

Supplementary Information for

SNAT7 regulates mTORC1 via macropinocytosis

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Supplementary Material and Methods

Cell lines and tissue culture

Human Embryonic Kidney 293A (HEK293A) cells (described in (1)), mouse embryonic fibroblasts (MEFs; described in (1)), MIA PaCa-2 human pancreatic cancer cells (gift from Dr. Kim Orth), and HCT116 colon cancer cells (gift from Dr. Angelique Whitehurst) were cultured in high-glucose DMEM (#D5796 from Sigma). NCI-H2030 lung cancer cells (gift from Dr. Angelique Whitehurst) were cultured in RPMI 1640 (#R8758 from Sigma). HAP1 human near-haploid cells were cultured in IMDM (#I3390 from Sigma). All cell lines were cultured in media supplemented with 10% FBS (#F2442 from Sigma) (except for H2023 cells, which were cultured with 5% FBS) and penicillin/streptomycin (#P0781 from Sigma, 100 units penicillin and 100 µg/mL streptomycin) and maintained at 37°C with 5% CO₂. RagA/B knockout (KO) MEF and HEK293A cells were generated previously (1). HAP1 Raptor-GFP cells were a generous gift from Dr. Nicholas Ktistakis in Babraham Institute, UK (2). RagA/B KO or SNAT7 KO MIA PaCa-2 cells, RagA/B KO HAP1 Raptor-GFP cells were generated by CRISPR/Cas9 genome editing (3).

Antibodies

The following antibodies were purchased from Cell Signaling Technology and used at the indicated dilution for western blot analysis: phospho-S6K1 T389 (#9234, 1:1000), S6K1 (#9202, 1:1000), mTOR (#2983, 1:1000), mLST8 (#3274, 1:1000), phospho-4EBP1 S65 (#9451, 1:1000), 4EBP1 (#9452, 1:1500), phospho-ULK1 S758 (#6888, 1:1000), ULK1 (#8054, 1:1000), RagA (#4357, 1:1000), RagC (#3360s, 1:1000), Sin1 (#12860s, 1:1000), LAMTOR1 (#8975s, 1:1000), LAMTOR2 (#8145s, 1:1000), LAMTOR3 (#8168s, 1:1000), LAMTOR4 (#12284s, 1:1000), ATG7 (#2631s, 1:1000), Vinculin (#4650, 1:1000), Actin (#3700, 1:100000), and Tubulin (#2144S, 1:1000) were from Cell Signaling. Raptor antibody (#A300-553A) was from Bethyl Laboratories. Flag (#F3165, 1:10000) and SNAT7 (#HPA041777, 1:1000) antibodies were obtained from Sigma Aldrich. GFP antibody (#632381, 1:2000) was obtained from Clontech. Arf1 (#sc-53168, 1:200), HA (#sc-7392 or #sc-805, 1:500), and Myc (#sc-40, 1:1,000) antibodies were obtained from Santa Cruz. Horseradish peroxidase (HRP) linked secondary antibodies (#NXA931V anti-mouse or #NA934V anti-rabbit, 1:4000) were from GE Healthcare. SNAT9 (#NBP1-69235, 1:1000), VoC (NBP1-59654, 1:1000) were from Novus Biologicals. VoD (#ab 202897, 1:1000) and LAMP2 (#25631, 1:1000) were from Abcam. TMEM192 (#28263-1-AP, 1:1000) was from

Proteintech. V1A (#GTX 110815, 1:1000) was from GeneTex.

Antibodies used for the immunofluorescent microscopy experiments: LAMP2 (#13524 or #25631, 1:200) was obtained from Abcam. HA (#sc-7392) was obtained from Santa Cruz. Alexa Fluor 488 and 555 secondary antibodies (1:200) were obtained from Invitrogen.

Chemicals

Brefeldin A (#B6542) and bovine serum albumin (BSA, #A1470) were from Sigma. Fatty acid free BSA (#A8806) was from Sigma. BSA from Fisher Scientific (#BP1600) was also used. LysoTracker Red DND-99 (#L7528) and dextran-TMR (#D1818) were from Thermo Fisher Scientific. Glutamine (#G3126) and asparagine (#A0884) were obtained from Sigma. Glutamine methyl ester (#CDS003704) were from Sigma, and asparagine methyl ester was from Santa Cruz (sc-279251). Cycloheximide (#C7698) and Torin1 (#475991) were from Sigma.

