed and analyzed as in (D), except using the indicated concentrations of arginine.
(F) Arginine regulates the interaction between the ACT domains of CASTOR1 but not CASTOR2 in cells. HEK-293T cells cotransfected with the indicated cDNAs in expression vectors were either deprived of arginine in the cell media for 50 min or starved and restimulated with arginine for 10 min. Anti-HA immunoprecipitates were prepared and analyzed as in (A).
Supplemental Figure 3

A

HEK-293T cell line stably expressing:

transfected eODNAs:

YHEK-293T cell line stably expressing:

GhGO2hCST

mlos

CASTOR1MA

H A-mtmp2

cell lysate

mlos

CASTOR2

B

IP antibody:

CASTOR1

CASTOR2

Seestrin2

mlos

WDR24

GSK3β

IP:

HA

Mlos

WDR24

GSK3β

C

amino acids: r,.

+ + +

IP:

mlos

HA

Mlos

WDR24

GSK3β

D

amino acids:

transfected cDNAs:

leucine: - + +

arginine: - + +

IP:

 CASTOR1 -

CASTOR2 -

mlos

mlos

S6K1

E

amino acid (400 μM) added to CASTOR1-HA & CASTOR2-FLAG immunoprecipitates:

mlos

IP:

CASTOR1-MA

F

HEK-293T cell fraction:

arginine:

CASTOR1

CASTOR2

S6K1

Lamp2

cathepsin

VDAC

Histone H3

29-389-S6K1
Unlike CASTOR1, amino acids only very weakly regulated the interaction of the CASTOR2 homodimer with GATOR2 (Figure 3A and B, Figure S3B). We suspected that the slight amino acid sensitivity of this complex might stem from the small fraction of CASTOR2 that binds to endogenous CASTOR1, thus forming an amino acid-responsive heterodimer. To test this possibility, we immunoprecipitated CASTOR2-GATOR2 complexes from cells depleted of CASTOR1 by stable coexpression of Cas9 and a guide RNA (sgRNA) targeting the CASTOR1 locus. Confirming our suspicions, CASTOR1 depletion eliminated the weak amino acid sensitivity of the CASTOR2-GATOR2 interaction (Figure S3C).

Notably, these findings also suggest that the weak interaction we initially detected between GATOR2 and CASTOR1 (Figure 1D) resulted from isolating these

Figure S3, related to Figure 3: Arginine regulates the interaction of GATOR2 with CASTOR1-containing complexes in cells and in vitro.

(A) RNAi-mediated depletion of CASTOR2 does not alter the level of endogenous GATOR2 that co-immunoprecipitates with recombinant CASTOR1. HEK-293T cells cotransfected with the indicated cDNAs and stably expressing the indicated shRNAs were deprived of amino acids in the cell media for 50 min. Anti-HA immunoprecipitates were analyzed by immunoblotting for the indicated proteins.

(B) Amino acids disrupt the interaction of GATOR2 with endogenous CASTOR1, but not CASTOR2. HEK-293T cells were starved or restimulated with amino acids, and immunoprecipitates were prepared from these cells using an antibody directed against GSK3β or WDR24. Immunoprecipitates were analyzed by immunoblotting for the indicated proteins.

(C) Loss of CASTOR1 abrogates the slight amino acid regulation of the interaction of CASTOR2 homodimers with GATOR2. Stable expression lines of HEK-293T cells stably expressing the denoted proteins were cotransfected with the indicated cDNAs and treated as in Figure 3A. Anti-HA immunoprecipitates were analyzed by immunoblotting for the levels of indicated proteins.

(D) Deprivation of arginine, but not leucine, is sufficient to promote the association of endogenous GATOR2 with recombinant CASTOR1-CASTOR2 heterodimers. HEK-293T cells cotransfected with the indicated cDNAs were treated and analyzed as in Figure 3C.

(E) Arginine disrupts the interaction between GATOR2 and the CASTOR1-CASTOR2 heterodimer in ice-cold detergent lysates of amino acid-starved cells. Anti-HA immunoprecipitates were prepared and analyzed as in Figure 3E.

(F) Arginine does not alter the distribution of CASTOR1 and CASTOR2 in subcellular fractions of HEK-293T cells. HEK-293T cells were starved and restimulated with arginine and subsequently separated into cytosolic, membrane/organellar, and nuclear/cytoskeletal fractions. Fractions were analyzed by immunoblotting for the indicated proteins. The arrow denotes the band corresponding to CASTOR2 while the asterisk denotes a non-specific band.
complexes from cells growing in DMEM media, which contains high levels of amino acids that would have disrupted most of the CASTOR1-GATOR2 complexes. In contrast, the CASTOR2-GATOR2 complexes were readily detectable as they are amino acid insensitive.

To determine whether a particular amino acid modulates the interaction of CASTOR1 with GATOR2, we focused on leucine and arginine, which have long been known to regulate mTORC1 signaling (Ban et al., 2004; Blommaart et al., 1995; Fox et al., 1998; Hara, 1998; Lynch et al., 2000). In HEK-293T cells, removal of leucine or arginine from the cell medium inhibited mTORC1 signaling to a comparable degree as that of all amino acids, as detected by phosphorylated S6K1, an established mTORC1 substrate (Figure 3C). Despite similar effects on mTORC1 signaling, only arginine removal recapitulated the ability of total amino acid starvation to promote the binding of GATOR2 to CASTOR1-containing complexes. Re-stimulation with arginine completely reversed the interaction (Figure 3C and Figure S3D). Arginine does not appear to regulate the subcellular distribution of CASTOR1 in HEK-293T cells, as it was present in both the cytosolic and organellar subcellular fractions upon the removal and re-addition of arginine to cells (Figure S3F). CASTOR2 was in the cytosolic fraction in cells starved for or stimulated with arginine. These results are consistent with the notion that both proteins are likely cytosolic, as they lack transmembrane domains and obvious localization signals.

Because CASTOR1 contains ACT domains, we considered the possibility that arginine might act directly on CASTOR1 to perturb its interaction with GATOR2. First, we assessed whether arginine could disrupt the interaction between CASTOR1 and GATOR2 immunopurified from amino acid starved cells. Indeed, the addition of 400 μM arginine to these purified complexes was sufficient to dissociate GATOR2 from both the CASTOR1 homodimer and CASTOR1-CASTOR2 heterodimer, with half-maximal disruption occurring at an arginine concentration of 20-40 μM (Figure 3D and 3E and Figure S3E). Arginine does so with remarkable specificity as none of the other 15 amino acids tested had the same effect.
In light of previous structural studies that reveal ligand-induced association of ACT domains (Cross et al., 2013; Cross et al., 2011; Lang et al., 2014; Tan et al., 2008), we tested whether arginine might mediate its effects on CASTOR1 by regulating the interaction between its ACT domains. We divided the CASTOR proteins in half to generate two ACT domain-containing fragments denoted as ACT1 and ACT2, and performed co-immunoprecipitation analyses in arginine-starved and -replete cells. Intriguingly, the CASTOR1 ACT domains interact with each other only when arginine is present, with arginine withdrawal from the cell medium leading to rapid dissociation of the two CASTOR1 halves. In contrast, the CASTOR2 ACT domains bound constitutively to each other, irrespective of arginine (Figure 3F). The ability of the CASTOR2 ACT domains to interact without a ligand is at odds with the fact that ligand binding induces ACT domain association, and suggests that separating the ACT domains may enable them to exhibit novel interactions that are not possible in the context of intact CASTOR2. Overall, these data are consistent with the notion that differences between the CASTOR1 and CASTOR2 ACT domains underlie the ability of arginine to regulate the interaction of GATOR2 with CASTOR1, but not CASTOR2. Taken together these data reveal a role for arginine as a regulator of the CASTOR1-GATOR2 interaction.

The CASTOR1 homodimer and CASTOR1-CASTOR2 heterodimer bind arginine with a dissociation constant of around 30 μM

Because arginine specifically disrupts the purified CASTOR1-GATOR2 complex and modulates the interaction between the CASTOR1 ACT domains, we tested the possibility that arginine directly binds to CASTOR1. We used an equilibrium binding assay to assess whether immunopurified CASTORs from HEK-293T cells bind radiolabelled arginine. Indeed, tritiated arginine bound the CASTOR1 homodimer, but not the CASTOR2 homodimer or a control protein Rap2A, in a manner that was competed by excess nonradiolabelled arginine (Figure 4A and B). The CASTOR1-CASTOR2 heterodimer bound roughly half as much arginine as the CASTOR1 homodimer, reflecting the fact that within this complex only CASTOR1 can bind arginine (Figure 4B). Furthermore, neither radiolabelled leucine nor lysine bound to CASTOR1,
Figure 4

A

B

C

D

E

F

transfected cDNAs: CASTOR1-FLAG CASTOR1-FLAG
arginine added to cell media (μM): 0 0 1 3 5 10 20 30 50 100 350 500 1000

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Kₐ = 0.2 μM

arginine bound (cpm)

arginine added to cell media (μM): 0 1 10 100 1000

arginine added to cell media (μM): 0 1 10 100 1000

arginine added to cell media (μM): 0 1 10 100 1000
consistent with the previously observed specificity for arginine for disrupting the CASTOR1-GATOR2 complex (Figure 4A).

It remained a formal possibility that arginine binds to an unidentified protein in the mammalian preparations of the CASTOR1 homodimer and CASTOR1-CASTOR2 heterodimer. To provide orthogonal evidence that CASTOR1 binds arginine, we purified the CASTOR complexes from *E. coli*, which do not encode a CASTOR homolog. The CASTOR1 homodimer and heterodimer, but not Sestrin2, bound arginine to a comparable degree as the complexes prepared from human cells, demonstrating that arginine binds directly to CASTOR1 and not a co-purifying contaminating protein (Figure 4C).

A competition binding assay with increasing amounts of cold arginine revealed

---

**Figure 4:** The CASTOR1 homodimer and CASTOR1-CASTOR2 heterodimer bind arginine with a $K_d$ of around 30 µM

(A) Radiolabelled arginine, but not radiolabelled leucine or lysine, binds to CASTOR1 homodimers. FLAG-immunoprecipitates were prepared from HEK-293T cells cotransfected with the indicated cDNAs, and binding assays were performed with these immunoprecipitates as described in the methods. Unlabelled amino acids were added where indicated. Values are mean ± SD of three technical replicates from one representative experiment (n.s., not significant).

(B) Arginine binds to CASTOR1-containing homo- and heterodimers, but not the CASTOR2 homodimer. FLAG immunoprecipitates of the indicated complexes were prepared from HEK-293T cells and analyzed as in (A). Equal volumes of eluants from immunoprecipitates of the denoted complexes were loaded and analyzed in SDS-PAGE, followed by Coomassie blue staining.

(C) Arginine binds to bacterially produced CASTOR1-containing complexes, but not the CASTOR2 homodimer or the control protein Sestrin2. Proteins purified from bacteria were analyzed as in (A) and (B).

(D) Arginine binds to the CASTOR1 homodimer with a dissociation constant of 34.8 µM. Binding assays were performed as in (A) with the indicated concentrations of unlabelled arginine. A representative experiment is shown, and each point represents the mean ± SD for three experiments. The $K_d$ was calculated from four experiments.

(E) Arginine binds to the CASTOR1-CASTOR2 heterodimer with a dissociation constant of 24.2 µM. FLAG-immunoprecipitates were prepared from HEK-293T cells and analyzed as in (D).

(F) The concentration of arginine that half-maximally activates the mTORC1 pathway correlates with the concentration of arginine that disrupts half of the complexes of GATOR2 and CASTOR1 homodimers. HEK-293T cells were transfected with the indicated cDNAs and immunoprecipitates and lysates analyzed as in Figure 3C.

---

157
Supplemental Figure 4

A

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that the $K_d$ of arginine for CASTOR1 in the homodimer is $34.8 \pm 5.9 \, \mu M$, which is similar to its $K_d$ in the heterodimer of $24.2 \pm 4.1 \, \mu M$ (Figure 4D and E). These affinities correlate well with the half maximal concentration of arginine that disrupts the interaction of GATOR2 with CASTOR1-containing complexes in vitro (Figure 3E) and activates mTORC1 in cells (Figure 4F and S4A). In combination, these data strongly support the notion that arginine binds directly to CASTOR1 to regulate its interaction with GATOR2.

