nature portfolio

https://doi.org/10.1038/s41586-021-03768-w

Supplementary information

SAR1B senses leucine levels to regulate mTORC1 signalling

In the format provided by the authors and unedited

Methods

Antibodies

Antibodies used in this study were: anti-Flag (Sigma, F7425; 1:10,000 dilution in immunoblotting), anti-HA (CST, 3724; 1:10,000 dilution in immunoblotting), anti-GFP (Abcam, 290; 1:10,000 dilution in immunoblotting), anti-p-S6K1 (CST, 9234; 1:1,000 dilution in immunoblotting), anti-S6K1 (CST, 9202; 1:1,000 dilution in immunoblotting), anti-mTOR (CST, 2983; 1:100 dilution in immunostaining), anti-LAMP2 (Abcam, 25631; 1:100 dilution in immunostaining), anti-SAR1A (Proteintech, 22291-1-AP; 1:1,000 dilution in immunoblotting), anti-SAR1B (Proteintech, 22292-1-AP; 1:1,000 dilution in immunoblotting; 1:50 dilution in immunoprecipitation), anti-GAPDH (Abcam, 128915; 1:10,000 dilution in immunoblotting), anti-NPRL2 (Sigma, SAB1305758; 1:1,000 dilution in immunoblotting), anti-NPRL3 (Sigma, HPA011741; 1:1,000 dilution in immunoblotting), anti-DEPDC5 (Sigma, SAB1302644; 1:500 dilution in immunoblotting), anti-Sec13 (Santa Cruz, 514308; 1:1,000 dilution in immunoblotting), anti-Mios (CST, 13557; 1:1,000 dilution in immunoblotting), anti-WDR24 (Proteintech, 20778-1-AP; 1:1,000 dilution in immunoblotting; 1:50 dilution in immunoprecipitation), anti-WDR59 (CST, 53385; 1:1,000 dilution in immunoblotting), anti-Sestrin2 (Proteintech, 10795-1-AP; 1:5,000 dilution in immunoblotting), anti-Sestrin1 (Proteintech, 21668-1-AP; 1:1,000 dilution in immunoblotting), anti-Sestrin3 (Abgent, AP12471c; 1:1,000 dilution in immunoblotting), anti-MYL1 (Proteintech, 15814-1-AP; 1:1,000 dilution in immunoblotting), anti-CKM (Proteintech, 18712-1-AP; 1:1,000 dilution in immunoblotting), anti-MYOD1 (Proteintech, 18943-1-AP; 1:1,000 dilution in immunoblotting), anti-α-Tubulin (CST, 2144; 1:10,000 dilution in immunoblotting), anti-β-Actin (Sigma, A5441; 1:10,000 dilution in immunoblotting), anti-p-S6 (CST, 4858; 1:100 dilution in immunohistochemistry), anti-LC3B (Novus Biologicals, NB100-2220; 1:100 dilution in immunostaining and immunohistochemistry) and anti-Ki-67 (Abcam, 16667; 1:100 dilution in immunohistochemistry).

Cell culture

HEK293T (ATCC CRL-3216), HFL1 (ATCC CCL-153), IMR-90 (ATCC CCL-186), NCI-H650 (ATCC CRL-5835), and NCI-H1581 (ATCC CRL-5878) cells were cultured in DMEM high glucose medium (Gibco), F-12K medium (ATCC), EMEM medium (ATCC), RPMI-1640 medium (Gibco), and RPMI-1640 medium (Gibco) respectively, supplemented with 10% FBS (Gibco) and penicillin/streptomycin (HyClone). Cells were maintained at 37 °C with 5% CO_2. All cell lines were authenticated by STR analysis and validated to be free of mycoplasma contamination before usage in this study.

Amino acid and insulin treatments

Amino acid treatments were conducted as previously described[20]. In brief, cells were starved of all amino acids or a single kind of amino acid for 50 min, and then treated with or without the corresponding amino acids for 10 min. Treatments with leucine analogues were similar to leucine treatment. In Extended Data Fig. 3a, MG132 was supplied to prevent DEPDC5 degradation as previously described[20]. For insulin treatments in Extended Data Fig. 1d, cells were cultured in standard DMEM medium (Gibco) for 50 min, and then stimulated with or without 150 nM insulin (Sigma, I9278) for 10 min.

C2C12 and 3T3-L1 differentiation into myotubes and adipocytes

C2C12 cells were maintained in DMEM/F-12 (1:1) medium (Gibco) supplemented with 10% FBS and penicillin/streptomycin. C2C12 cells at 90% confluence were differentiated into myotubes by culturing in differentiation medium (DMEM/F-12 (1:1) containing 100 mg/L BSA, 4 mg/L transferrin, 30 nM sodium selenite, 2.1 μM vitamin E, 0.1 mM 2-mercaptoethanol, 8.2 μM ethanolamine, 0.5 mg/L insulin, and penicillin/streptomycin) for 8-10 days. 3T3-L1 cells were maintained in DMEM medium supplemented with 10% FBS and penicillin/streptomycin. For differentiation, cells at 100% confluence were cultured in differentiation medium (DMEM medium supplemented with 0.5 mM isobutylmethylxanthine, 10 μg/mL insulin and 0.25 mM dexamethasone) for 48 hours, and then continuously cultured in a maintenance medium (DMEM medium containing 10 μg/ml insulin) for 6-8

days until lipid droplets formed.