Plasmids

The cDNA for human SLC38A7 (NM_018231) was obtained from the McDermott Center for Human Genetics at UT Southwestern Medical Center, amplified using Q5 high-fidelity DNA polymerase (#M0492L from New England Biolabs) and subcloned into *Sall* and *EcoRI* sites of pRK7 for transient transfection, or *NotI* and *EcoRI* sites of pQCXIP for retrovirus packaging, or *XbaI* and *BamHI* sites of pLentiCas9-blast (replacing Cas9 encoding sequence) for lentivirus packaging. An HA-tag was fused to the N-terminus of the protein product. Truncated version of SLC38A7 was generated similarly. HA-tagged SLC38A1, SLC38A2, SLC38A3, SLC38A4, SLC38A10 and SLC25A5 in pRK7 backbone were generated similarly. pRK5-Flag-SNAT9 was a gift from Dr. David Sabatini (Addgene #71855; (4)). pANT7-SLC35G1 (#HsCD00630132), pLX304-SLC1A5 (#HsCD00436374), pLX304-SLC38A5 (#HsCD00434194), pLX304-SLC38A6 (#HsCD00440302) and pLX304-SLC38A11 (#HsCD00443413) were purchased from DNASU. pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene #62988; (3)), lentiCRISPR v2 (Addgene #52961; (5)), and lentiCas9-Blast (Addgene #52962; (5)) were kind gifts from Dr. Feng Zhang. All plasmids constructed in this paper were verified by DNA sequencing.

cDNA transfection

Cells were transfected with plasmid DNA using PolyJet™ DNA *In Vitro* Transfection Reagent (#SL100688 from SignaGen Laboratories) according to manufacturer's

instructions. For immunofluorescence experiment, RagA/B KO MEF or HEK293A cells were seeded in 24-well plate and transfected with 0.5 μ g HA-tagged SNAT7, SNAT7 1-150, or SNAT7 21-C for 24 hours. For co-immunoprecipitation experiment in Figure 2, RagA/B KO HEK293A cells were transfected with 18 μ g of HA-SNAT7 or controls in 15 cm dishes for 24 hours and collected for immunoprecipitation. Other transfection experiments were performed similarly.

RNA interference

Protein expression silencing was done by siRNA. ON-TARGET plus SMARTpool siRNAs for Arf1 or non-targeting control were from Dharmacon and used in knock down experiments. siRNAs were delivered into cells using DharmaFECT 1 transfection reagent (#T-2001-03 from GE Healthcare Dharmacon) according to manufacturer's instructions. 48 hours post transfection the cells were harvested and analyzed via Western blot analysis. Also, MIA PaCa-2 cells were seeded at 4×10^4 per well in complete media in 12 wells. 24 hours later, cells were transfected with DsiRNA ATG7 (hs.Ri. ATG7.13 from Integrated DNA Technologies) using TriFECTa® RNAi Kit (#SLC38A7 from Integrated DNA Technologies) according to the manufacturer's instructions. Cells were then transfected again 48 hours later with ATG7 siRNA1 (hs.Ri. ATG7.13.1) or control. Fresh medium was added 6 hours after each transfection. 24 hour following transfection, cells were treated with media without glutamine (#11960069 from Thermo Fisher Scientific) and cell lysates were collected after 17 hours of glutamine starvation.