CASTOR1 functions in parallel with SLC38A9 to regulate arginine sensing by mTORC1

Given the ability of arginine to bind to CASTOR1 and to modulate its interaction with GATOR2, we reasoned that CASTOR1 can affect the capacity of the mTORC1 pathway to respond to arginine. Indeed, transient overexpression of CASTOR1 driven by the strong CMV promoter inhibited mTORC1 activation by amino acids to a similar extent as expression of the dominant negative Rag GTPases mutants (Figure 5A).

 Conversely, in HEK-293T and HEC59 cells, CASTOR1-depletion mediated by expression of either shRNAs or Cas9 with sgRNAs made the mTORC1 pathway substantially insensitive to deprivation of arginine (Figure 5B and C, Figure S5B), but not to deprivation of leucine or all amino acids (Figure S5D and E). To determine if the RNAi-mediated effects are on target, we stably expressed an RNAi-resistant CASTOR1 cDNA in the CASTOR1 knockdown cells. To avoid inhibition of the mTORC1 pathway, we used the Sestrin2 promoter to express CASTOR1 at levels lower than those obtained with the CMV promoter. At this reduced level of expression, reintroduction of

Figure S4, related to Figure 4: The CASTOR1 homodimer and CASTOR1-CASTOR2 heterodimer bind arginine with a $K_d$ of around 30 $\mu M$. (A) The concentration of arginine required to half-maximally activate the mTORC1 pathway correlates with the concentration required to disrupt half the complexes of GATOR2 and CASTOR1-CASTOR2 heterodimers. HEK-293T cells were transfected with the indicated cDNAs and immunoprecipitates and lysates analyzed as in Figure 3C.

159
Figure 5

A

transfected cell lysates

FLAG

IP

amino acids:

T389-S6K1

CASTOR2 aa

CASTOR1 aa

B

HEK-293T stably expressing:

T389-S6K1

S6K1

CASTOR1

CASTOR2

C

HEK-293T stably expressing arginine:

T389-S6K1

S6K1

CASTOR1

raptor

D

HEK-293T stably expressing arginine:

T389-S6K1

S6K1

CASTOR1

CASTOR2

raptor

E

HEK-293T cell line:

wild type

SLC38A9 KO

hairpin:

GFP

CASTOR1_2

GFP CASTOR1

arginine:

T389-S6K1

S6K1

CASTOR1

CASTOR2

raptor
CASTOR1 into the CASTOR1 knockdown cells restored the arginine responsiveness of the mTORC1 pathway, demonstrating that the RNAi effects are on target (Figure S5A). Despite the use of a weaker promoter, the level of recombinant CASTOR1 still greatly exceeded that of the endogenous protein (Figure S5A). However, this fact does not alter our conclusions because the overexpressed CASTOR1 restored, not inhibited, the arginine responsiveness of the mTORC1 pathway. Overall, these findings indicate that CASTOR1 is a negative regulator of the mTORC1 pathway.

Unlike CASTOR1, CASTOR2 constitutively associates with GATOR2 and does not bind arginine, and thus appears to be an arginine-insensitive version of CASTOR1. RNAi-mediated depletion of CASTOR2 slightly increased mTORC1 activity in the arginine-replete conditions (Figure 5D), and the knockdown of CASTOR2 together with that of CASTOR1 had a similar effect (Figure S5C). CASTOR2 levels are also partially reduced by expression of shCASTOR1_2 in HEK-293T cells, which may contribute to the slight increase in mTORC1 activation observed with this hairpin under arginine-

**Figure 5: CASTOR1 functions in parallel with SLC38A9 to regulate arginine signaling to mTORC1**

(A) Transient overexpression of recombinant CASTOR2 and CASTOR1 inhibits mTORC1 activation in response to amino acids. HEK-293T cells were cotransfected with the indicated cDNAs. Cells were treated as in Figure 3A and anti-FLAG immunoprecipitates analyzed by immunoblotting for the indicated proteins.

(B) RNAi-mediated depletion of CASTOR1 in HEK-293T cells renders the mTORC1 pathway partially insensitive to arginine deprivation. HEK-293T cells stably expressing the indicated shRNAs were starved of arginine in the cell media for 50 min or starved and restimulated with arginine for 10 min. Lysates were analyzed via immunoblotting for the indicated proteins and phosphorylation states.

(C) CRISPR/Cas9 mediated depletion of CASTOR1 in HEK-293T cells confers resistance of the mTORC1 pathway to arginine deprivation. HEK-293T cells stably coexpressing Cas9 with the indicated guide RNAs were treated as in (B) and lysates were analyzed by immunoblotting for indicated proteins.

(D) Loss of CASTOR2 slightly increases mTORC1 activity in response to arginine. HEK-293T cells stably expressing the indicated shRNAs were treated as in (B) and lysates were analyzed by immunoblotting for indicated proteins. The normalized phosphorylated S6K1 signal under arginine stimulation for shCASTOR2_1 and shCASTOR2_2 expressing cells is 1.4 fold and 1.1 fold of shGFP expressing cells, respectively, as quantified with ImageJ.

(E) CASTOR1 and SLC38A9 likely function in parallel to signal arginine availability to the mTORC1 pathway. Wild type or SLC38A9 knockout HEK-293T cells expressing the indicated shRNAs were treated as in (B) and lysates were analyzed by immunoblotting for indicated proteins.
Supplemental Figure 5

A
HEK-293T cell line stably expressing:
- CASTOR1
- arginine
- hairpin: G
- T389-M6K1
- S6K1
- CASTOR1-
- CASTOR1-
- raptor

B
HEK59 stably expressing:
- arginine
- hairpin: G
- T389-M6K1
- S6K1
- CASTOR1
- raptor

C
HEK-293T stably expressing:
- arginine
- hairpin: G
- T389-M6K1
- S6K1
- CASTOR1
- CASTOR2
- raptor

D
HEK-293T stably expressing:
- arginine
- hairpin: G
- T389-M6K1
- S6K1
- CASTOR1
- CASTOR2
- raptor

E
HEK-293T stably expressing:
- arginine
- hairpin: G
- T389-M6K1
- S6K1
- CASTOR1
- CASTOR2
- raptor

F
HEK-293T stably expressing:
- arginine added to cell media (µM)
- hairpin: G
- T389-M6K1
- S6K1
- CASTOR1
- CASTOR2

HEK-293T stably expressing:
- arginine added to cell media (µM)
- hairpin: G
- T389-M6K1
- S6K1
- CASTOR1
- CASTOR2
replete conditions (Figure 5B). These results support the notion that CASTOR2, due to its inability to bind to arginine, dampens mTORC1 activity when arginine is present. However, under arginine withdrawal, loss of CASTOR2 does not affect mTORC1 activity because CASTOR1 is still present to inhibit the mTORC1 pathway. Further corroborating an inhibitory role for CASTOR2, its transient overexpression abrogated mTORC1 activation by amino acids even more potently than overexpression of CASTOR1 (Figure 5A). Thus, both CASTOR1 and CASTOR2 are negative regulators of arginine signaling to mTORC1.

Consistent with the differential sensitivities of CASTOR1 and CASTOR2 to arginine, mild overexpression of each in HEK-293T cells had distinct effects on the response of the mTORC1 pathway to arginine. Overexpression of CASTOR2 blunted the maximal level of arginine-induced mTORC1 activity, while that of CASTOR1 reduced the sensitivity of the pathway to arginine but did not affect its maximal activity.

Figure S5, related to Figure 5: CASTOR1 functions as a negative regulator of arginine signaling to mTORC1, and overexpression of the CASTOR proteins affects the response of the mTORC1 pathway to arginine.

(A) Reintroduction of CASTOR1 into CASTOR1 knockdown cells rescues the ability of the mTORC1 pathway to respond to arginine deprivation. HEK-293T cells stably expressing the indicated shRNAs and cDNA constructs were treated as in Figure 3B and lysates were analyzed by immunoblotting for indicated proteins.

(B) CRISPR/Cas9 mediated depletion of CASTOR1 in HEC59 cells confers resistance of the mTORC1 pathway to arginine deprivation. HEC59 cells stably expressing Cas9 with the indicated guide RNAs were treated as in Figure 3B and lysates were analyzed by immunoblotting for indicated proteins.

(C) RNAi-mediated depletion of CASTOR2 increases mTORC1 activity in response to arginine in cells depleted of CASTOR1. HEK-293T cells stably expressing the indicated shRNAs were treated as in Figure 3B and lysates were analyzed by immunoblotting for indicated proteins.

(D) RNAi-mediated depletion of CASTOR1 does not confer resistance of the mTORC1 pathway to deprivation of leucine or total amino acids. HEK-293T cells stably expressing the indicated shRNAs were treated as in Figure 3B and lysates were analyzed by immunoblotting for the indicated proteins.

(E) CRISPR/Cas9-mediated depletion of CASTOR1 does not confer resistance of the mTORC1 pathway to deprivation of leucine or total amino acids. HEK-293T cells stably expressing the indicated shRNAs were treated as in Figure 3B and lysates were analyzed by immunoblotting for the indicated proteins.

(F) Overexpression of CASTOR1 and CASTOR2 alters the response of the mTORC1 pathway to arginine. HEK-293T cells stably expressing the indicated cDNAs were stimulated with the increasing concentrations of arginine in the cell media, and lysates were analyzed by immunoblotting for the proteins shown.
Figure 6

(A) Graph showing the binding of (14C)-arginine to CASTOR1 and CASTOR1Δ26A in the presence of 10 mM arginine.

(B) Western blot analysis of transfected cDNAs: arginase, CASTOR1, and CASTOR1Δ26A in cell lysates.

(C) Western blot analysis of HEK-293T cells stably expressing various constructs, including CASTOR1, CASTOR1Δ26A, and CASTOR1Δ2Δ466, comparing the expression levels of dihydrotestosterone receptor (DHT-R), CASTOR1, and CASTOR1Δ2Δ466.

(D) Schematic diagram illustrating the interaction between CASTOR1 and arginine in the context of lysosomal membrane proteins and ATPase activity.
(Figure S5F). Like is the case for the Sestrins (Budanov et al., 2004; Chen et al., 2010; Lee et al., 2010; Ouyang et al., 2012), changes in CASTOR protein levels may be mediated by stress responsive transcription factors such as p53 and FOXO1. Consistent with this possibility, analysis of publicly available ChIP-seq data indicates that FOXO1 binds upstream of and may regulate the expression of CASTOR2 in addition to SESN3 (Ouyang et al., 2012).

Finally, we probed the relationship between CASTOR1 and SLC38A9, a putative lysosomal arginine sensor that is required to signal the presence of arginine to mTORC1 (Wang et al., 2015). Consistent with the established role of SLC38A9, arginine-induced activation of mTORC1 signaling was severely blunted in HEK-293T cells lacking SLC38A9 (Figure 5E). RNAi-mediated depletion of CASTOR1 in SLC38A9-null cells renders the mTORC1 pathway insensitive to arginine: cells neither activated mTORC1 when arginine was present nor inactivated mTORC1 when arginine was withdrawn (Figure 5E). While other models are possible, it is likely that CASTOR1 and SLC38A9 function in parallel to enable arginine to regulate mTORC1, and in their absence, arginine signaling is almost fully defective.

The binding of arginine to CASTOR1 is necessary for it to activate mTORC1

To test whether the activation of mTORC1 by arginine requires the arginine-binding capacity of CASTOR1, we used alanine scanning mutagenesis of the

Figure 6: Arginine must be able to bind to CASTOR1 for it to activate mTORC1

(A) The CASTOR1 I280A mutant does not bind arginine. Binding assays were performed with FLAG immunoprecipitates of the indicated complexes as in Figure 4A.

(B) Arginine does not regulate the interaction of CASTOR1 I280A with GATOR2. HEK-293T cells cotransfected with the indicated cDNAs in expression vectors were treated as in Figure 5B and anti-HA immunoprecipitates were analyzed by immunoblotting for levels of the indicated proteins.