LC-MS assay for leucine analogues

HEK293T cells treated with indicated leucine analogues were collected and resuspended in 200 μl cold PBS. The cell suspension was subjected to 2 rounds of sonication (VCX 130 PB, SONICS) at 35% Ampl for 20 seconds per round, followed by centrifugation (14000 rpm, 4 °C, 10 min) to yield a clarified supernatant. Molecules smaller than 3 kD were filtered from the supernatant by a Centrifugal Filter (Millipore) and then subjected to LC-MS assay for the indicated leucine analogues. The LC-MS assays were performed by the Metabolic Platform in the Institute of Molecular Medicine (IMM) at Peking University.

cDNA, siRNA or shRNA expression in cells

cDNAs were transiently transfected into cells by polyethylenimine (PEI), or stably integrated into genomes by lentivirus infection as previously described[13]. siRNAs were transfected into cells by RNAiMAX transfection reagent (Thermo, 13778150). shRNAs were delivered into cells by lentivirus infections. For all SAR1B knockdown assays, cells were assayed 72 hr after siSAR1B or shSAR1B delivery, and used once to avoid any chance of compensation.

The cDNA sequence of SAR1B was optimized so that it encodes mRNA resistant to SAR1B knockdown. The optimized region is:

Native human SAR1B: *5'……GCGAGAGATGTTTGGTTTATA……3'*

Codon-optimized SAR1B: *5'……GCG**C**GA**A**ATGTT**C**GG**C**TTATA……3'*

shRNA plasmids were generated by annealing the paired oligonucleotides listed below and cloning them into AgeI/EcoRI-digested pLKO.1 vector (Addgene):

Sec23A:

5' CCGGCGCTAGAAATAAAGACCTCAATCGATTGAGGTCTTTATTTCTAGCGTTTTTT 3'

3' GCGATCTTTATTTCTGGAGTTAGCTAACTCCAGAAATAAAGATCGCAAAAAATTAA 5'

Sec23B:

5’CCGGCCTGTTCACAAGATTGATATTCGAATATCAATCTTGTGAACAGGTTTTTT 3’

3’GGACAAGTGTTCTAACTATAAGCTTATAGTTAGAACACTTGTCCAAAAAATTAA 5’

Sec24A:

5’CCGGGCTACTACACCAATGCCTTCTTCGAAGAAGGCATTGGTGTAGTAGCTTTTTT 3’

3’CGATGATGTGGTTACGGAAGAAGCTTCTTCCGTAACCACATCATCGAAAAAATTAA 5’

Sec24B:

5’CCGGGCCCAGATTCATTTCGGTGTATCGATACACCGAAATGAATCTGGGCTTTTTT 3’

3’CGGGTCTAAGTAAAGCCACATAGCTATGTGGCTTTACTTAGACCCGAAAAAATTAA 5’

Sec24C:

5’CCGGCCAAGCCCTATTCAGGTCATTTCGAAATGACCTGAATAGGGCTTGGTTTTTT 3’

3’GGTTCGGGATAAGTCCAGTAAAGCTTTACTGGACTTATCCCGAACCAAAAAATTAA 5’

Sec24D:

5’CCGGCGGATTCACAATCTTGGCTTATCGATAAGCCAAGATTGTGAATCCGTTTTTT 3’

3’GCCTAAGTGTTAGAACCGAATAGCTATTCGGTTCTAACACTTAGGCAAAAAATTAA 5’

Sec13:

5’CCGGGTGTTCATTTGGACCTGTGATTCGAATCACAGGTCCAAATGAACACTTTTTT 3’

3’CACAAGTAAACCTGGACACTAAGCTTAGTGTCCAGGTTTACTTGTGAAAAAATTAA 5’

Sec31A:

5’CCGGCCTCATTAGCATGTTTGCATATCGATATGCAAACATGCTAATGAGGTTTTTT 3’

3’GGAGTAATCGTACAAACGTATAGCTATACGTTTGTACGATTACTCCAAAAAATTAA 5’

Sec31B:

5’CCGGGCCATTCCTTCTGTGATGATATCGATATCATCACAGAAGGAATGGCTTTTTT 3’

3’CGGTAAGGAAGACACTACTATAGCTATAGTAGTGTCTTCCTTACCGAAAAAATTAA 5’

SAR1A:

5'CCGG GCAATTAATGGGATTGTCTTTTCGAAAAGACAATCCCATTAATTGCTTTTTT 3'

3'CGTTAATTACCCTAACAGAAAAGCTTTTCTGTTAGGGTAATTAACGAAAAAATTAA 5'

SAR1B:

5'CCGGGCGAGAGATGTTTGGTTTATATCGATATAAACCAAACATCTCTCGCTTTTTT 3'

3'CGCTCTCTACAAACCAAATATAGCTATATTTGGTTTGTAGAGAGCGAAAAAATTAA 5'

Mios:

5'CCGG GCAGCTACATTACTGTCAATATCGATATTGACAGTAATGTAGCTGCTTTTTT 3'

3'CGTCGATGTAATGACAGTTATAGCTATAACTGTCATTACATCGACGAAAAAATTAA 5'

Sense strand targeting sequences of siRNAs duplexes used in this study are:

Luciferase siRNA (siCtrl): *5'CGUACGCGGAAUACUUCGA 3'*

siSAR1B: *5'CGAGAGAUGUUUGGUUUAU 3'*

Generation of knockout cells

Knockout cells were generated by the CRISPR/Cas9 technique. The 20-nucleotide targeting sequences of the guide RNAs were designed online (*chopchop.cbu.uib.no*) and cloned into the CRISPR vectors. The 20-nucleotide targeting sequences used in this study were:

sgGFP (sgCtrl): *5'GCTGAAGCACTGCACGCCGT 3'*

sgSAR1B-1: *5'TTTCTTGGATTGGATAATGC 3'*

sgSAR1B-2: *5'TCCGAAGAACTGACCATTGC 3'*

sgSestrin1: *5'GGATAACATTACGTTAGTGA 3'*

sgSestrin2: *5'TCCACGGGGATGAAGGCGCT 3'*

sgSestrin3: *5'ACCACAGCATGTACCAGTTC 3'*

sgMios: *5'TTGAACTCCCTTGTACAAGA 3'*

To knock out SAR1B, Mios, or Sestrin1/2/3 in HEK293T cells, the 20-nucleotide oligos were cloned

into the pBC2 CRISPR vector. CRISPR vectors were transfected into cells by PEI. Cells were selected by hygromycin (Invitrogen). After selection, single cells were sorted into 96-well plates by flow cytometry and expanded for two weeks. Clones were validated for knockout of the indicated genes by genome extraction and genotyping (TianGen Biotech, KG203).

For the lung fibroblasts IMR-90 and HFL1, which could hardly form any colonies from single cells, we generated pooled SAR1B knockout cells and Sestrin1/2/3 triple knockout cells through the lentivirus infection approach. The 20-nucleotide oligoes were cloned into pLentiCRISPR v2 vectors (Addgene). CRISPR vectors were packaged into lentiviruses and delivered into cells by infection. Uninfected cells were eliminated by puromycin (Gibco) selection. Pooled knockout cells were validated for the loss of the indicated proteins by immunoblotting.

Cell lysis and immunoprecipitations

Immunoprecipitations were performed as previously described[20]. In brief, cells were lysed in TX-100 Lysis Buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1% Triton X-100 and protease inhibitors). The cell lysate was clarified by centrifugation. An 80 μl aliquot of the clarified lysate was prepared as the total cell lysate sample. The remaining lysate was combined with anti-FLAG or anti-HA resin (Sigma) for immunoprecipitation. Resins were briefly washed, and the proteins immobilized on resins were denatured and dissolved in Laemmli Sample Buffer (Bio-Rad) by heating. For the immunoprecipitation of endogenous proteins, cell lysates were first incubated with primary antibodies at 4 °C overnight. On the second day, protein A/G resins (Pierce) were supplied to capture antibody-protein complexes by rotation at 4 °C for 1 hr. Resins were then washed and protein samples were prepared as described above.

Protein extraction from mouse tissues

The indicated tissues were rapidly dissected from an adult male mouse and placed on ice. For each ~40 mg piece of tissue, 500 μl of ice-cold Tissue Lysis Buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1

mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate and protease inhibitors) was added. The tissues were homogenized by an electric homogenizer and then further lysed by rotation at 4°C for 2 hr. Tissue lysates were clarified by centrifugation (12,000 rpm, 4°C, 20 min). Protein concentrations were determined by BCA Assay kit (Pierce) and adjusted to 4 mg/ml. Samples were denatured and dissolved in Laemmli Sample Buffer by heating.

Mass spectrometry analysis

Five 15-cm dishes of HEK293T cells stably expressing Mios-Flag were starved of amino acids for 50 min (-AAs). Another five dishes of cells were starved and then re-stimulated with amino acids for 10 min (+AAs). The two groups of cells were then lysed in TX-100 Lysis Buffer. Cell lysates were clarified by centrifugation. Clarified lysate from each group was incubated with 300 µl anti-Flag resin (Sigma) by rotating at 4 °C for 2 hr. Proteins immobilized on the resins were denatured and dissolved in Laemmli Sample Buffer by heating. Proteins were separated by SDS-PAGE, silver-stained (Silver staining kit, Sigma), and identified by mass spectrometry.

Immunostaining

Immunostaining was performed as previously described[20]. In brief, cells plated on polylysine-coated glass coverslips (Corning) were sequentially processed: fixed in a PBS solution containing 4% paraformaldehyde, briefly washed in PBS, permeabilized in PBS containing 0.1% Triton X-100, blocked in PBS containing 5% BSA, and sequentially incubated with primary and fluorophore-labeled secondary antibodies. The coverslips were finally washed and mounted onto slides using an antiquench mounting buffer (Thermo). Cells were imaged by a Zeiss Confocal Microscope (LSM 710). Secondary antibodies used in this study were anti-rabbit Alexa fluor 488 (Thermo, A21206, 1:100 dilution) and anti-mouse Alexa fluor 555 (Thermo, A31570, 1:100 dilution). Colocalization of mTOR with lysosomes (Extended Data Fig. 4c) was quantitated by ImageJ.