GFP nanobody purification

For GFP nanobody purification, 6X-His-TEV-GFP nanobody plasmid was transformed into Rosetta DE3 cells and cells were grown in Luria Bertani (LB) media until an OD_{600} of 0.6 – 0.8. Protein expression was induced with 0.4 mM IPTG at room temperature overnight. Cells were spun down and pellets were lysed in 20 mM Tris pH 7.9, 500 mM NaCl, 0.5 mM PMSF, 0.5% TritonX-100 and 5 mM Imidazole by sonication. Cell lysates were spun down at 20,000 x g for 1 hour at 4°C and supernatant was incubated with washed Ni-NTA beads for 1 hour at 4°C. Beads were washed with 20 mM Tris pH 7.9, 500 mM NaCl and 20 mM Imidazole. Proteins were eluted with 20 mM Tris pH 7.9, 500 mM NaCl and 0.4 M Imidazole, and collected by passing over a column. Proteins were concentrated followed by gel filtration chromatography as described above. Proteins were then dialyzed in 0.2 M NaHCO_3 pH 8.3, 0.5 M NaCl, coupled to NHS-activated sepharose (GE Healthcare)

according to manufacturer's instruction, and stored in 20% of ethanol at 4°C until use.

Cell lysis and immunoprecipitation

Cells were rinsed with either ice-cold PBS and lysed in ice-cold CHAPS lysis buffer (40 mM HEPES pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM Orthoranaolate, 0.3% CHAPS, and one tablet of EDTA-free protease inhibitors (#11873580001 from Roche) per 25 ml) or Triton lysis buffer (same as CHAPS lysis buffer but with 1% Triton X-100 instead of 0.3% CHAPS). The only immunoprecipitation performed with 1% Triton X-100 was Supplemental Figure 4. The soluble fractions from cell lysates were isolated by centrifugation at 13,000 rpm for 10 minutes in a microfuge at 4°C. 50 µl of cell lysate was taken as an input control. For immunoprecipitations, 30 µl of anti-HA (#PI88836 from Thermo Fisher Scientific) beads or 10 µl of GFP-nanobody coupled beads were washed twice using lysis buffer, added to each sample and incubated with rotation for 4 hours at 4°C. Immunoprecipitates were washed three times with lysis buffer. Immunoprecipitated proteins were denatured by adding 50 µl of sample buffer and boiling for 5 minutes (or kept at room temperature when probing for SNAT7), resolved by 10%–15% SDS-PAGE, and analyzed via Western blot analysis.

Subcellular fractionation

HEK293A cells were plated in 1 x 15 cm dishes per condition and allowed to grow overnight. Cells were harvested by trypsinization and spun at 3000 rpm for 5 mins at 4°C. The remaining steps were performed on ice or spun at 4°C, followed by lysing according to a previously published protocol (6). Cells were washed twice with cold 1X PBS and spun at 4000 rpm for 5 mins. Cells were resuspended in 1mL of HNMEK lysis buffer (20 mM HEPES pH 7.4, 50 mM NaCl, 2 mM MgCl₂, 2 mM EDTA, 10 mM KCl, 50 nM EGTA, protease inhibitors) and incubated for at least 20 mins, then lysed using a Dounce homogenizer. Lysates were centrifuged at 750 g for 10 mins to remove nuclei and cell debris. The supernatant was collected and centrifuged at 12500 g for 10 min to pellet organelle fraction (P2). Cytoplasmic (S2) fraction was collected and kept for analysis. The P2 fraction was washed twice in HNMEK lysis buffer, and centrifuged at 12500 g for 10 min. The pellet was resuspended in RIPA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA pH 8.0, 1% Nonidet P-40, 0.5% deoxycholate, protease inhibitors). Input (WCL), P2 and S2 fractions were collected or resuspended in equal volume.

Western blot

Cells were rinsed with PBS and lysed with Laemmli sample buffer (50 mM Tris pH 6.8, 2% SDS, 0.025% Bromophenol Blue, 10% glycerol, 5% BME) and boiled for 10 mins before separation by 10%-15% SDS-PAGE and transfer to polyvinylidene difluoride membranes (#162-0177 from Bio-Rad). Blots were then blocked in 5% milk for 1 hour, probed with primary antibodies and horseradish peroxidase (HRP) conjugated secondary antibodies, and developed with SuperSignal™ West Dura Substrate (#34075 from Thermo Fisher Scientific). Densitometric analysis were done using Image J software.

