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; [^{3}H]-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 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 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 Sestrin2 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 $MgCl_2$, 0.1% CHAPS, 1 mM TCEP, 200 µM leucine, and protease inhibitor tablets), which was then supplemented with lysozyme and crude DNAse. 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 $MgCl_2$, 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 $MgCl_2$) with the appropriate amount of [^{3}H]-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 $MgCl_2$, 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 [^{3}H]-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.

K_d/K_i calculations

Amino acid affinities to Sestrin2 were determined by first normalizing the bound [^{3}H]-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 IC_{50} value. K_d or K_i values were derived from the IC_{50} value using the equation: K_d or K_i = IC_{50} / (1 + ([[^{3}H]Leucine])/K_d).

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 *Physconitrella 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: caccgGACTACCTGCGGTTCGCCC
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 TAGGTAGAATGTGATACATGTGAAAAG

S2_promoter_R:GCGT GTCGAC GCACCACCACCACCACCCTTGTCATCGTCA TCCTTGTAGTCCATGGTGGCGGTGCGCGCCAGGACCCGGTCGCGG

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.

Figure S1: Leucine disrupts the interaction between GATOR2 and Sestrin1 but not Sestrin3

A) Effect of leucine on the interaction between endogenous GATOR2 and endogenous Sestrin2. HEK-293T cells were deprived of leucine for 1 hour, or starved for 50 minutes and restimulated with leucine for 10 minutes. Immunoprecipitates were prepared using an antibody to WDR24 or a control protein (GSK3β), and, along with cell lysates, analyzed via immunoblotting for the indicated proteins.

B) Effects of leucine starvation and restimulation on the interaction between GATOR2 and endogenous Sestrin1 (two isoforms), Sestrin2, or Sestrin3. HEK-293T cells stably expressing FLAG-metap2 or FLAG-WDR24 were deprived of leucine for 50 minutes, and, where indicated, restimulated with leucine for 10 minutes. FLAG immunoprecipitates were prepared and analyzed as in Figure 1A. Asterisks denote non-specific bands.

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 [^{3}H]Arginine with or without unlabeled arginine.

B) dSestrin (CG11299-PD) binds to leucine. Binding assays were performed and immunoprecipitates analyzed as in Figure 2A.

C) 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.

D) 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.

E) Effects of leucine or arginine on the melting temperature of another control protein, *Physconitrella patens* hydroxycinnamoyl transferase (PpHCT) in a thermal shift assay.

F) SDS-PAGE and Coomasie blue staining analyses of the bacterially prepared proteins (His-MBP-Sestrin2, ChAT, and PpHCT) used in the thermal shift assays.

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 signaling. 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 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).