(C) Reintroduction of the CASTOR1 I280A mutant into CASTOR1 knockdown cells renders the mTORC1 pathway unable to sense the presence of arginine. HEK-293T cells stably expressing the indicated shRNAs and cDNA constructs were treated as in Figure 5B and lysates analyzed by immunoblotting for indicated proteins.

(D) A model depicting how the cytosolic and lysosomal amino acid inputs impinge on CASTORs, Sestrins, and SLC38A9 to regulate mTORC1 activity.
Supplemental Figure 6

A

transfected cDNAs:

<table>
<thead>
<tr>
<th></th>
<th>FLAG CASTOR1 ACT1</th>
<th>+</th>
<th>+</th>
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<tr>
<td></td>
<td>HA-CASTOR1 ACT2</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>HA-CASTOR1 1290A ACT2</td>
<td>+</td>
<td>+</td>
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arginine:

- + - +

IP:

HA-CASTOR1 ACT2

FLAG:

FLAG CASTOR1 ACT1

cell lysate:

HA-CASTOR1 ACT2
CASTOR1 ACT domains to identify CASTOR1 mutants that no longer bind arginine. These efforts led to the identification of I280A, a mutation within the second ACT domain that fully abrogated the ability of CASTOR1 to bind arginine in vitro (Figure 6A).

Consistent with the notion that the binding of arginine to CASTOR1 leads to the disruption of the CASTOR1-GATOR2 complex, the arginine-binding mutant of CASTOR1 constitutively interacted with GATOR2 in cells, irrespective of arginine levels (Figure 6B). Notably, this mutant bound more strongly to GATOR2 than its wild type counterpart, confirming that arginine modulates the CASTOR1-GATOR2 interaction. In addition, while the isolated ACT domains of CASTOR1 associated only in the presence of arginine, an I280A change in ACT2 fully abrogated this regulation, and provides further support for an important role for this residue in the binding of arginine to CASTOR1 (Figure S6A). Further reflecting the importance of this residue in CASTOR1 function, I280 is highly conserved in orthologs of CASTOR1 and is present in nearly all bacterial and fungal ACT domains that share sequence homology with the CASTOR1 ACT domain (Figure 1C and Figure S1B, C and D).

Finally, if CASTOR1 is a bona fide arginine sensor for the mTORC1 pathway, abolishing its ability to bind arginine should in turn abolish the ability of arginine to activate mTORC1 in cells. To test this hypothesis, we compared the arginine sensitivity of the mTORC1 pathway in CASTOR1 knockdown cells that stably expressed either wild type CASTOR1 or the arginine-binding mutant of CASTOR1. Unlike reintroduction of wild type CASTOR1, which restores the ability of arginine to signal to mTORC1, expression of the CASTOR1 arginine-binding mutant rendered the mTORC1 pathway

---

Figure S6, related to Figure 6: Arginine must bind to CASTOR1 for it to regulate the interaction between the CASTOR1 ACT domains.
(A) Arginine does not regulate the interaction between ACT1 and ACT2 I280A in cells. HEK-293T cells cotransfected with the indicated cDNAs in expression vectors were either deprived of arginine in the cell media for 50 min or starved and restimulated with arginine for 10 min. Anti-FLAG immunoprecipitates were prepared and analyzed as in Figure 3F.
inactive and insensitive to the presence of arginine (Figure 6C). In combination, these findings establish that arginine must bind to CASTOR1 in order for the mTORC1 pathway to respond to arginine.

We establish the CASTOR1 homodimer and CASTOR1-CASTOR2 heterodimer as arginine sensors for the mTORC1 pathway. First, arginine binds to both complexes at affinities that are consistent with those that activate mTORC1 in cells. Second, CASTOR1 loss leads to insensitivity of the mTORC1 pathway to arginine deprivation. Third, expression in cells of an arginine-binding mutant of CASTOR1 prevents the mTORC1 pathway from sensing the presence of arginine.

The identification of CASTOR1 and Sestrin1 and 2 as sensors for the mTORC1 pathway reveal that GATOR2 is a critical hub of amino acid sensing, where leucine and arginine signals converge upstream of the Rag GTPases to regulate mTORC1 activity (Figure 6D). Leucine and arginine have long been appreciated to be important for mTORC1 activation (Ban et al., 2004; Blommaart et al., 1995; Fox et al., 1998; Hara, 1998; Lynch et al., 2000), and our findings highlight differences in how these two amino acids are sensed. The cytosolic Sestrin proteins are likely the primary leucine sensors because their loss confers complete insensitivity of the mTORC1 pathway to leucine deprivation (Saxton et al., 2015; Wolfson et al., 2015). In contrast, arginine sensing appears to be more complex and may have inputs from two distinct cellular compartments. Loss of function experiments suggests that CASTOR1 signals the absence of arginine to inhibit mTORC1. Because CASTOR1 lacks transmembrane domains and signal sequences it is likely a soluble protein that senses free arginine in the cytosol. In contrast, SLC38A9 is needed to signal the presence of arginine, presumably lysosomal, to mTORC1. Together, both proteins appear to form parallel sensing branches that relay arginine availability to mTORC1. In the absence of both CASTOR1 and SLC38A9, arginine no longer regulates the activity of the mTORC1 pathway.

Despite these insights, several key questions remain. While CASTOR1 and Sestrin2 both bind to and likely inhibit GATOR2, whether they operate through distinct
mechanisms can only be determined once the function of GATOR2 is elucidated. Furthermore, structural studies will provide insight into how the binding pocket of CASTOR1 achieves its remarkable specificity for arginine. Importantly, these structural studies may also reveal why arginine binds to CASTOR1, but not CASTOR2. Although I280 is critical for arginine binding to CASTOR1, this residue is also present in CASTOR2, suggesting that other unidentified residues must dictate the difference in arginine binding between these two proteins.

In addition, in vivo characterization of mice lacking the CASTOR genes will be needed to reveal how arginine sensing varies across tissues and during development. Because arginine differentially regulates each CASTOR complex, altering the expression of CASTOR1 versus CASTOR2 could serve as a means to modulate mTORC1 activity. CASTOR2 appears analogous to Sestrin3, as both are defective in amino acid binding and constitutively associate with GATOR2 to inhibit mTORC1 signaling. Thus, increased levels of CASTOR2 should blunt maximal mTORC1 signaling while those of CASTOR1 may alter the sensitivity of the pathway to arginine, as we observed in HEK-293T cells (Figure S5F).

It is likely that additional amino acid sensors exist to signal the presence of other critical amino acids for mTORC1 activity, such as glutamine (Jewell et al., 2015), as well as sensors that mediate the amino acid sensitive events upstream of additional mTORC1 regulators, such as Folliculin/FNIP (Petit et al., 2013; Tsun et al., 2013). Characterizing the evolutionary conservation of the amino acid sensors of the mTORC1/TORC1 pathway will provide insight into how varied are the amino acid inputs that drive mTORC1/TORC1 signaling in diverse organisms. For instance, budding yeast encodes a homolog of GATOR2, but not of the Sestrins or CASTORs, hinting at a divergence in the regulation of the upstream components of the nutrient sensing pathway. This divergence may be expected given that yeast, unlike mammals, can synthesize all amino acids and thus must sense the quality and abundance of nitrogen sources rather than the identity and availability of particular amino acids. Further identification and characterization of the amino acid sensors upstream of the Rag GTPases will guide us
towards a comprehensive understanding of how nutrients regulate the mTORC1 pathway.
Materials and Methods

Materials

Reagents were obtained from the following sources: HRP-labeled anti-mouse and anti-rabbit secondary antibodies, lamp and cathepsin antibodies from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, S6K1, Sestrin2, Histone H3, VDAC and mios from Cell Signaling Technology; antibody to the HA epitope from Bethyl laboratories; antibody to raptor from Millipore; FLAG M2 antibody, FLAG M2 affinity gel, ATP, and amino acids from Sigma Aldrich; HA magnetic beads and RPMI without leucine, arginine, or lysine from Pierce; DMEM from SAFC Biosciences; XtremeGene9 and Complete Protease Cocktail from Roche; Inactivated Fetal Calf Serum (IFS) from Invitrogen; amino acid-free RPMI from US Biologicals; [3H]-labeled amino acids from American Radiolabeled Chemicals. The WDR24, Mios, CASTOR1, and CASTOR2 antibodies were generously provided by Jianxin Xie (Cell Signaling Technology).

Cell lines and tissue culture

All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% inactivated fetal calf serum supplemented with 2 mM glutamine. All cell lines were maintained at 37°C and 5% CO2.

Preparation of cell lysates and immunoprecipitates

Cell lysate preparation, cell lysis, and immunoprecipitations were performed as described in Supplemental Experimental Procedures.

Where indicated, for transient cotransfection experiments, 2 million HEK-293T cells were plated in 10 cm dishes and transfected 24 hrs later using the polyethylenimine method (Boussif et al., 1995) with the indicated pRK5-based expression vectors: 300 ng HA-metap2, 40 ng CASTOR1-HA, 80 ng CASTOR1-FLAG, 40 ng CASTOR2-HA, 80 ng CASTOR2-FLAG; 100 ng WDR24-FLAG, 100 ng WDR59, 100 ng mios, 150 ng sec13, 150 ng seh1L, 10 ng or 40 ng or 100 ng or 600 ng of myc-Sestrin2 or myc-CASTOR2; 2 ng FLAG-S6K1, 15 ng or 60 ng HA-CASTOR2, 75 ng and
175 ng of CASTOR1-HA. The total amount of plasmid DNA in each transfection was normalized to 5 μg with empty pRK5. Thirty-six hours after transfection, cells were harvested as described above.

For experiments that required leucine, arginine or amino acid starvation or restimulation, cells were treated as previously described (Wolfson et al., 2015). Briefly, cells were incubated in leucine, arginine, or amino acid free RPMI for 50 minutes and then restimulated with the indicated amino acid(s) for 10 minutes.

**Arginine binding assay**

Four million HEK-293T cells were plated in a 15 cm plate four days prior to the experiment. Forty-eight hours after plating, the cells were transfected via the polyethylenimine method (Boussif et al., 1995) with the pRK5-based cDNA expression plasmids indicated in the figures in the following amounts: 15 μg FLAG-Rap2A, 400 ng CASTOR1-FLAG or CASTOR2-FLAG with 1200 ng CASTOR1-HA or 1200 ng CASTOR2-HA, 400 ng CASTOR1 I280A-FLAG with 1200 ng CASTOR1 I280A-HA. The total amount of plasmid DNA in each transfection was normalized to 15 μg with empty pRK5. Forty-eight hours after transfection cells were lysed and binding assays performed and analyzed as previously described (Wolfson et al., 2015).

**In vitro CASTOR-GATOR2 dissociation assay**

HEK-293T cells cotransfected with 40 ng CASTOR1-HA and either 80 ng CASTOR1-FLAG (CASTOR1 homodimer) or 80 ng CASTOR2-FLAG (CASTOR1-CASTOR2 heterodimer) were starved for all amino acids for 50 minutes, lysed and subjected to anti-HA immunoprecipitation as described previously. The CASTOR-GATOR2 complexes immobilized on agarose beads were washed once in Triton wash buffer, three times in Triton wash buffer supplemented with 500 mM NaCl, and then incubated for 20 minutes in 1 mL of ice-cold Triton wash buffer supplemented with 500 mM NaCl and the indicated concentrations of individual amino acids. The amount of GATOR2 that remained bound to CASTOR complexes was assayed by SDS-PAGE and immunoblotting as previously described.
Statistical analysis

Two-tailed t tests were used for comparison between two groups. All comparisons were two-sided, and P values of less than 0.001 were considered to indicate statistical significance.

Extended Experimental Procedures

Sequence alignments

Indicated protein sequences were obtained from the NCBI protein database and aligned via the T-coffee multiple sequence alignment program on EMBL-EBI. Alignments were annotated using JalView.