RNA extraction, reverse transcription, and real-time PCR

Cells were washed with cold PBS and subjected to RNA extraction using a RNeasy Plus mini kit (#74104 from Qiagen). RNA samples (1 µg) were reverse transcribed to complementary DNA using iScript Reverse Transcription Supermix (#1708841 from Bio-Rad). Complementary DNA was then diluted and used for quantification (with GAPDH gene as an internal control) by real-time PCR, which was performed using iTaq Universal SYBR Green Supermix (#172-5121 from Bio-Rad) and the CFX96 real-time PCR system (Bio-Rad) using predesigned primers from Sigma Aldrich.

Generation of stable cell lines

For SNAT7 overexpression stable cell lines, retro viruses were packaged in HEK293A cells by transfecting the cells with pQCXIP-HA-SNAT7 and packaging vector pCL10A. Alternatively, lentiviruses were packaged in HEK293A cells using pLenti-Blast vectors in which Cas9 cDNA sequence was replaced by SNAT7 cDNA sequence, together with packaging plasmids pMD2.G and pSPAX2. The produced recombinant retro/lentiviruses were collected from the medium 48 hrs after transfection. Viruses were spun down at 500 x g for 10 minutes, filtered through a 0.45 µm filter, and used to infect RagA/B KO HEK293A cells, WT MIA PaCa-2 cells, or SNAT7 KO MIA PaCa-2 cells with polybrene (8 µg/ml; TR-1003-G from Millipore), followed by selection of infected cells using puromycin (5 µg/ml; #ant-pr-1 from InvivoGen) or blasticidin (10 µg/ml; #A1113903 from Thermo Fisher Scientific) until all non-infected cells died.

Generation of RagA/B and SNAT7 knockout cells using CRISPR/Cas9 genome editing

RagA/B or SNAT7 KO MIA PaCa-2 cells and RagA/B KO Hap1 Raptor-GFP cells were generated using CRISPR/Cas9 genome editing. The 20 nucleotide guide sequences targeting human *SLC38A7* (the encoding gene of SNAT7), *RRAGA* and *RRAGB* (the encoding genes of RagA and RagB) were designed according to Hsu et al. (7). Guide RNA sequences for *RRAGA* and *RRAGB* were cloned into a bicistronic expression vector (pX459) containing human codon optimized Cas9 and the RNA components ((3); Addgene #62988). Guide RNA sequence for *SLC38A7* was cloned into a lentiviral version of CRISPR vector (lentiCRISPR v2; addgene #52961;(7). The guide sequence targeting exon 1 and exon 2 of human *SLC38A7*, exon 1 of human *RRAGA* and exon 1 of human *RRAGB* are shown below.

SLC38A7-1 5' -ATCGTCGTC AACGCGTGCCT-3'
SLC38A7-2 5' - GCTGACAGGTGTGCTATGTG-3'
RRAGA 5' -ATTACATTGCTCGCGACACC-3'
RRAGB 5' -CCACCACTAGGGGAACCGGA-3'

The single guide RNAs (sgRNAs) in the pX459 vector (1 µg) were transfected into HEK293A cells using PolyJet™ DNA *In Vitro* Transfection Reagent according to manufacturer's instructions. 24 hours post transfection, the cells were treated with 5 µg/mL puromycin (#ant-pr-1 from InvivoGen) for another 24 hours until all the control cells without transfection died. The sgRNAs in the lentiCRISPR v2 vector were transfected into HEK293A cells together with packaging vectors pSPAX2 and pMG2D using PolyJet™ DNA *In Vitro* Transfection Reagent. Lentiviruses were collected 48 hrs post transfection, spun down at 500 x g for 10 minutes, filtered through a 0.45 µm filter, and used to infect MIA PaCa-2 cells with 8 µg/ml polybrene (TR-1003-G from Millipore), followed by selection of infected cells using puromycin (5 µg/ml; #ant-pr-1 from InvivoGen). Cells are allowed to recover for a few days prior to single cell FACs sorting into 96-well plates in DMEM containing 30% FBS (UT Southwestern; Flow Cytometry Core). Single clones were expanded, and screened for RagA, RagB or SNAT7 by protein immunoblotting.

Patient, tissue samples and immunohistochemistry

Patients enrolled in the University of Texas Southwestern study provided written consent allowing the use of discarded surgical samples for research purposes according to an Institutional Board approved protocol. Human pancreatic ductal adenocarcinoma (PDAC)

tissues were obtained from the University of Texas, Southwestern Tissue Repository under Institutional Board Review (IRB) 102010-051.

