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

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Abstract

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.

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

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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.

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

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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-293T cells co-immunoprecipitated GATOR2, but not GATOR1 or the metap2 control protein (Figure 1B). When stably expressed in HEK-293T cells, FLAG-Sestrin2 coimmunoprecipitated endogenous GATOR2 as detected through its Mios component (Figure 1C). The reciprocal was also true because stably expressed FLAG-WDR24 coimmunoprecipitated 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.

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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 shorthairpin 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 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 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

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

Discussion

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

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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 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 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,

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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.

Experimental Procedures

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 Hepes pH 7.4, 2.5 mM $MgCl_2$ 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, HASeh1L, 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

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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: caccgCGGGGGAGATGGCGGCGCGA

sgWDR59_1AS: aaacTCGCGCCGCCATCTCCCCCGc

sgSesn2_1S: caccgAGAGCCTCGAGCAGCACCTG

sgSesn2_1AS: aaacCAGGTGCTGCTCGAGGCTCTc

sgSesn2_2S: caccGGACTACCTGCGGTTCGCCC

sgSesn2_2AS: aaacGGGCGAACCGCAGGTAGTCC

sgSesn2_3S: caccGCCACAGCCAAACACGAAGG

sgSesn2_3AS: aaacCCTTCGTGTTTGGCTGTGGC

sgGFP_1S: caccgTGAACCGCATCGAGCTGAA

sgGFP_1AS: aaacTTCAGCTCGATGCGGTTCAc

sgNprl3_1S: caccGGCTTTCAGGCTCCGTTCGA

sgNprl3_1AS: aaacTCGAACGGAGCCTGAAAGCC

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.

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Supplementary Material

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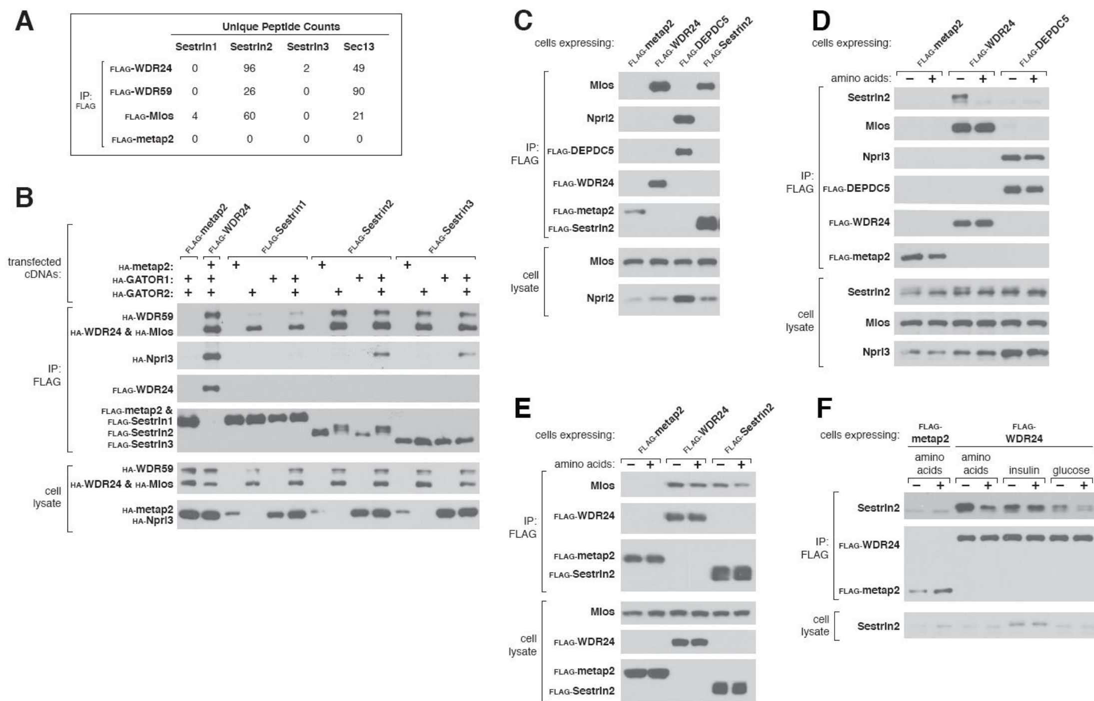

Figure 1. The Sestrins interact with GATOR2, but not GATOR1, in an amino acid-sensitive fashion

(A) GATOR2 interacts with the Sestrins. Mass spectrometric analyses identify Sestrinderived peptides in immunoprecipitates from HEK-293T cells stably expressing FLAGtagged 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 acidsensitive 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).