Preparation of cell lysates and immunoprecipitates

Cells were rinsed once with ice-cold PBS and immediately lysed with Triton lysis buffer (1% Triton, 10 mM β-glycerol phosphate, 10 mM pyrophosphate, 40 mM Hepes pH 7.4, 2.5 mM MgCl₂ and 1 tablet of EDTA-free protease inhibitor [Roche] (per 25 ml buffer). The cell lysates were cleared by centrifugation at 13,200 rpm at 4°C in a microcentrifuge for 8 minutes. For anti-FLAG and anti-HA immunoprecipitations, the FLAG-M2 or HA affinity gel was washed 3 times with Triton wash buffer (1% Triton, 40 mM Hepes pH 7.4, 2.5 mM MgCl₂). 30 μl of a 50/50 slurry of the FLAG-M2 affinity gel or 25 μl of the HA affinity gel was then added to clarified cell lysates and incubated with rotation for 90 minutes at 4°C. Following immunoprecipitation, the beads were washed one time with Triton wash buffer and 3 times with Triton wash buffer containing 500 mM NaCl. Immunoprecipitated proteins were denatured by the addition of 50 μl of sample buffer, resolved by 8%–16% SDS-PAGE, and analyzed by immunoblotting.

Subcellular fractionation

Five million HEK-293T cells were seeded on a 10 cm plate 24 hrs before harvesting. Cells were pelleted in cold PBS at 1,000 g for 2 minutes, and subsequently fractionated with the Cell Fractionation Kit (CST) according to the manufacturer’s instructions. Briefly, cell pellets were resuspended in 500 μl cytoplasmic isolation buffer (CIB), vortexed for 5 minutes and incubated on ice for 5 minutes. Following a 5 minute spin at 500 g, an aliquot of the supernatant was taken (cytosolic fraction). The remaining pellet was resuspended in 500 μl of membrane isolation buffer (MIB), vortexed for 15 seconds, and incubated on ice for 5 minutes. Following a 5 minute spin at 8,000 g, an aliquot of the supernatant was taken (membrane and organellar
fraction). The remaining pellet was resuspended in 250 µl of cytoskeletal/nuclear isolation buffer (CyNIB) and sonicated to yield the cytoskeletal and nuclear fraction.

**Mammalian lentiviral production and transduction**

Lentiviral short hairpin RNAs (shRNAs) were obtained from the TRC. Guide RNAs (sgRNAs) targeting CASTOR1, CASTOR2, or a control AAVSI locus were cloned into pLentiCRISPR v2. The target sequences are described below.

To generate HEK-293T cells with RNAi-mediated loss of CASTOR2 and/or CASTOR1, the following shRNAs were transfected into viral HEK-293Ts:

Human shCASTOR1_1: TRCN0000284010  
Human shCASTOR1_2: TRCN0000269399  
Human shCASTOR2_1: TRCN0000352396  
Human shCASTOR2_2: TRCN0000337256  
Human shCASTOR2_3: TRCN0000352387

The following sense (S) and antisense (AS) oligonucleotides encoding the guide RNAs were cloned into a pLentiCRISPR vector:

sgCASTOR1_1S: caccgTGTAGAGCCAGAGACCGGGA  
sgCASTOR1_1AS: aaacTCCCGGTCTCTGGCTCTACAc  
sgCASTOR1_2S: caccgGAGCAGCTTGATGAGCGGGT  
sgCASTOR1_2AS: aaacACCCGCTCATCAAGCTGCTCc  
sgCASTOR1_3S: caccgGACACGTGGCTCGGCCAG  
sgCASTOR1_3AS: aaacCTGGCCGAGCACCACGTGTCc

Lentiviruses were produced by transfection of viral HEK-293T cells with either pS2JC6-CASTOR1-FLAG (wild-type or mutant) constructs or shRNA constructs in combination with the VSV-G envelope and CMV ΔVPR packaging plasmids. Twenty-four hours after transfection, the media was changed to fresh DMEM with 20% IFS. Forty-eight hours after transfection, the virus-containing supernatant was collected from the cells and passed through a 0.45 µm filter. To generate the indicated stable cell lines overexpressing CASTOR1, 300,000 cells were plated in 6-well plates containing 1.5 mls DMEM 10% IFS with 8 µg/mL polybrene and infected with virus containing media. Twenty-four hours later, the media was changed to fresh media containing 5 µg/ml blasticidin for selection.
To generate the indicated knockdown lines, 3 million cells were plated in 6-well plates containing 2 ml s DMEM 10% IFS with 8 μg/mL polybrene and infected with virus containing media. Cells were spun at 2,200 rpm for 45 minutes at 37°C. Twelve hours later, the media was changed to fresh DMEM 10% IFS, and 7-10 hours later, cells were trypsinized and selected with puromycin.
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We thank all members of the Sabatini lab for helpful suggestions, in particular Monther Abu-Remaileh and Walter W. Chen for experimental insight and advice. We are grateful to Jianxin Xie of Cell Signaling Technologies for generously providing many antibodies, in particular the CASTOR1 and CASTOR2 anti-sera. This work has been supported by grants from the US NIH (R01CA103866 and AI47389) the Department of Defense (W81XWH-07-0448) to D.M.S. Fellowship support was provided by the John Reed UROP Fund to S.M.S.; the National Defense Science & Engineering Graduate Fellowship (NDSEG) to G.A.W.; and by the NIH to L.C. (F31 CA180271); to T.W. (F31 CA189437); U41 HG006673 to S.P.G and J.W.H., and GM095567 to J.W.H. K.S. is a Pfizer Fellow of the Life Sciences Research Foundation. D.M.S is an investigator of the Howard Hughes Medical Institute.
References


CHAPTER 6

The KICSTOR complex targets GATOR1 to the lysosomal surface and is necessary for nutrient starvation to suppress mTORC1

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Experiments in Figures 1-4, S1-S4 were performed by RW and LC, with assistance from SMS and XG. Experiments in Figure 2 were performed by JK. Experiments in Figure S2 were performed by KC.
Summary

The mechanistic target of rapamycin complex 1 kinase (mTORC1) is a central regulator of cell growth that responds to diverse environmental signals. Amino acids are a key input, and act through the Rag GTPases to promote the translocation of mTORC1 to the lysosomal surface, its site of activation. Multiple protein complexes regulate the Rag GTPases in response to amino acids, including GATOR1, a GTPase activating protein for RagA, and GATOR2, a positive regulator of unknown molecular function. Here, we identify a four-membered protein complex composed of KPTN, ITFG2, c12orf66, and SZT2 (KICSTOR) as required for amino acid deprivation to inhibit mTORC1. Several KICSTOR components are lost in neurological diseases associated with hyperactive mTORC1 signaling. KICSTOR binds to GATOR1; localizes it, but not GATOR2, to the lysosomal surface; and is necessary for the GATOR1-GATOR2 interaction. Thus, KICSTOR is a key negative regulator of mTORC1 signaling that, like GATOR1, is mutated in disease.
Introduction

The mechanistic target of rapamycin complex 1 (mTORC1) protein kinase is a central controller of cell growth that is deregulated in a number of human diseases, including cancer (Laplante and Sabatini, 2012). Recent evidence also reveals that several neurological disorders, including focal cortical dysplasia (FCD), epilepsy, and hemimegalencephaly, are associated with mutations in components of the mTORC1 pathway, including PI3K, Akt, mTOR, the TSC complex, and GATOR1 (Baldassari et al., 2016; Crino, 2011; DeGama et al., 2015; Lim et al., 2015; Mirzaa et al., 2016).

mTORC1 regulates anabolic processes that promote cell growth in response to diverse environmental signals (Laplante and Sabatini, 2012). Many of these inputs, including growth factors, cellular stress, and oxygen levels, impinge on the nucleotide state of Rheb, a lysosomal bound GTPase and essential activator of mTORC1 (Brugarolas et al., 2004; Garami et al., 2003; Inoki et al., 2003; Long et al., 2005; Sancak et al., 2008; Saucedo et al., 2003; Stocker et al., 2003; Tee et al., 2002). Growth factors drive the translocation of the TSC complex, the GTPase activating protein (GAP) for Rheb, off the lysosomal surface (Buerger et al., 2006; Dibble et al., 2012; Menon et al., 2014; Saito et al., 2005). This renders Rheb GTP-loaded and able to activate mTORC1, but only if mTORC1 also localizes to the lysosomal surface.

Nutrients, specifically amino acids, promote mTORC1 activity by stimulating its translocation to the lysosomal surface. This process depends on the Rag GTPases, which are obligate heterodimers of RagA or RagB bound to RagC or RagD (Kim et al., 2008; Sancak et al., 2008). In response to nutrients, numerous protein complexes regulate the nucleotide loading state of the Rag GTPases, including FLCN-FNIP2 (Petit et al., 2013; Tsun et al., 2013), a GAP for RagC/D; GATOR1, a GAP for RagA/B (Bar-Peled et al., 2013); and Ragulator, which controls the nucleotide loading state and lysosomal localization of the Rag GTPases (Bar-Peled et al., 2012; Sancak et al., 2010b). Ragulator is a part of a lysosomal supercomplex, including the vacuolar H⁺-
ATPase (v-ATPase), a positive regulator of the pathway, and SLC38A9, a transporter for and putative sensor of lysosomal amino acids (Wang et al., 2015).

In the cytosol, Sestrin1/2 and CASTOR1 serve as leucine and arginine sensors, respectively (Chantranupong et al., 2016; Chantranupong et al., 2014; Wolfson et al., 2015). These cytosolic sensors interact with GATOR2, a pentameric complex and a positive regulator of unknown function, which lies upstream of or parallel to GATOR1. Together, these proteins participate in the cytosolic amino acid sensing arm of the mTORC1 pathway. It remains unknown how these protein complexes dock on the lysosome and if amino acid sensing through the Rag GTPases requires additional components.

Here, we identify KICSTOR, a complex of four proteins that is necessary for nutrient starvation to suppress mTORC1 signaling. Three of the KICSTOR components are lost in brain malformation disorders similar to those associated with mutations in known mTORC1 pathway members. KICSTOR interacts with and tethers GATOR1, but not GATOR2, to the lysosomal surface. Thus, we identify a key regulatory complex that is necessary to inhibit mTORC1 signaling in the absence of amino acids.
Results and Discussion

KICSTOR is a GATOR1-interacting complex

To begin to search for GATOR1-interacting proteins that may have escaped prior identification, we used the CRISPR/Cas9 system to engineer HEK-293T cells to express a FLAG-tagged version of DEPDC5, a GATOR1 component, from the endogenous gene. Mass spectrometric analysis of FLAG-immunoprecipitates prepared from these cells revealed the presence of GATOR2, an established GATOR1-interacting complex, as well as four proteins of unknown function encoded by the KPTN, ITFG2, c12orf66, and SZT2 genes (Fig. 1A). We named this complex KICSTOR for KPTN, ITFG2, c12orf66, and SZT2-containing regulator of mTORC1.

In co-immunoprecipitation experiments using HEK-293T cells transiently expressing KICSTOR members, we explored how the four proteins interact with each other. As KPTN and ITFG2 associated in the absence of c12orf66 and SZT2 (Fig. 1B),
Figure 1

<table>
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<td>c12orf66 isoform 2</td>
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<td>WDR59</td>
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<td>WDR24</td>
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</table>

**B**

Transfected cDNAs:
- MYcTFG2:
- cNs:
- HA-KPTN:
- FLA-KPTN,
- FLA-metp2

**C**

Transfected cDNAs:
- wrc-KPTN:
- KPTN
- ITFG2:
- FLA-KPTN, FLA-metp2

**D**

HEK-293E cells expressing:
- FLA-WDR24
- WDR59
- Npr3

**E**

HEK-293 clone: Npr3-null
- KPTN
- RagC
- RagA
- Npr3
- sestrin2

**F**

HEK-293 clone: Npr3-null
- KPTN
- RagC
- RagA
- Npr3
- sestrin2

**G**

HEK-293 clone: wild-type
- KPTN
- RagC
- RagA
- Npr3
- sestrin2

**H**

HEK-293 clone: wild-type
- KPTN
- RagC
- RagA
- Npr3
- sestrin2
Supp. Figure 1

A

<table>
<thead>
<tr>
<th>Transfected cDNAs</th>
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<th>WDR24 null</th>
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<td>+</td>
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C

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<td>Flag-SZT2</td>
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<tr>
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D

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Figure S1: related to Figure 1. Loss of the GATOR complexes does not affect the interaction between KICSTOR components, and GATOR1 interacts at a distinct site on SZT2 than KPTN-ITFG2 and c12orf66.