Purification of recombinant protein from bacteria

Tag-free SAR1B was prepared from *E. coli*-expressed His-GFP-SAR1B by removing the His-GFP tag. A vector expressing His-GFP-SAR1B was kindly provided by Dr. Lei Chen (Peking University). The vector was delivered into BL21 (DE3) *E. coli* by transformation. 500 ml bacterial cultures were grown at 37 °C until reaching an optical density (OD) of 0.8, at which point the temperature was lowered to 16 °C. The cultures were then induced with 0.5 mM IPTG at 16 °C overnight. Bacteria were pelleted by centrifugation at 5,000 rpm for 15 min, resuspended in 10 ml His Lysis Buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM GDP, 1 mM TCEP, 1 mg/ml lysozyme and protease inhibitors), and lysed by incubation on ice for 40 min. The lysate was homogenized by 10 rounds of sonication (VCX 130 PB, SONICS) at 60% Ampl for 10 seconds per round, then centrifuged (1,2000 rpm, 10 min, 4 °C). The supernatant was then incubated with 1 ml Ni-NTA (Qiagen) resin by rotating at 4 °C for 30 min. Proteins immobilized on the resin were washed and eluted into 5 ml Elution Buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM GDP, 300 mM imidazole) by rotating at 4 °C for 10 min. Since there is a Human Rhinovirus 3C protease cleavage site (H3C site) between the His-GFP tag and SAR1B, 0.2 mg/ml homemade H3C protease was added into the Elution Buffer to cleave His-GFP-SAR1B, yielding His-GFP and tag-free SAR1B. Anion-exchange (HiTrap Q HP, GE Healthcare) and gel-filtration (Superdex 200, GE Healthcare) chromatography were then sequentially applied to remove His-GFP from the protein solution. Tag-free SAR1B was then concentrated and dialyzed against Cytosolic Buffer (40 mM HEPES pH 7.4, 0.1% Triton X-100, 10 mM NaCl, 150 mM KCl, 2.5 mM $MgCl_2$, 1 mM GDP) for immediate *in vitro* binding assay or storage at -80 °C (adding 20% glycerol). The protocol for purification of His-tagged Rap2a, Sestrin2 and SAR1B from bacteria was essentially the same as that described above for the purification of tag-free SAR1B, excluding the protease cleavage and anion-exchange chromatography steps.

Purification of recombinant proteins from mammalian cells

Plasmids carrying indicated cDNAs were transfected into HEK293T cells using PEI transfection reagent with 27 μg plasmids per 15-cm dish. Twenty 15-cm dishes of cells were used for one purification. After transfection, cells were cultured until 80% confluent and subjected to leucine starvation treatment. Cells were lysed in Purification Buffer (40 mM HEPES pH 7.4, 1% TX-100, 100 mM NaCl, 2.5 mM $MgCl_2$, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate and protease inhibitors). The lysate was clarified by centrifugation at 1,2000 rpm for 10 min at 4 °C (Eppendorf 5810 R). The clarified cell lysate was re-adjusted to 80 ml with Purification Buffer and incubated with a 1.25 ml slurry of GSH resin (GE Healthcare) at 4 °C for 2 hr. Resins were washed 3 times in Purification Buffer and then balanced with GSH-free Elution Buffer. Recombinant proteins on resins were eluted into 12 ml Elution Buffer (20 mM HEPES, 150 mM NaCl, 0.005% Triton X-100 and 10 mM GSH, adjusted to pH 8.0). The protein solution was concentrated to 500 μl using a Centrifugal Filter (Millipore) and further purified by gel-filtration chromatography (Superdex 200, GE Healthcare). It should be noted that as a membrane protein, SAR1B sometimes becomes aggregated during purification even in the presence of detergent, which will result in a failure of the leucine binding assay. Therefore, the purification process should be fast, and the purified recombinant proteins should be assayed immediately.

***In vitro* SAR1B-GATOR2 and Sestrin2-GATOR2 binding assays**

cDNAs encoding human GATOR2 subunits were cloned into pGEX4T-1 vectors containing an N-terminal GST tag. Plasmids were transformed into BL21 (DE3) bacteria, and the bacteria were cultured and pelleted as described above. Bacterial pellets were resuspended in GST Lysis Buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% NP40, 1 mM TCEP and protease inhibitors) and incubated on ice for 40 min. The lysates were homogenized and clarified as described above. 1 ml aliquot of each clarified lysate was incubated with 15 μl GSH resin (GE Healthcare) by rotating at 4 °C for 1 hr. The resin was then washed and resuspended in 1 ml Cytosolic Buffer.

For binding assays, 1 μg tag-free SAR1B was added to Cytosolic Buffer containing GST-GATOR2 subunit proteins immobilized on the resin (prepared as described above) and rotated at 4 °C for 2 hr. The resin was then washed with Cytosolic Buffer and denatured by heating at 100 °C in Laemmli Sample Buffer. Proteins were separated by SDS-PAGE and assayed by Coomassie staining (GATOR2 subunits) and immunoblotting (tag-free SAR1B).