Formalin-fixed, paraffin-embedded tissues of 20 PDAC samples were cut into 4 μ m-thick sections. Immunohistochemical analysis was performed on a Dako Autostainer Link 48 system. Briefly, the slides were baked for 20 minutes at 60°C, then deparaffinized and hydrated before the antigen retrieval step. Heat-induced antigen retrieval was performed at pH 6 for 20 mins in a Dako PT Link. The tissue was incubated with peroxidase block and then an antibody incubation for 20 minutes (the SNAT7 antibody was used at 1:350 dilution). The sections incubated with albumin instead of the primary antibody were used as negative controls. The staining was visualized using the GenFLEX visualization system. Hematoxylin and eosin (H&E) staining was performed to verify the morphological and cellular features of the tissue.

Immunohistochemistry scoring

Semiquantitative scoring was performed by a pathologist in a blinded manner, based on percentage of tumor cells presenting cytoplasmic staining (0-100%) and staining intensity (0-3+). H-scores were calculated using the following equation: $H\text{-score} = 3 \times (\% \text{ of } 3+ \text{ intensity cells}) + 2 \times (\% \text{ of } 2+ \text{ intensity cells}) + 1 \times (\% \text{ of } 1+ \text{ intensity cells})$ (8).

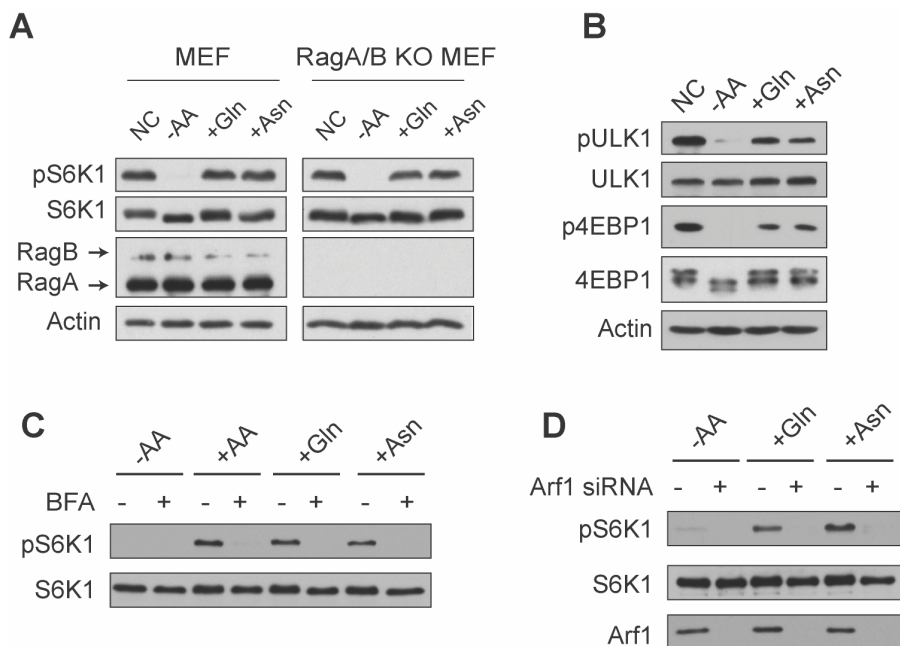


Fig. S1: Glutamine and asparagine signal to mTORC1 in the absence of the Rag GTPases. (A) Glutamine (Gln) and asparagine (Asn) signal to mTORC1 in the absence of the Rag GTPases. Wildtype (WT) or RagA/B knockout (KO) mouse embryonic fibroblasts (MEFs) were starved of amino acids (-AA) for 2 hr and stimulated with 4mM Gln (+Gln) or Asn (+Asn) for 2 h. NC denotes normal condition. mTORC1 activity was analyzed by phosphorylation of S6K1 at threonine 389 (pS6K1). S6K1 and Actin were probed for as loading controls. Protein levels of RagA/B were also confirmed by immunoblotting. (B) Gln and Asn activate