A) The KICSTOR complex remains intact in the absence of GATOR1 and B) GATOR2. Anti-FLAG immunoprecipitates were prepared from wild-type, Nprl3-null, or WDR24-null HEK-293T cells expressing the indicated cDNAs and analyzed, along with cell lysates, by immunoblotting for the relevant epitope tags.

C) c12orf66 binds to SZT2 at a distinct site from KPTN-ITFG2. HEK-293T cells expressing the indicated cDNAs were treated and analyzed as in (A).

D) GATOR1 interacts with the first and second regions of SZT2. HEK-293T cells expressing the indicated cDNAs were treated and analyzed as in (A).

they can form a heterodimeric complex on their own. Consistent with this interpretation, loss of ITFG2 severely reduced KPTN protein levels, and vice versa, while that of c12orf66 did not affect either (Fig. 2B, C). The KPTN-ITFG2 heterodimer co-immunoprecipitated c12orf66 only in the presence of SZT2 (Fig. 1B, C), and thus these four proteins form a complex in which SZT2 serves as the link between the other three components. SZT2 binds both the KPTN-ITFG2 heterodimer and c12orf66, but apparently at different sites as increasing amounts of KPTN-ITFG2 did not displace c12orf66 from SZT2 (Fig. 1C).

We readily detected endogenous KICSTOR in anti-FLAG immunoprecipitates from HEK-293E cells stably expressing FLAG-Nprl2, a GATOR1 component (Fig. 1D). In contrast, the interaction of KICSTOR with GATOR2, an established GATOR1-interacting complex, is weaker, as FLAG-tagged WDR24 co-immunoprecipitated lower

Figure 2: The KICSTOR complex is required for amino acid deprivation to inhibit mTORC1.

A) SZT2-null cells are insensitive to amino acid starvation. Wild-type and SZT2-null HeLa cells generated with the CRISPR/Cas9 system were starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min. Immunoblotting of cell lysates allowed for the analysis of the levels and phosphorylation states of the indicated proteins.

B) CRISPR/Cas9-mediated depletion of KPTN, (C) ITFG2, and (D) c12orf66 renders cells insensitive to amino acid deprivation. HEK-293T cells stably expressing the indicated sgRNAs were treated and analyzed as in (A).

E) KICSTOR loss results in constitutive mTORC1 localization to the lysosomal surface. Wild-type and KPTN-, ITFG2-, or SZT2-null HEK-293T cells created with the CRISPR/Cas9 system were starved or starved and restimulated with amino acids for the indicated times prior to processing for immunofluorescence. In all images, insets depict selected fields that were magnified 3.24X and their overlays. Scale bars represent 10 μm.
Figure 2

A

HEK-293T stably expressing amino acids:

\( \text{T389-S6K1} \)

\( \text{S6K1} \)

\( \text{Nprl3} \)

\( \text{miros} \)

\( \text{raptor} \)

B

HEK-293T stably expressing amino acids:

\( \text{T389-S6K1} \)

\( \text{S6K1} \)

\( \text{Nprl3} \)

\( \text{KPTN} \)

\( \text{IFG2} \)

C

HEK-293T stably expressing amino acids:

\( \text{T389-S6K1} \)

\( \text{S6K1} \)

\( \text{Nprl3} \)

\( \text{KPTN} \)

\( \text{IFG2} \)

D

HEK-293T stably expressing amino acids:

\( \text{T389-S6K1} \)

\( \text{S6K1} \)

\( \text{Nprl3} \)

\( \text{KPTN} \)

\( \text{IFG2} \)

E

HEK-293T clone wild-type KPTN-null ITFG2-null SZT2-null

antibody mTOR LAMP2 mTOR LAMP2 mTOR LAMP2 mTOR LAMP2

-AA for 90 min

+AA for 10 min

-AA for 60 min
Supp. Figure 2

A. HeLa clone: HeLa cells expressing: insulin: ei®-T389-S6K1 amino acids: -T308-Akt impairments: Akt

B. HeLa cells stably expressing: insulin: ei®-T389-S6K1 amino acids: -K1 T369-S61

C. HeLa cells stably expressing: insulin: ei®-T389-S6K1 amino acids: -K1 T369-S61

D. HeLa stably expressing: amino acids: ei®-T389-S6K1 S6K1 KPTN ITFG2 raptor

E. HeLa stably expressing: amino acids: ei®-T389-S6K1 S6K1 KPTN ITFG2 raptor

F. HeLa stably expressing: amino acids: ei®-T389-S6K1 S6K1 KPTN ITFG2 raptor

G. HeLa stably expressing: insulin: amino acids: ei®-T389-S6K1 raptor

H. HEK-293T cell line: amino acids: ei®-T389-S6K1 raptor

I. HEK-293T cell line: amino acids: ei®-T389-S6K1 raptor

J. HEK293T cell clones: amino acids: ei®-T389-S6K1 S6K1 WDR24 SZT2

K. HEK-293T cells: Wild-type & SZT2 null

L. HEK293T cell clones: amino acids: ei®-T389-S6K1 S6K1 WDR24 Npr3 raptor
levels of SZT2 and KPTN than did FLAG-Nprl2 (Fig. 1D). GATOR1 likely mediates the
GATOR2-KICSTOR interaction because GATOR1 loss severely reduced it (Fig 1E),
while GATOR2 loss had little effect on the GATOR1-KICSTOR interaction (Fig. 1F).
Notably, the absence of GATOR1 or GATOR2 did not impair the interaction between
the four components of KICSTOR (Fig S1A, B). Furthermore, loss of any KICSTOR
member did not alter the expression levels of Nprl3 or mios, GATOR1 and GATOR2
components, respectively (Fig. 2A-C). Altogether, these results are most consistent with
KICSTOR being a distinct four-protein complex that interacts with GATOR1, which in
turn binds to GATOR2.

In the absence of SZT2, the KPTN-ITFG2 dimer did not co-immunoprecipitate
GATOR1 or GATOR2 (Fig. 1G). In contrast, the interaction of SZT2 with GATOR1 did
not require KPTN, ITFG2, or c12orf66 (Fig. 1H), indicating that SZT2 links GATOR1 to
the other KICSTOR components. As SZT2 is a 378 kDa protein that is nearly eight
times the mass of the other KICSTOR subunits, we divided SZT2 into three fragments
based on an evolutionary conservation. Consistent with the notion that KPTN-ITFG2 and
c12orf66 bind different sites on SZT2 (Fig 1C), KPTN-ITFG2 interacted with all three
fragments while c12orf66 strongly co-immunoprecipitated only with the C-terminal third

Figure S2: related to Figure 2. The KICSTOR complex is not required for insulin
depression to inhibit mTORC1, and it functions downstream of or in parallel to GATOR2
to inhibit mTORC1 activation.
(A) – (G) KICSTOR-null cells are sensitive to amino acid deprivation, but not growth factor
depression. Wild-type, SZT2-null HeLa cells, or HeLa cells stably expressing sgRNAs targeting
KPTN, ITFG2, and c12orf66 were starved of amino acids or growth factors for 50 min or starved
and restimulated with amino acids or insulin, where indicated, for 10 min. Immunoblotting of cell
lysates allowed for the analysis of the levels and phosphorylation states of the indicated
proteins.
H) CRISPR/Cas9-mediated depletion of ITFG2 and (I) KPTN renders cells insensitive to amino
acid deprivation. Lysates from the indicated HEK-293T cells were treated and prepared for
immunoblotting as in (A)
J) KICSTOR functions downstream of or in parallel to GATOR2. Wild-type, SZT2-null, or SZT2- and
WDR24-null HEK-293Ts were treated and analyzed as in (A).
K) KICSTOR functions upstream of the Rag GTPases to regulate the mTORC1 pathway. Wild-
type or SZT2-null HEK-293Ts expressing the indicated cDNAs were treated and analyzed as in
(A).
L) GATOR1 functions downstream of or in parallel to GATOR2. Wild-type, WDR24-null, or Nprl3- and
WDR24-null HEK-293Ts were treated and analyzed as in (A).
Figure 3

A
HeLa clone: wild-type SZT2-null

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<th>cells expressing:</th>
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<tbody>
<tr>
<td>FLAG-metap2</td>
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</tr>
<tr>
<td>WDR24</td>
<td>+</td>
</tr>
<tr>
<td>Nprl2</td>
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</table>

| mI04 | |
| Npr13 | |

[Western blot images of RagA, RagC, and Npr13 with high exposure and low exposure]

IP: FLAG

B
HEK-293T cell line stably expressing: metap2, WDR24, Nprl2

<table>
<thead>
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<th>amino acids:</th>
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<tbody>
<tr>
<td>- + +</td>
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| Npr13 | |
| RagC | |

[Western blot images of Npr13 and RagC with high exposure and low exposure]

C
HEK-293T cell line stably expressing: metap2, WDR24, Nprl2

<table>
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<tbody>
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<td>- + +</td>
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| Npr13 | |
| RagC | |

[Western blot images of Npr13 and RagC with high exposure and low exposure]

D
HEK-293T cell line stably expressing: metap2, WDR24, Nprl2

<table>
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<td>- + +</td>
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| Npr13 | |
| RagC | |

[Western blot images of Npr13 and RagC with high exposure and low exposure]
Meanwhile, GATOR1 interacted with the first and second regions of SZT2 (Fig. S1D). Taken together, these results indicate that KPTN-ITFG2 and c12orf66 bind to SZT2 at distinct sites from GATOR1.

**KICSTOR is necessary to suppress mTORC1 signaling in the absence of nutrients**

Given the strong interaction between KICSTOR and GATOR1, a key inhibitor of the Rag GTPases, we reasoned that KICSTOR may be required for the control of the mTORC1 pathway by amino acids. Indeed, nutrient deprivation did not fully inhibit mTORC1 signaling in KICSTOR-null HEK-293T and HeLa cells, independently of which subunit was absent (Fig. 2A-D, S2B, D, F, I, and H). Consistent with this signaling defect, loss of KICSTOR increased the amount of lysosomally localized mTORC1 upon amino acid starvation (Fig. 2E). KICSTOR specifically regulates the nutrient sensing branch of mTORC1, as KICSTOR-null HeLa cells were still sensitive to insulin deprivation (Fig. S2A, C, E, and G).

To better define where KICSTOR acts in the mTORC1 pathway, we performed epistasis experiments between KICSTOR and established components of the pathway. Overexpression of the dominant negative Rag GTPases (RagB^T54N-RagC^Q120L) still inhibited mTORC1 in SZT2-null cells, indicating that KICSTOR is upstream of the Rag GTPases (Fig. S2J). Furthermore, GATOR1 overexpression partially suppressed the constitutive mTORC1 signaling of SZT2-null cells, while fully suppressing it in wild-type

---

**Figure 3: KICSTOR is required for the interaction of GATOR1 with GATOR2.**

A) Loss of SZT2 disrupts the interaction of GATOR2 with GATOR1, but not with the Rag GTPases. Anti-FLAG immunoprecipitates were prepared from wild-type or SZT2-null HeLa cells stably expressing the indicated cDNAs that had been starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min. Immunoprecipitates and cell lysates were analyzed by immunoblotting for the indicated proteins.

B) KPTN mediates the interaction of GATOR2 with GATOR1, but not with the Rag GTPases. HEK-293T cells stably expressing the indicated sgRNAs were treated and analyzed as in (A).

C) ITFG2 loss disrupts the GATOR1-GATOR2 interaction. Cells were treated and analyzed as in (A).