Sestrin2-GATOR2 binding assays were conducted in essentially the same way, using 2 μg His-Sestrin2 recombinant proteins purified from bacteria.

SAR1B dissociation from lysosomes *in vitro*

Purification of lysosomes was performed as previously described[20]. In this study, lysosomes were immuno-isolated from leucine-starved cells. Lysosomes immobilized on resins were washed and resuspended in 300 μl Fractionation Buffer supplemented with the indicated concentrations of leucine. Tubes were shaken at 650 rpm for 10 min at room temperature on a thermomixer (Eppendorf). Lysosomes immobilized on resin were washed with Fractionation Buffer and dissolved in Laemmli Sample Buffer by heating at 100 °C.

Dissociation of SAR1B and Sestrin from GATOR2 *in vitro*

Indicated cells were starved of amino acids or leucine for 50 min. Starved cells were lysed in TX-100 Lysis Buffer, and the lysates were subjected to immunoprecipitations. The resins carrying immobilized SAR1B-GATOR2 or Sestrin-GATOR2 protein complexes were washed once in TX-100 Lysis Buffer and once in Cytosolic Buffer. Washed resins were then incubated in 1 ml Cytosolic Buffer supplemented with the indicated amino acids or leucine analogues at the indicated concentrations for 10 min at room temperature. SAR1B and Sestrins that remained associated with GATOR2 were assayed by immunoblotting.

Radiolabeled Leucine-binding assays

Bacterially prepared recombinant proteins were combined with Ni-NTA resins in Binding Buffer (20 mM Tris pH 7.4, 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM GDP, 0.1% CHAPS). Recombinant proteins purified from HEK293T cells were combined with GSH resins (GE Healthcare) in Purification Buffer. Proteins immobilized on resins were washed for 3 times in the corresponding buffers, and then balanced and resuspended in 200 μl Cytosolic Buffer supplemented with [^{3}H]-labelled leucine (PerkinElmer) together with unlabeled leucine where indicated. Binding assays were conducted as previously described[6]. Radioactivity was quantified using a TriCarb Scintillation Counter (PerkinElmer).

Microscale thermophoresis (MST) assay

Recombinant proteins dialyzed against MST Buffer (25 mM HEPES pH 7.4, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.025% NP-40) were labeled with MST fluorescence dye (NanoTemper MO-L018) according to manufacturer's instructions. The binding of labeled proteins to leucine, arginine or isoleucine was measured on a Monolith NT.115 machine (NanoTemper) with the software MO.Control. Protein concentration was set as 50 nM. Amino acid gradient was set as: 1 mM, $1/2^1$ mM, $1/2^2$ mM, $1/2^3$ mM, $1/2^4$ mM, $1/2^5$ mM, $1/2^6$ mM, $1/2^7$ mM, $1/2^8$ mM, $1/2^9$ mM, $1/2^{10}$ mM, $1/2^{11}$ mM, $1/2^{12}$ mM, $1/2^{13}$ mM, $1/2^{14}$ mM and $1/2^{15}$ mM. Data from three independent biological replicates were analyzed by Graphpad Prism 8.0.

Circular dichroism (CD) assay

Indicated proteins prepared from HEK293T cells were dialyzed against PBS containing 0.01% Triton X-100. Protein solutions supplemented with or without leucine were loaded into a 1 mm path-length quartz cell and then subjected to circular dichroism assays (BioLogic MOS-500) at room temperature. Data were collected and analyzed by the software Bio-Kine 32. Ellipticity data were converted into mean residue ellipticity. Mean residue ellipticity [θ] = ellipticity (mdeg) x 1000 / (protein concentration (mM) x light path (mm) x number of amino acid residues). The curves were generated by Graphpad Prism 8.0.

Capture of SAR1B in cells by LeuProbe

HEK293T cells were starved of leucine for 50 min and then re-stimulated for 10 min with the leucine probe (LeuProbe), or LeuProbe together with increasing amounts of native leucine. Cells were irradiated for 10 min by 365 nm ultraviolet (UV) light, resulting in a covalent linkage between LeuProbe and its binding proteins. Cells were then lysed in lysis buffer (50 mM triethanolamine, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS; adjusted pH to 7.4 and supplied with EDTA-free proteinase inhibitors). The lysates were homogenized by sonication and clarified by centrifugation. Click chemistry was subsequently conducted to label LeuProbe with a biotin tag. Proteins were precipitated from the lysate by incubation in methanol at -20 °C overnight. After a brief wash, proteins were resuspended in 1 ml PBS supplemented with 1.2% SDS. Streptavidin resins (Pierce) were supplied to enriched biotinylated proteins. Resins were then sequentially washed in 1 ml (1) PBS + 2% SDS, (2) 8 M urea + 250 mM ammonium bicarbonate, (3) PBS + 2.5 M NaCl, and (4) PBS. Immobilized proteins were denatured and dissolved in Laemmli Sample Buffer containing 1 mM biotin by heating.