phosphorylation of multiple mTORC1 substrates. Like (A), mTORC1 activity in RagA/B KO MEFs was analyzed by phosphorylation of ULK1 at Serine 758 and 4EBP1 at Serine 65. Actin, ULK1, and 4EBP1 were probed for as loading controls. (C) Pharmacological inhibition of Arf1 diminishes Gln and Asn signaling to mTORC1 in the absence of the Rag GTPases. RagA/B knockout (KO) mouse embryonic fibroblast (MEF) cells were starved of amino acids (-AA) for 2 hrs, pretreated with or without 10 uM brefeldin A (BFA) for 1 hr, and then amino acids (+AA), Gln (+Gln) or Asn (+Asn) were added for 2 h at 4 mM. mTORC1 activity was analyzed by immunoblotting for the phosphorylation status of S6K1 (pS6K1) at threonine 389. S6K1 was used as a loading control. (D) Genetic knockdown of Arf1 blocks Gln and Asn signaling to mTORC1 in the absence of the Rag GTPases. RagA/B KO human embryonic kidney 293A (HEK293A) cells were transfected with control siRNA (-) or siRNA against Arf1 (+) for 48 h, starved for amino acids (-AA) for 2 h, and then Gln (+Gln) or Asn (+Asn) were added for 2 h at 4 mM. mTORC1 activity was analyzed as described in (A).

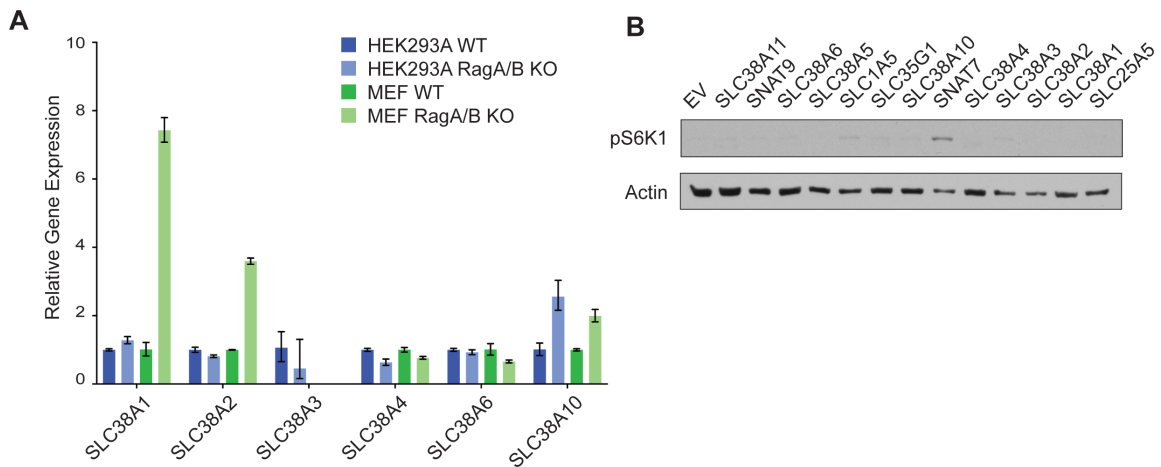


Fig. S2: Screening of solute carriers involved in mTORC1 signaling. (A) mRNA levels of SLC38 family amino acid transporters in both wildtype (WT) and RagA/B knockout (KO) cells. SLC38A3 in mouse embryonic fibroblast (MEF) cells were not detectable; SLC38A5, SLC38A8, SLC38A11 in both human embryonic kidney 293A (HEK293A) and MEF cells were not detectable. See Figure 1A for *P* values. (B) Over-expression of SNAT7 renders mTORC1 resistant to amino acid starvation. Empty vector (EV) or tagged SLC transporters were expressed in HEK293A cells for 72 h. Cells were starved of amino acids for 1 h, lysed and mTORC1 activity was analyzed by immunoblotting for the phosphorylation status of S6K1 (pS6K1) at threonine 389. Actin was used as a loading control.