D) c12orf66 is necessary for the GATOR1-GATOR2, but not the GATOR2-Rag interaction. Cells were treated and analyzed as in (A).
Supp. Figure 3

A

cells expressing:

amino acids:

mice

IP: FLAG3

mlos

Npr13

mlos -A U

Nprt3

AE O-

SZT2

HeLa

HeLa SGT2-null (1.1)

FLAG- metap2 WDR24 Npr12

FLAG- metap2 WDR24 Npr12

FLAG-

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cells, suggesting that KICSTOR is required for GATOR1 to function normally (Fig. S2J). Finally, while loss of the WDR24 component of GATOR2 strongly inhibited amino acid-induced activation of mTORC1, loss of WDR24 had no effect on the constitutive signaling of cells lacking SZT2 (Fig. S2K). Thus, KICSTOR, likely acting together with GATOR1, functions downstream of or in parallel to GATOR2 to negatively regulate nutrient signaling to mTORC1 (Fig. S2L).

KICSTOR is required for GATOR1 to interact with GATOR2

We explored several possible mechanisms for why the suppression of mTORC1 signaling upon nutrient deprivation requires KICSTOR. One possibility is that GATOR1 requires KICSTOR for its stability, but loss of any KICSTOR component did not affect Nprl3 protein levels (Fig. 2A-D).

Alternatively, as a GATOR1-interacting protein, KICSTOR could mediate the interaction of GATOR1 with GATOR2. Consistent with this possibility, GATOR2 failed to co-immunoprecipitate GATOR1 in cells lacking SZT2 (Fig. 3A, S3A). Similarly, loss of any of the other three members of KICSTOR also disrupted the GATOR1-GATOR2 interaction (Fig. 3B-D, S3A). In contrast, loss of any component of KICSTOR did not greatly impair the interaction of GATOR2 with the Rag GTPases, implying that GATOR1 does not fully mediate the GATOR2-Rag interaction (Fig. 3A-D, S3A). Thus, the interaction of GATOR2 with GATOR1 requires KICSTOR, but that of GATOR2 with the Rag GTPases does not.

Figure S3, related to Figure 3: SZT2 is required for the interaction of GATOR1 with GATOR2.
A) Loss of SZT2 disrupts the interaction of GATOR1 and GATOR2. Anti-FLAG immunoprecipitates were prepared from wild-type or SZT2-null HeLa cells stably expressing the indicated cDNAs that had been starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min. Immunoprecipitates and cell lysates were analyzed by immunoblotting for the indicated proteins.
Figure 4

A HeLa cells expressing:

- AA for 50 min
- AA for 50 min
- AA for 10 min

B HeLa cells expressing:

GFP-Nprl2 - AA for 50 min
GFP-mios - AA for 50 min
GFP-WDR24 - AA for 50 min
GFP-mios - AA for 50 min
GFP-WDR24 - AA for 50 min
GFP-WDR24 - AA for 50 min

C HeLa cells expressing:

GFP-Nprl2 - AA for 50 min
GFP-mios - AA for 50 min
GFP-WDR24 - AA for 50 min
GFP-mios - AA for 50 min
GFP-WDR24 - AA for 50 min
GFP-WDR24 - AA for 50 min

D HeLa cell clone:

- AA for 50 min
- AA for 50 min
- AA for 10 min

E HeLa cell clone:

- AA for 50 min
- AA for 50 min
- AA for 10 min

F HeLa cell clone:

- AA for 50 min
- AA for 50 min
- AA for 10 min

G HeLa cell clone:

- AA for 50 min
- AA for 50 min
- AA for 10 min
GATOR1, but not GATOR2, requires KICSTOR for its localization to the lysosomal surface

Consistent with the well-established interaction of GATOR1 with GATOR2, both protein complexes have been shown to localize to same subcellular compartment – the lysosomal surface. However, it remains unclear how they are tethered there. The disruption of the interaction between GATOR1 with GATOR2 caused by loss of KICSTOR suggests that KICSTOR may function as a lysosomal scaffold for GATOR1. The absence of KICSTOR would mislocalize GATOR1 and thus prevent it from interacting with GATOR2.

To test this hypothesis, we first determined the subcellular localization of KICSTOR by stably expressing green fluorescent protein (GFP) tagged components of KICSTOR (KPTN and ITFG2) at levels similar to the endogenous proteins in HeLa cells. By immunofluorescence analysis KICSTOR localized to the lysosomal surface in a fashion not regulated by amino acids (Fig. 4G). Because KICSTOR is on the lysosomal surface and binds to GATOR1, we reasoned that KICSTOR might be necessary to

---

**Figure 4: KICSTOR localizes GATOR1 to the lysosomal surface.**

A) GATOR1 localizes to the lysosome regardless of amino acid levels. Nprl2-null HeLa cells were reconstituted with GFP-tagged Nprl2. Cells were starved or starved and restimulated with amino acids for the indicated times prior to processing for immunofluorescence.

B) GATOR2 localizes to the lysosomal surface regardless of amino acid levels, as assessed by the localization of GFP-tagged mios. Wild-type HeLa cells stably expressing GFP-tagged mios were treated and processed as in (A).

C) Amino acids do not control the localization of GATOR2 to the lysosomal surface. Wild-type HeLa cells stably expressing GFP-tagged WDR24, a component of GATOR2, were treated and processed as in (A).

D) SZT2 loss renders GATOR1 dispersed throughout the cytoplasm. Nprl2-null HeLa cells were reconstituted with GFP-Nprl2 and subsequently genetically modified using the CRISPR/Cas9 system to create SZT2-null cells. These cells were treated and analyzed as in (A).

E) KICSTOR loss disrupts the localization of GATOR1 to the lysosomal surface. Cells were generated as in (D) and treated and analyzed as in (A).

F) KICSTOR loss partially disrupts GATOR2 localization to lysosomes. Wild-type HeLa cells stably expressing GFP-tagged WDR24 were modified using the CRISPR/Cas9 system with the indicated sgRNAs and treated and analyzed as in (A).

G) KISCTOR is localized to the lysosome. Wild-type HeLa cells stably expressing GFP-tagged ITFG2 were modified using the CRISPR/Cas9 system with the indicated sgRNAs and treated and analyzed as in (A).
Supp. Figure 4

A

HEK-293T stably expressing amino acids:
- KPTN
- Nprl3
- FLAG
- Mice
- KPTN
- Mice

B

HEK-293T stably expressing amino acids:
- KPTN
- Nprl3
- FLAG-DEPDC5
- Mice
- KPTN
- Mice

C

IP antibody: FLAG

D

E

wild-type

SZT2 null

- AA for 50 min
- AA for 50 min
- AA for 10 min

merge

merge

merge
target GATOR1 to the lysosome. To test this hypothesis, we generated HeLa cells stably expressing GFP-tagged components of GATOR1 (Nprl2) and GATOR2 (mios and WDR24) (Fig S4D). As shown previously, both GATOR1 and GATOR2 colocalized with the LAMP2 lysosomal marker in a manner that is not regulated by amino acids (Fig. 4A-C). In agreement with this finding, amino acids did not regulate the interaction of KICSTOR with either GATOR1 or GATOR2 (Fig 1D, S4A-C).

Consistent with the KICSTOR complex being necessary to target GATOR1 to the lysosome, loss of SZT2, c12orf66, or KPTN rendered GFP-tagged Npr12 dispersed throughout the cytoplasm (Fig. 4D-E). In contrast, GFP-tagged WDR24 and the endogenous Rag GTPases localized to the lysosome independently of KICSTOR, as assessed in KPTN-null or SZT2-null cells, respectively (Fig. 4F, S4E). Thus, KICSTOR is necessary for the targeting of GATOR1 to the lysosomal surface.

Figure S4, related to Figure 4: Amino acids do not regulate the interaction of KICSTOR with the GATOR complexes, and KICSTOR is not required for the Rag GTPases to localize to the lysosome.
A) and (B) GATOR1 and GATOR2 do not exhibit amino acid regulated interactions with KICSTOR. Anti-FLAG immunoprecipitates were prepared from HEK-293T cells expressing the indicated endogenously tagged GATOR component that had been starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min. Immunoprecipitates and cell lysates were analyzed by immunoblotting for the indicated proteins.
C) Endogenous KICSTOR isolated from cells does not interact with GATOR1 or GATOR2 in an amino acid-regulated manner. Anti-GSK3B and anti-KPTN immunoprecipitates were prepared from HEK-293T cells treated and analyzed as in (A)
D) Loss of SZT2 confers resistance to amino acid starvation. Cell lysates were prepared from the indicated cell lines and analyzed by immunoblotting for the indicated proteins.
E) Rag GTPases localize to the lysosome regardless of KICSTOR levels. Wild-type and SZT2-null HeLa cells were starved or starved and restimulated with amino acids for the indicated times prior to processing for immunofluorescence as in Figure 4A.
Discussion

The Rag and Rheb GTPases, which reside on the lysosomal surface, control mTORC1 localization and kinase activity, respectively, in response to multiple inputs, centering this important growth regulatory pathway at the interface between the cytosol and lysosome (Laplante and Sabatini, 2012). Nutrients control the nucleotide loading state of the Rags through a variety of protein complexes, including GATOR1 (Bar-Peled et al., 2013). Here, we identify a novel GATOR1-interacting complex, KICSTOR, which negatively regulates the mTORC1 pathway and is necessary for the localization of GATOR1 to the lysosomal surface. Furthermore, we find that constitutive targeting of GATOR1 to the lysosomal surface in KICSTOR-null cells rescues the amino acid deprivation insensitivity of these cells, indicating that a key function of KICSTOR in the nutrient sensing pathway is to target GATOR1 to lysosomes.

While the identification of KICSTOR adds an additional critical component to the pathway through which amino acids regulate the mTORC1 pathway, many outstanding questions remain. First, our work indicates that GATOR1 and the Rag GTPases interact independently with GATOR2. Until the function of GATOR2 is deduced, it remains unclear whether the biochemical interaction of either of these complexes with GATOR2 is functionally significant. Because KICSTOR is not necessary for the lysosomal localization of GATOR2, perhaps the Rags mediate this localization, but further work is needed to test if this is the case.

Second, while it is clear that KICSTOR is necessary for targeting GATOR1 to lysosomes, how the KICSTOR complex itself is tethered to lysosomes remains unclear. None of the KICSTOR subunits contain predicted transmembrane domains or lysosomal targeting sequences so perhaps currently unidentified protein interactions mediate its localization.

In addition, our discovery of four novel regulators of the nutrient sensing pathway indicates that there may be additional upstream components which regulate KICSTOR
and the mTORC1 pathway. Indeed, while multiple nutrients impinge on mTORC1 activity, sensors have only been identified for arginine and leucine (Chantranupong et al., 2016; Wang et al., 2015; Wolfson et al., 2015). The lack of an amino acid regulated interaction between KICSTOR and GATOR1 hints that other inputs might regulate their function. Other environmental inputs, including glucose, have been reported to impinge on the mTORC1 pathway upstream of the Rag GTPases (Efeyan et al., 2012), but how these signals control the Rag nucleotide state remains unknown. It is tantalizing to image that the four proteins of KICSTOR may have more functions than simply constitutively localizing GATOR1 to the lysosomal surface, likely mediating the effects of other inputs on the mTORC1 pathway.

Finally, multiple loss of function mutations in KPTN, c12orf66, and SZT2 have been identified in patients with epilepsy and brain malformation disorders, such as macrocephaly (Baple et al., 2014; Basel-Vanagaite et al., 2013; McCormack et al., 2015). Consistent with the phenotypes observed in humans, SZT2-null mice that survive to adulthood also suffer from severe epilepsy (Frankel et al., 2009). The mutations in KICSTOR genes likely lead to aberrantly increased mTORC1 signaling, which has been previously linked to these brain diseases (Crino, 2011; DeGama et al., 2015). In future work it will be important to understand if patients with these mutations might benefit from pharmacological inhibition of mTORC1. Careful sequencing of KICSTOR genes in patients with brain development abnormalities and epilepsy of unknown etiology may reveal additional causative mutations that would further strengthen the link between deregulated mTORC1 activity and brain malformation and epilepsy disorders.
Materials and Methods

Materials

Reagents were obtained from the following sources: LAMP2 H4B4, ITFG2, and HRP-labeled anti-mouse and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, S6K, mTOR, RagC, Mios and myc, and FLAG epitope from Cell Signaling Technology; antibodies to the HA epitope from Bethyl laboratories; antibody to KPTN from ProteinTech. RPMI, FLAG M2 affinity gel, and amino acids from Sigma Aldrich; DMEM from SAFC Biosciences; XtremeGene9 and Complete Protease Cocktail from Roche; Alexa 488 and 568-conjugated secondary antibodies; Inactivated Fetal Calf Serum (IFS) from Invitrogen; amino acid-free RPMI from US Biologicals. The WDR24 antibody was generously provided by Jianxin Xie (Cell Signaling Technology).