***C. elegans* strains and treatments**

C. elegans strains (wild type, N2; MAH240, *hlh-30p::hlh-30::gfp*; VC2792, *sar-1* (*ok3513*) IV/nT1 [qIs51]) were provided by the *Caenorhabditis Genetics Center* (CGC) and maintained at 20 °C.

For gene expression in worms, plasmids carrying desired cDNAs were microinjected into the syncytial gonad of hermaphrodite worms. In this study, plasmids expressing *hlh-30p::hlh-30::mCherry*, human SAR1B::EGFP (driven by *rpl-28* promoter), *sar-1p::mCherry* and *sesn-1p::gfp* were used. Human SAR1B sequence was codon-optimized by a webtool (*C. elegans* Codon Adapter) to improve protein expression in *C. elegans*. Imaging of *sar-1p::mCherry* and *sesn-1p::gfp* was performed on a Zeiss Microscope (Axio Image M2) with the software ZEN 2012.

For gene knockdown, synchronized larva stage 1 (L1) worms were raised until the L4 stage on control or indicated RNAi bacteria (Ahringer Library, Source BioScience). The sense strand sequences

(cloned from genomic DNA) for expressing dsRNAs are:

F39C12.1 (CeMios): *5'ACAAGTTCCGAT… (to) …GTGTGAAGGAGC 3'*

npp-18 (CeSeh1l): *5' AAAAATGCAAGA… (to) …ACTGAAACCCGA 3'*

let-363 (CeTOR)*: 5'AGTGAGCTGAAA… (to) …GCAAAGCTCGAT 3'*

sar-1: 5'CACACTGCGTCT… (to) …aaacatccccat 3'

sesn-1: 5'AATTCTGCCAAC… (to) …ACTGACGTCACG 3'

sec-23: 5'AATTCCTACAGC… (to) …GCTGACTACAAA 3'

sec-24.1: 5'CGACTGCATGGC… (to) …AAAGCAAATCTT 3'

sec-24.2: 5'AACACTTCATCC … (to) …ATGCTGTCAAAT 3'

sec-31: 5'CTACAGGAAGGA … (to) …TCAACAGCCACC 3'

Nutrient starvation and repletion treatments were conducted as previously described[20,21]. In brief, worms at the L4 stage were subjected to nutrient starvation or repletion for 4 hr. OP50 bacteria (worms' standard food) at the concentration of 5 x 10^8/ml and 5 x 10^{11}/ml were used as starvation and repletion food, respectively. 5 mg/ml of carbenicillin and 2.5 mg/ml of kanamycin were supplied to prevent continuous bacterial growth. Treated worms were subjected to imaging of HLH-30::GFP (three biological replicates with 30 worms per replicate) by Zeiss Microscope (Axio Image M2) or immunoblotting of p-RSKS-1 (RSKS-1 is the orthologue of human S6K1) with antibodies against p-S6K1 (CST, 9234).

For treatments with a single type of amino acid, worms at L4 stage were subjected to leucine, arginine or isoleucine starvation or stimulation treatment. Starvation treatment was performed by culturing worms on empty plates without any food for 4 hr. Stimulation was achieved by supplying 5 mM leucine, arginine or isoleucine into empty plates and then culturing worms on the indicated plates for 4 hr.

C. elegans lifespan assays

Worms were raised on plates with OP50 bacteria (Fig. 3d) or the indicated RNAi bacteria (Extended Data Fig. 9n) until day 7. Nutrient restriction and repletion treatments were started at the end of the reproductive period (day 7), because starvation during the reproductive period would cause worm death. Worms were switched to fresh bacterial cultures for starvation or repletion every other day. Animals that did not move when gently prodded were scored as dead. Animals that crawled off the plate or died owing to vulva bursting were omitted. For lifespan assays with RNAi soaking (Extended Data Fig. 9q), experiments were conducted following the indicated workflow. RNAi soaking was performed as previously described[22]. The sense strand sequence of *let-363* dsRNA is displayed below. Source Data Figure 3 and Source Data Extended Data Figure 7 show the raw data of three biological replicates with 30 worms per replicate for lifespan assays.

The sense strand sequence of *let-363* dsRNA for RNAi soaking:

5'GCAAUCGAUGGACGAACAGA…(to)…CUGAUCAACGAUUCGAACUC 3'

Analysis of SAR1B alterations and gene expression levels in human lung cancer datasets

TCGA datasets of lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD) are available at *cancergenome.nih.gov* with a website tool cBioPortal. SAR1B homozygous or hemizygous focal deletions were identified by analyzing the copy number alteration data based on Affymetrix SNP 6.0 microarray data. Analyses of mRNA expression levels in LUSC and LUAD were based on the Illumina HiSeq_RNASeqV2 data.

Associations between SAR1B expression levels and lung cancer patient prognoses were analyzed by the website tool Kaplan-Meier-Plotter (*kmplot.com*).

Bioinformatic analysis of SAR1B mRNA expression in murine tissues

The mRNA expression data for murine tissues was obtained from the Gene Expression Database in MGI (*www.informatics.jax.org/gxd*). The heatmap graph was plotted using the Pheatmap package in R.