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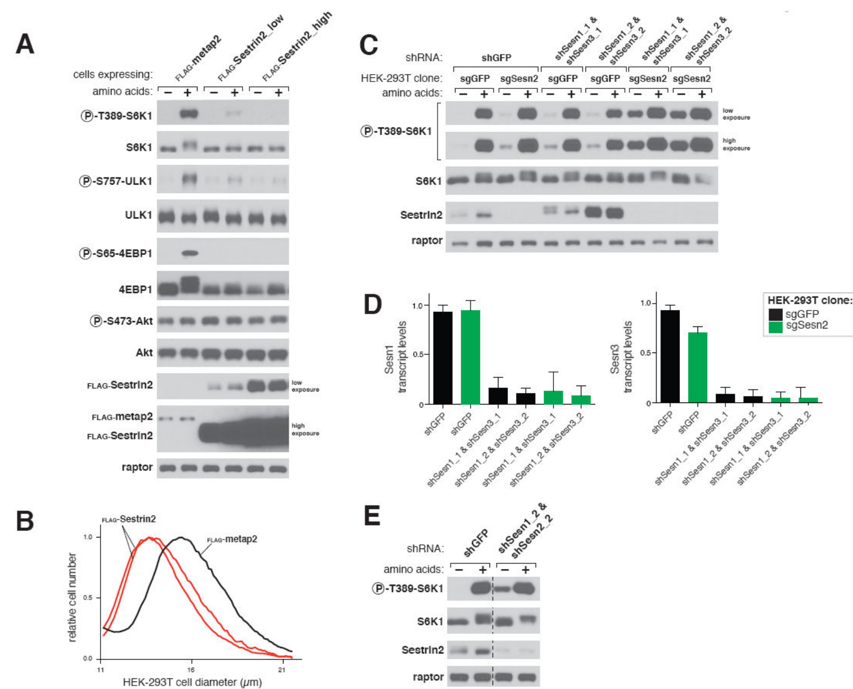

Figure 2. The Sestrins are negative regulators of the amino acid sensing pathway upstream of mTORC1

(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).

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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 $RagB^{99L}$-$RagC^{75N}$ or the dominant negative $RagB^{54N}$-$RagC^{121L}$ 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.

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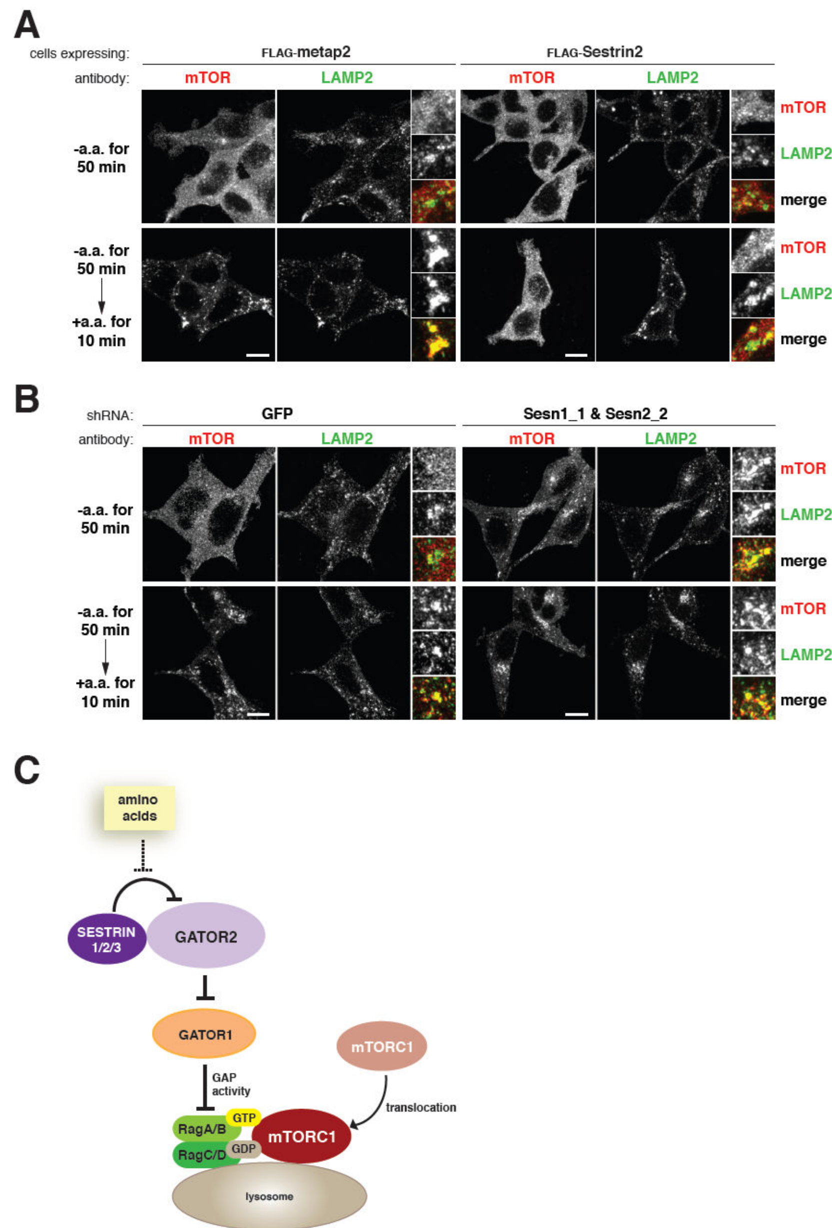

Figure 4. The Sestrins control mTORC1 localization in response to amino acids

(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).