Cell lines and tissue culture

HEK-293T and HeLa cells were cultured in DMEM 10% IFS supplemented with 2 mM glutamine. All cell lines were maintained at 37°C and 5% CO2.

Cell lysis and immunoprecipitation

Cells were rinsed once with ice-cold PBS and lysed immediately with Triton lysis buffer (1% Triton, 10 mM β-glycerol phosphate, 10 mM pyrophosphate, 40 mM Hepes pH 7.4, 2.5 mM MgCl2 and 1 tablet of EDTA-free protease inhibitor [Roche] (per 25 ml buffer). The cell lysates were clarified by centrifugation at 13,000 rpm at 4°C in a microcentrifuge for 10 minutes. For anti-FLAG-immunoprecipitations, the FLAG-M2 affinity gel was washed with lysis buffer 3 times. 30 µl of a 50% slurry of the affinity gel was then added to cleared cell lysates and incubated with rotation for 2 hours at 4°C. The beads were washed 3 times with lysis buffer containing 500 mM NaCl. In the case of transient cotransfection assays to explore the interaction of the Sestrins with GATOR2, beads were incubated in the final salt wash for 30 minutes to reduce non-specific binding. Immunoprecipitated proteins were denatured by the addition of 50 µl of sample buffer and boiling for 5 minutes as described (Kim et al., 2002), resolved by
8%-16% SDS-PAGE, and analyzed by immunoblotting.

For co-transfection experiments in HEK-293T cells, 2 million cells were plated in 10 cm culture dishes. Twenty-four hours later, cells were transfected via the polyethylenimine method (Boussif et al., 1995) with the pRK5-based cDNA expression plasmids indicated in the figures. The total amount of plasmid DNA in each transfection was normalized to 5 μg with empty pRK5. Thirty-six hours after transfection, cells were lysed as described above.

For experiments which required amino acid starvation or restimulation, cells were treated as previously described (Tsun et al., 2013). Briefly, cells were incubated in amino acid free RPMI for 50 minutes and then stimulated with amino acids for 10 minutes. For glucose starvation, cells were incubated in RPMI media lacking glucose but containing amino acids and dialyzed serum for 50 minutes, followed by a 10 minute restimulation with 5 mM D-Glucose. For insulin deprivation, cells were incubated in RPMI without serum for 50 minutes and restimulated with 1 ug/ml insulin for 10 minutes.

**Generation of CRISPR/Cas9 genetically modified cells**

To generate HEK-293T or HeLa cells with loss of GATOR2, GATOR1, or KICSTOR components, sense (S) and antisense (AS) oligonucleotides encoding the guide RNAs were cloned into the pX330 vector (Petit et al., 2013).

sgSZT2_1S: caccgCTCTGGACCCGCCTCTCTCTCTG
sgSTZ2_1AS: aaacCAGAGGAGGCGGGTCCAGAGc

sgWDR24_1S: caccgACCCAGGGCTGTGGTCACAC
sgWDR24_1AS: aaacTCAGGAGTACTCGCAGAGGTc

sgGFP_1S: caccgTGAACCGCATCGAGCTGAA
sgGFP_lAS: aaacTTCAGCTCGATGCGGTTCAc
On day one, 200,000 cells were seeded into 6 wells of a 6-well plate. Twenty-four hours post seeding, each well was transfected with 250 ng shGFP pLKO, 1 ug of the pX330 guide construct, 0.5 ug of empty pRK5 using XtremeGene9. The following day, cells were trypsinized, pooled in a 10 cm dish, and selected with puromycin to eliminate untransfected cells. Forty-eight hours after selection, the media was aspirated and replenished with fresh media lacking puromycin. The following day, cells were single cell sorted with a flow cytometer into the wells of a 96-well plate containing 150 ul of DMEM supplemented with 30% IFS. Cells were grown for two weeks and the resultant colonies were trypsinized and expanded. Clones were validated for loss of the relevant protein via immunoblotting.

To generate cells stably expressing the indicated sgRNAs, virus was first generated. On day one, 750,000 HEK-293T or HeLa cells were seeded in a 6 well plate in DMEM supplemented with 20% inactivated fetal bovine serum (IFS). Twenty-fours hours later, the cells were transfected with sgRNA pLenti encoding plasmids indicated below alongside the Delta VPR envelope and CMV VSV-G packaging plasmids using XtremeGene9 transfection reagent.

The following oligos were used to generate pLenti viral vectors encoding sgRNAs targeting KPTN, ITFG2, and c12orf66:

\[
\begin{align*}
\text{sgNprl3} \_1S & : \text{caccGGCTTTCAGGCTCCGTTCGA} \\
\text{sgNprl3} \_1AS & : \text{aaacTCGAACGGAGCCTGAAAGCC} \\
\text{sgKPTN} \_1S & : \text{caccgATCACATCAGTAAACATGAG} \\
\text{sgKPTN} \_1AS & : \text{aaacCTCATGTTTACTGATGTGATc} \\
\text{sgITFG2} \_1S & : \text{caccgACCCAGGGCTGTGGTCACAC} \\
\text{sgITFG2} \_1AS & : \text{aaacGTGTGACCACAGCCCTGGGTc} \\
\end{align*}
\]

\[
\begin{align*}
\text{sgKPTN} \_1S & : \text{caccgATCACATCAGTAAACATGAG} \\
\text{sgKPTN} \_1AS & : \text{aaacCTCATGTTTACTGATGTGATc} \\
\text{sgITFG2} \_1S & : \text{caccgACCCAGGGCTGTGGTCACAC} \\
\text{sgITFG2} \_1AS & : \text{aaacGTGTGACCACAGCCCTGGGTc} \\
\end{align*}
\]
sgKPTN_2S: caccgGCAGAGCAATGTGTACGGGC
sgKPTN_2AS: aaacGCCCGTACACATTGCTCTGCc
sgKPTN_3S: caccgGAGCACCTTGCCTTTAAGGG
sgKPTN_3AS: aaacCCCTTAAAGGCAAGGTGCTCc
sgKPTN_6S: caccgGTCAAGGTTGTACTCAGAGC
sgKPTN_6AS: aaacGCTCTGAGTACAACCTTGACc
sgITFG2_1S: caccgGGTGGGAGACACCAGCGGGA
sgITFG2_1AS: aaacTCCCGCTGGTGTCTCCCACCc
sgITFG2_2S: caccgGAAGTTAAATGAACTGGTGG
sgITFG2_2AS: aaacCCACCAGTTCATTTAACTTCc
sgITFG2_3S: caccgAAAATGATGACAGTCGGCCA
sgITFG2_3AS: aaacTGGCCGACTGTCATCATTTTc
sgcl2orf66_1S: caccgCGAGAGGCCAACAAGAGCGC
sgcl2orf66_1AS: aaacGCGCTCTTGTTGGCCTCTCGc
sgcl2orf66_3S: caccgGGCTAAGGACAATGTGGAGA
sgcl2orf66_3AS: aaacTCTCCACATTGTCTCTTGGCCA
sgcl2orf66_5S: caccgCTGTTCCACCGGGACCGGGG
sgcl2orf66_5AS: aaacCCCCGTCCCCGTTGGAACACGc

Twelve hours post transfection, the old media was aspirated and replaced with 2
ml fresh media. Virus-containing supernatants were collected 36 hours after replacing media and passed through a 0.45 micron filter to eliminate cells. Four million cells in the presence of 8 μg/ml polybrene (Millipore) were infected with 1 ml of virus for each construct in the case of single knockdown or with 500 ul of virus in the case of double or triple knockdown in 2 ml total volume of media and then spun at 2,200 rpm for 45 minutes at 37°C. Forty-eight hours after selection, cells were trypsinized and selected with puromycin and seeded on the 10th day for signaling experiments, as described.

Identification of KICSTOR by mass spectrometry

Immunoprecipitates from HEK-293T cells expressing endogenously tagged FLAG-DEPDC5 were prepared using Triton lysis buffer without crosslinking as described earlier. Proteins were eluted with the FLAG peptide (sequence DYKDDDDK) from the FLAG-M2 affinity gel, resolved on 4-12% NuPage gels (Invitrogen), and stained with simply blue stain (Invitrogen). Each gel lane was sliced into 10-12 pieces and the proteins in each gel slice digested overnight with trypsin. The resulting digests were analyzed by mass spectrometry as described (Sancak et al., 2008). Peptides corresponding to KICSTOR components were detected in the FLAG-DEPDC5 immunoprecipitates, while no peptides were detected in negative control immunoprecipitates of FLAG-Metap2.

Immunofluorescence assays

Immunofluorescence assays were performed as described in (Sancak et al., 2010a). Briefly, 300,000 HEK-293T or HeLa cells were plated on fibronectin-coated glass coverslips in 6-well tissue culture plates. Twenty-four hours later, the slides were rinsed with PBS once and fixed for 15 min with 4% paraformaldehyde in PBS at room temperature. The slides were rinsed three times with PBS and cells were permeabilized with 0.05% Triton X-100 in PBS for 5 min. After rinsing three times with PBS, the slides were blocked for 1 hour in Odyssey blocking buffer, and then incubated with primary antibody in Odyssey blocking buffer for 1 hr at room temperature, rinsed three times with PBS, and incubated with secondary antibodies produced in donkey (diluted 1:1000 in Odyssey blocking buffer) for 45 minutes at room temperature in the dark and washed.
three times with PBS. Slides were mounted on glass coverslips using Vectashield (Vector Laboratories) and imaged on a spinning disk confocal system (Perkin Elmer).
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References


CHAPTER 7

DISCUSSION AND FUTURE DIRECTIONS

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mTORC1 is a master growth controller that integrates diverse environmental inputs to coordinate many anabolic and catabolic processes in cells. Critical for mTORC1 activity are amino acids, which promote the translocation of mTORC1 to the lysosomal surface, its site of activation. Here, we have described the identification of multiple proteins that further our understanding of how amino acids signal to the mTORC1 pathway. GATOR1 is a GTPase activating protein for RagA/B and is required for amino acid withdrawal to inhibit mTORC1. Necessary to anchor GATOR1 on the lysosome is KICSTOR, a four-membered protein complex. GATOR2 functions upstream of or in parallel to GATOR1 to positively regulate the mTORC1 pathway. Importantly, GATOR2 is a critical integrator of cellular amino acid availability, as it interacts with Sestrin1/2, a leucine sensor, and CASTOR1, an arginine sensor for the mTORC1 pathway (Figure 1). Despite these advances, several key questions remain to be addressed.

7.1 What is the function of GATOR2?

The identification of Sestrin1/2 and CASTOR1 as GATOR2-interacting amino acid sensors reveals GATOR2 as a hub of amino acid sensing (Chantranupong et al., 2016; Wolfson et al., 2015). Whether Sestrin1/2 and CASTOR1 operate through distinct mechanisms to inhibit GATOR2 can only be determined once the function of GATOR2 is elucidated.