Luciferase assay

The luciferase assay for detecting SAR1A transcription was performed with the Dual-Luciferase Reporter Assay System (Promega). The SAR1A promoter sequence (1 kb upstream of the SAR1A transcription start site) was amplified from human genomic DNA and cloned into the pGL4.17 vector to drive the expression of firefly luciferase. Renilla luciferase controlled by the CMV promoter in the pRL-CMV vector was constitutively expressed and served as the internal control. SAR1A transcription activity was determined by normalizing the firefly luciferase activity to Renilla luciferase activity. Experiments were performed according to the manufacturer's instructions.

Soft agar colony formation assays

Soft agar colony formation assay was performed as previously described[20]. 7,500 lung fibroblast cells (IMR-90 or HFL1) were supplied in the top layer soft-agar medium. Where indicated, 100 nM rapamycin was also supplemented in the top-layer medium. Colonies were stained with 0.005% crystal violet in 5% methanol, imaged and quantified using ImageJ.

Rapamycin sensitivity assays

To test cellular sensitivity to rapamycin by soft-agar assays, the top-layer medium containing 7,500 IMR-90 or HFL1 cells per well was supplemented with varying concentrations of rapamycin (0 mM, 10^{-9} mM, 10^{-8} mM, 10^{-7} mM, 10^{-6} mM, 10^{-5} mM, 10^{-4} mM, 10^{-3} mM, 10^{-2} mM, 10^{-1} mM, and 1 mM). Colonies were stained and quantified as described above. Under each indicated concentration of rapamycin, the colony number was normalized to the colony number in the control well (no rapamycin). Three biological replicates were performed. Average values were analyzed for IC50 by Graphpad Prism 8.0.

Subcutaneous xenograft tumour growth

The subcutaneous xenograft tumour model was established as previously described[20]. In brief, trypsinized IMR-90 or HFL1 cells were mixed with Matrigel (Corning), and then subcutaneously injected

into the right back flank of female BALB/c nude mice (Charles River). Rapamycin at 1.5 mg/kg mouse weight (low dose) or 4.5 mg/kg mouse weight (high dose) was applied via intraperitoneal injection where indicated. According to the rules of IACUC at Pony Testing International Group, where we conducted our study, all tumours were kept under 20 mm at their largest dimension. Any mouse with an evidently ulcerated tumour before the last day was euthanized. The sample size of N = 10 mice for each group was determined according to the literature. Mice were randomly allocated into groups. Researchers who collected the data were blinded to genotypes and treatments. During the whole experimental period, mice were kept under ~20 °C with ~50% humidity in a 14-hour light/10-hour dark cycle.

Lung orthotopic xenograft tumour growth

Trypsinized IMR-90 or HFL1 cells were washed twice with PBS and concentrated to 10^7 cells per 40 µl in PBS. Cell suspensions were then mixed well with equal volumes of Matrigel (Corning). An 8-week-old female NU/NU nude mouse (Charles River) was anesthetized. A small incision (3 mm) was generated in the area of the left lateral thorax of the mouse using surgical scissors. 80 µl of cell suspension mixed with Matrigel was loaded into a 1 ml insulin syringe (BD) and rapidly transplanted into the left lobe of the lung by quickly advancing the needle to a depth of approximately 5 mm and injecting. The wound was closed by two 9 mm surgical clips (BD), and the mouse was allowed to rest on a heating lamp until fully recovered. Surgical clips were removed after around 10 days when the incision site was healed. Mice transplanted with SAR1A/B-deficient cells were subjected to rapamycin treatment (4.5 mg/kg mouse weight via intraperitoneal injection) every other day, starting on day 6. Mice transplanted with HFL1 were sacrificed on day 30, while mice injected with IMR-90 cells were sacrificed on day 35. The tumour lesion surface on each left lung lobe was measured by a caliper, and the lesion area was calculated as width/2 x length/2 x 3.14. Mice with cells incorrectly injected into the thoracic cavities instead of left lung lobes were removed from the study. The entire lung of one mouse from each experimental group was taken out without perfusion for photographing. The lungs of three mice from each group were

perfused with PBS, and the tumours in the left lung lobes were removed for protein extraction and western blotting. The lungs of the remaining mice from each group were perfused with PBS and then fixed in 4% PFA. Fixed left lung lobes were sectioned and subjected to H&E and immunohistochemistry (IHC) staining. For the study of Sestrin1/2/3 knockout in HFL1 and xenograft tumour growth, mice were sacrificed on day 35. Other experimental procedures were essentially the same as described above. Lung orthotopic xenograft experiments were approved by IACUC at Peking University. According to the institutional rules, all tumour lesion areas were kept under 40 mm^2. For each group, the sample size was N = 12 mice. Mice were chosen in an unbiased fashion to ensure randomization. During the whole experimental period, mice were kept under ~20 °C with ~50% humidity in a 14-hour light/10-hour dark cycle.

mRNA quantifications by Q-PCR

Cells or worms were lysed with TRIzon Reagent (CoWin Biosciences). Total RNAs were isolated by chloroform extraction and isopropanol precipitation. 500 ng total RNAs were subjected to reverse transcription using a cDNA synthesis kit (TransGen Biotech). cDNAs of the indicated genes were quantified by real-time quantitative PCR (Q-PCR) using SYBR Green dye (Vazyme Biotech) on CFX96^{TM} Real-Time System (Bio-Rad). Data were collected and analyzed with the software CFX Manager. Mammalian cell transcript quantifications were normalized to GAPDH. *C. elegans* transcript quantifications were normalized to *rpl-32*. The following primers were used:

SAR1B fwd: *5'CTACCTTCCTGCTATCAATGG*; rev: *5'ATGGCTTCAGGTCTGTCGATC*

SAR1A fwd: *5'TGGACTGTGCAGATCATTCTC*; rev: *5'CTTTCCTGTGGTCTGTCCAT*

Sec13 fwd: *5'CAGATGGACTACTATGGCAC*; rev: *5'CCTCTCTCCAGATAATGAC*

Sec31A fwd: *5'CATGGCATCCTGATGTTGC*; rev: *5'GAGCAGAGAATCTTAGCATC*

Sec31B fwd: *5'CAGAGGACATCAAGGCACTG*; rev: *5'GAAGTCGATCATCCTCTGAG*

Sec23A fwd: *5'CCTAGTTATGCTGGTATATC*; rev: *5'CCTTCACATCCAAGTTCATG*

Sec23B fwd: *5'GTCACGCATTATCAGCACTC*; rev: *5'GATGAGTTGTCGGTCCAGC*

Sec24A fwd: *5'CACATGACACCATCCACTGAC*; rev: *5'CCTCATGACTGCCTCAAAGC*

Sec24B fwd: *5'CAGGCCTTCCATCGACTCAAG*; rev: *5'CTGAGGTCACAGGCTCAACTG*

Sec24C fwd: *5'CATGATTGACGTCTCCTAC*; rev: *5'GTTGACCAGGAAGCCATCCAG*

Sec24D fwd: *5'CTCTACCAATGTACAGACCAG*; rev: *5'GATAGAGTCAGGATCCAGC*

GAPDH fwd: *5'ACCACAGTCCATGCCATCAC*; rev: *5'TCCACCACCCTGTTGCTGTA*

sar-1 fwd: *5'ATGGAGTGCTCAACATGCTGG*; rev: *5'CGTGACCACCGAGATCGTATG*

sec-23 fwd: *5'CAACCATTGAGTACACGCTTCG*; rev: *5'CTGAGTGTTGAGCTCATGCAG*

sec-24.1 fwd: *5'ACAGCCGCAGTTTCAGTCAAACG*; rev: *5'ATTGCTGTGGTGGAGCTCCGG*

sec-24.2 fwd: *5'ACTGCAACGACTTCGCAAGAGC*; rev: *5'TACTTGGAGCTGCTGAACCAAC*

sec-31 fwd: *5'TGCATTGGAAAGCGATGGTTG*; rev: *5'ATGAGAAGTTCATTGGCGAAG*

sesn-1 fwd: *5'GAGTATACGGATACACTGACG;* rev: *5'TTCACGGAATGCAGCAGTGTC*

let-363 fwd: *5'CAGTTCTCATGCAGCTTCTAC;* rev: *5'TGTGTGGCATGTACATCTGG*

F39C12.1(CeMios) fwd: *5'CCTGACATTGGATTTGATGAC*; rev: *5'AACGCGTCCATCGGATGCATG*

npp-18(CeSeh1L) fwd: *5'TGTCATGGTGGAGCAGTTTGG*; rev: *5'GATATCGGTGACGTCTGAACG*

rpl-32 fwd: *5'AGGGAATTGATAACCGTGTCCG*; rev: *5'TAGGACTGCATGAGGAGCATGT*

Statistical analyses and reproducibility

Sample size (N), replicates (*n*), quantifications and statistics are indicated in the figures, figure legends and methods. All columns, spots, boxes and curves (except those in Extended Data Fig. 10e) were drawn by Graphpad Prism 8.0. Curves in Extended Data Fig. 10e were generated by the Kaplan-Meier-Plotter webtool. All statistical analyses were performed with Graphpad Prism 8.0. Statistical evaluation results are presented as p values. *p* values adjustments (p_{adj}) were made for multiple comparisons. All experiments were repeated independently at least three times with similar results, with the exception that the following four sets of experiments were performed one time: subcutaneous xenograft tumour growth,

lung orthotopic xenograft tumour growth, identification of GATOR2 interaction proteins, and evaluations of leucine analogue transport and hydrolysis.

Data availability

The authors declare that all data supporting the findings of this study are available within the paper. Uncropped gel blots are shown in Supplementary Figure 1. Raw data for all animal-related experiments in main and extended figures (Fig. 3, Fig. 4, Extended Data Fig. 9, Extended Data Fig. 11 and Extended Data Fig. 12) are provided. Publicly available datasets generated by others used in this study are as following:

Database of lung cancers: www.cbioportal.org

Gene expression database of murine tissues: www.informatics.jax.org

Database of lung cancer patient prognosis: www.kmplot.com

Code availability

There is no custom code generated or applied in this study.

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