Insight into the function of GATOR2 comes from analysis of the conserved

Figure 1: Updated model of amino acid sensing by mTORC1.
A) Under amino acid deprivation, GATOR1 inhibits the mTORC1 pathway by functioning as a GTPase activating protein for RagA/B. GATOR1 is anchored to the lysosome by a four-membered protein complex we term KICSTOR, which consists of KPTN (K), ITFG2 (I), c12orf66 (C), and SZT2 (S). Upstream or in parallel to GATOR1 is GATOR2, a pentameric complex which, under leucine and arginine deprivation, is bound to and inhibited via unknown mechanisms by Sestrin1/2 and CASTOR1, the cytosolic leucine and arginine sensors, respectively.
B) Upon stimulation of cells with leucine and arginine, GATOR2 is activated upon the dissociation of Sestrin1/2 and CASTOR1, and GATOR1 is inhibited. The Rag GTPases are in the appropriate nucleotide state to recruit mTORC1 to the lysosomal surface, where it can in turn bind to Rheb and become an active kinase.
Figure 1 A

B
Figure 2

A. Models of WD40 and Metap2 proteins with RING domains.

B. Summary of protein expression and conjugate formation.

C. Electrophoretic analysis of conjugate formation with WT and C746A mutants.

D. Electrophoretic analysis of conjugate formation with WT and C788A mutants.

E. Electrophoretic analysis of conjugate formation with WT and serotonin (5-HT) induced mutants.

F. Electrophoretic analysis of conjugate formation with WT and C927A mutants.

G. Summary table of protein expression and conjugate formation with various mutants and HEK-293T clones.

Legend:
- WT: Wild Type
- C927A: Mutant with C927A substitution
- C746A: Mutant with C746A substitution
- C788A: Mutant with C788A substitution
- 5-HT: Serotonin
- HEK-293T clone: Mos or GFP

*Note: The diagram and text are highly detailed and require specific knowledge of molecular biology and protein conjugation for full comprehension.*
protein domains within this complex. Three GATOR2 components - WDR24, WDR59, and Mios - contain C-terminal RING domains (Figure 2A and B), which define a class of enzymes called E3 ubiquitin ligases that mediate the covalent conjugation of ubiquitin to a lysine of a protein substrate (Deshaies and Joazeiro, 2009). E3 ligases are preceded by an E1 activating enzyme and an E2 conjugating enzyme, which are required to sequentially activate ubiquitin prior to its transfer to a substrate (Deshaies and Joazeiro, 2009). Ubiquitylation can have diverse and profound functional consequences, depending on the nature of the ubiquitin conjugation. Whereas some ubiquitin modifications target a protein to the proteasome for degradation, others modulate protein activity or interactions (Komander and Rape, 2012).

Several lines of evidence indicate that GATOR2 is an E3 ligase whose activity is necessary for the mTORC1 pathway to sense amino acids. First, all three RING-containing GATOR2 components immunopurified from HEK-293T cells mediate autoubiquitylation (Figure 2C). To address the possibility that this activity stems from a contaminating E3 ligase present in the mammalian preparations of GATOR2, we expressed and purified isolated RING domains of WDR24, mios and WDR59 from bacteria, which lack a ubiquitylation system. Autoubiquitylation and/or ubiquitylation of associated bacterial proteins persisted, demonstrating that each RING-containing

Figure 2: The E3 ubiquitin ligase activity of GATOR2 is necessary for the mTORC1 pathway to sense the presence of amino acids.
A) Three components of GATOR2 contain C-terminal RING domains. A schematic representing the conserved protein domains within the GATOR2 complex.
B) Sequence alignments of the RING domains within GATOR2, in comparison with an established RING E3 ligase, Rnf4. Predicted critical zinc coordination sites are highlighted in yellow.
C) GATOR2 has autoubiquitylation activity. Anti-FLAG immunoprecipitates were prepared from HEK-293T cells transfected with the indicated cDNAs. Immunoprecipitates were incubated at 25°C for 90 minutes with the indicated components. Immunoprecipitates were boiled in 1% SDS and the supernatant was subjected to a second round of FLAG. Immunoblotting was performed for the indicated proteins.
D) GATOR2 RING domains exhibit ubiquitylation activity. The RING domains in (B) were fused to GST and expressed in BL21 bacteria. Purified RING domains were incubated with the indicated components and the reactions subjected to immunoblotting for the indicated proteins.
E) The E3 ligase activity of mios is necessary for amino acids to signal to mTORC1. HEK-293T cells were starved of amino acids, or starved for 50 mins and stimulated with amino acids for 10 mins, where indicated. Anti-FLAG immunoprecipitates were prepared and analyzed by immunoblotting for the indicated proteins.
component of GATOR2 has E3 ligase activity (Figure 2D-F). To test whether activation of mTORC1 by amino acids requires the E3 ligase activity of GATOR2, we used alanine scanning mutagenesis of conserved residues of the GATOR2 RING domains to identify a putative E2 binding mutant of mios, L787A, and a zinc coordination defective mutant, C788A. Reintroduction of either mios mutant into mios-null HEK-293T cells fails to rescue the amino acid signaling defect of these cells (Figure 2G), demonstrating that the E3 activity of mios is necessary for amino acids to signal to mTORC1.

To determine how the E3 ligase activity of GATOR2 regulates the mTORC1 pathway, identification of the GATOR2 substrate(s) is necessary. One possibility is that GATOR2 ubiquitylates a known regulator of the nutrient sensing branch of the mTORC1 pathway. GATOR1 and the Rag GTPases, which both interact with and function genetically downstream of GATOR2, are two prime candidate substrates. In one model, GATOR2 may ubiquitylate and inhibit GATOR1 in the presence of amino acids by targeting GATOR1 to the proteasome or by blocking its GAP activity towards RagA/B. In an alternate model, GATOR2 may ubiquitylate the Rag GTPases and block its ability to function as a substrate for GATOR1. Unfortunately, numerous efforts to detect ubiquitylated species of GATOR1 and the Rags that are generated in an amino acid- and GATOR2-dependent manner have been unsuccessful.

To address the alternate possibility that GATOR2 ubiquitylates an unidentified component of the mTORC1 pathway, we explored unbiased proteomics approaches for substrate identification (Kim et al., 2011). In collaboration with Dr. Steve Gygi’s lab at Harvard, we used a a di-glycine antibody to enrich for ubiquitylated peptides in wild type and GATOR2-null mammalian cells and performed mass spectrometry to identify changes in the ubiquitin-modified proteome. However, these efforts did not yield candidate substrates. Collectively, these results suggest the following: GATOR2 is not an E3 ubiquitin ligase and activates the mTORC1 pathway through other mechanisms, or GATOR2 is an E3 ligase, but technical issues have hindered our ability to detect proteins in the pathway that are ubiquitylated in a GATOR2-dependent manner. Future efforts are needed to thoroughly address these possibilities.
Once the function of GATOR2 is elucidated, we can address several pressing questions. First, what is the function of each RING-containing component in GATOR2? Does each component ubiquitylate a separate substrate, or do all RINGs act cooperatively to ensure the ubiquitylation of a common substrate? Heterodimeric and homodimeric RING ligases are prevalent in mammalian cells (Budhidarmo et al., 2012), and structural analysis of these complexes has revealed that multimerization of the RING domains enhances the efficiency of ubiquitin transfer from the E2 enzyme to the substrate (Dou et al., 2012; Plechanovová et al., 2011). Furthermore, what is the functional effect of GATOR2 ubiquitylation on the substrate? This modification may alter protein activity, levels or interactions with key regulators (Deshaies and Joazeiro, 2009). Finally, how do Sestrin1/2 and CASTOR1 inhibit GATOR2 upon leucine and arginine deprivation, and are their mechanisms distinct? These sensors may competitively block the ability of a substrate or an E2 enzyme to bind to GATOR2, and thus interfere with GATOR2 function. Alternatively, these sensors may indirectly impinge on GATOR2 function by recruiting negative regulators of GATOR2. Undoubtedly, we must determine the function of GATOR2 to clarify our understanding of amino acid signaling to mTORC1.

7.2 What are the roles of the amino acid sensors in vivo?

While the requirement of Sestrin1/2 and CASTOR1 to sense leucine and arginine is well established in mammalian cell culture, it remains to be determined whether these proteins signal amino acid availability to mTORC1 in vivo. Characterization of mice lacking Sestrin or CASTOR genes is needed to reveal if mTORC1 signaling is deregulated in the absence of these sensors. To complement these findings, it will be meaningful to profile the GATOR2-Sestrin1/2 and GATOR2-CASTOR1 interaction in tissues from mice that have been fasted or refed and to concomitantly determine the amino acid levels within these tissues. These studies will reveal whether amino acid levels in vivo fluctuate within the range required for Sestrin1/2 and CASTOR1 to regulate GATOR2. They may also reveal if the affinity of Sestrin1/2 and CASTOR1 for leucine and arginine differs in vivo from those determined in vitro.
If Sestrin1/2 and CASTOR1 are required to sense amino acid levels in vivo, another question of interest is how the requirements for leucine and arginine sensing vary across distinct tissues and during development. It is clear that amino acid sensing is critical for early postnatal survival, as neonates expressing constitutively active Rag GTPases rapidly die after birth from profound hypoglycemia and a decline in plasma amino acid concentrations (Efeyan et al., 2012). Characterization of mice lacking Sestrin and CASTOR in different tissues such as the muscle or liver will provide insight into the physiological effects of a specific defect in leucine and arginine sensing. In addition, characterization of how the expression of the Sestrin and CASTOR genes vary in these tissues upon fasting and refeeding may reveal additional mechanisms to modulate mTORC1 activity. We hypothesize that tissues may alter the expression of CASTOR1 versus CASTOR2 or Sestrin1/2 versus Sestrin3 to control mTORC1 activity, as amino acids differentially regulate each Sestrin and CASTOR protein. CASTOR2 and Sestrin3 are both defective in amino acid binding and constitutively associate with GATOR2 to inhibit mTORC1 signaling. Thus, increased CASTOR2 or Sestrin3 levels should blunt maximal mTORC1 signaling in tissues while increased CASTOR1 and Sestrin1/2 may alter the arginine or leucine sensitivity of the pathway.

7.3 Do other amino acid sensors exist for the TORC1/mTORC1 pathway?

In addition to leucine and arginine, other amino acids such as glutamine are critical for mTORC1 activity (Jewell et al., 2015), and distinct sensors may be required to relay their availability to mTORC1. Furthermore, sensors may be needed to mediate amino acid-sensitive events upstream of other mTORC1 regulators, such as folliculin/FNIP, the GAP for RagC/D (Petit et al., 2013; Tsun et al., 2013). We may expect these sensors to detect amino acids in the cytosol or lysosome, or to sense downstream metabolites of the amino acid, rather than the amino acid itself. A comprehensive IP/MS of mTORC1 pathway regulators from contexts where the amino acid sensing requirements differ may identify putative sensors and provide insight into the diversity of amino acid sensing mechanisms.
Another question of interest is how well conserved are the amino acid inputs that drive mTORC1/TORC1 signaling in organisms other than mammals. Characterizing the evolutionary conservation of the amino acid sensors of the mTORC1/TORC1 pathway will provide insight into this question. For instance, budding yeast encodes a homolog of GATOR2, but not of the Sestrins or CASTORs, hinting at a divergence in the regulation of the upstream components of the nutrient sensing pathway. This divergence may be expected given that yeast, unlike mammals, can synthesize all amino acids and thus must sense the quality and abundance of nitrogen sources rather than the identity and availability of particular amino acids. Further identification and characterization of the amino acid sensors upstream of the Rag GTPases in different organisms will guide us toward a comprehensive understanding of how nutrients regulate the mTORC1 pathway.

### 7.4 What regulates KICSTOR?

Other environmental inputs such as glucose have been reported to control the mTORC1 pathway upstream of the Rag GTPases, but their mechanisms remain unknown. One tempting hypothesis is that these inputs could control the interaction between KPTN/ITFG2 and c12orf66 with SZT2 to affect GATOR1 localization. First, it will be meaningful to establish whether the sole function of KICSTOR is to localize GATOR1. If so, constitutive targeting of GATOR1 to the lysosome in KICSTOR-null cells should bypass the requirement for KICSTOR and rescue the signaling defect of these cells. If not, KISCTOR likely has other functions, and it will be of interest to test the hypothesis that inputs such as glucose starvation, osmotic stress, or oxidative stress may affect KICSTOR function, as assessed by GATOR1 localization and mTORC1 signaling. In addition, identification of KICSTOR interacting proteins by IP/MS may provide further insight into the upstream regulation of this complex.

### Concluding remarks

In the past several years, we have witnessed exciting advances in our understanding of how amino acids control cellular growth. The multiple molecular
players on the lysosome and in the cytosol illustrate the complexity of this pathway and emphasize the importance of nutrient signaling in cells. Future efforts focused on characterizing GATOR2, KICSTOR, and the amino acid sensors will further clarify our understanding of the fundamental process of growth control and how it is deregulated in disease.
References