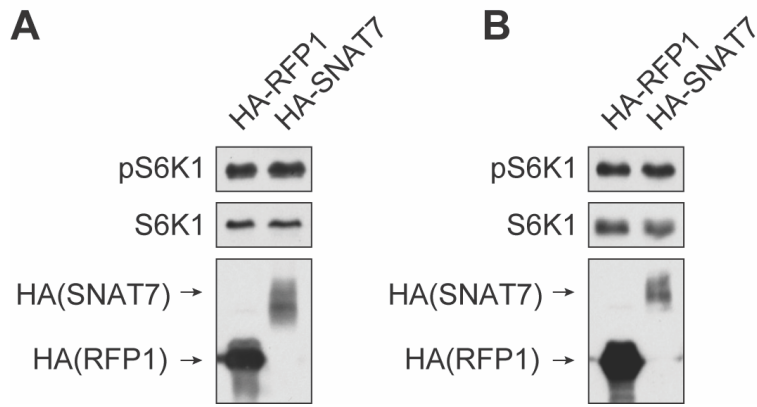


Fig. S3: Expression of SNAT7 in amino acid rich conditions. (A) Expression of SNAT7 doesn't further enhance mTORC1 activation under nutrient rich conditions. Wildtype (WT) human embryonic kidney 293A (HEK293A) cells were transfected with HA-tagged SNAT7 or HA-tagged RFP1 for 48 hrs. mTORC1 activity was analyzed by immunoblotting for the phosphorylation status of S6K1 (pS6K1) at threonine 389. S6K1 was used as loading control. Overexpression of HA-tagged (RFP1 and SNAT7) proteins was confirmed by Western blotting. **(B)** RagA/B KO HEK293A cells were analyzed similar to (A).

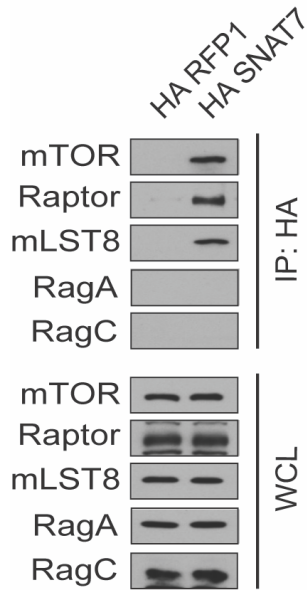


Fig. S4: SNAT7 interacts with mTORC1 using 1% Triton X-100. MIA PaCa-2 cells overexpressing HA-tagged SNAT7, or HA-tagged RFP (control) were immunoprecipitated with anti-HA beads, and then analyzed for indicated proteins (mTOR, Raptor, mLST8, RagA, and RagC).

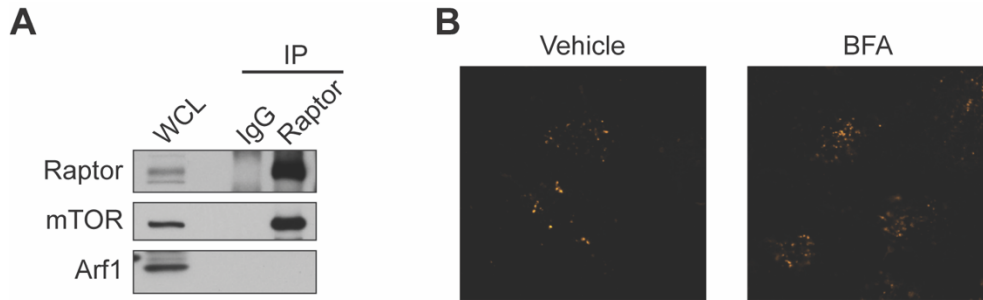


Fig. S5: Arf1 does not bind mTORC1 or alter macropinocytosis. (A) Arf1 does not interact with mTORC1. MIA PaCa-2 cells were immunoprecipitated using anti-Raptor antibody and the immunoprecipitates were probed for Raptor, mTOR and Arf1 by Western blot analysis. **(B)** Arf1 does not alter macropinocytosis. MIA PaCa-2 cells were starved of amino acids for 2 h, treated with 10 μ M BFA or vehicle for 40 mins, and then treated with 1 mg/ml dextran-TMR (orange) for 30 mins, followed by immunofluorescence microscopy.

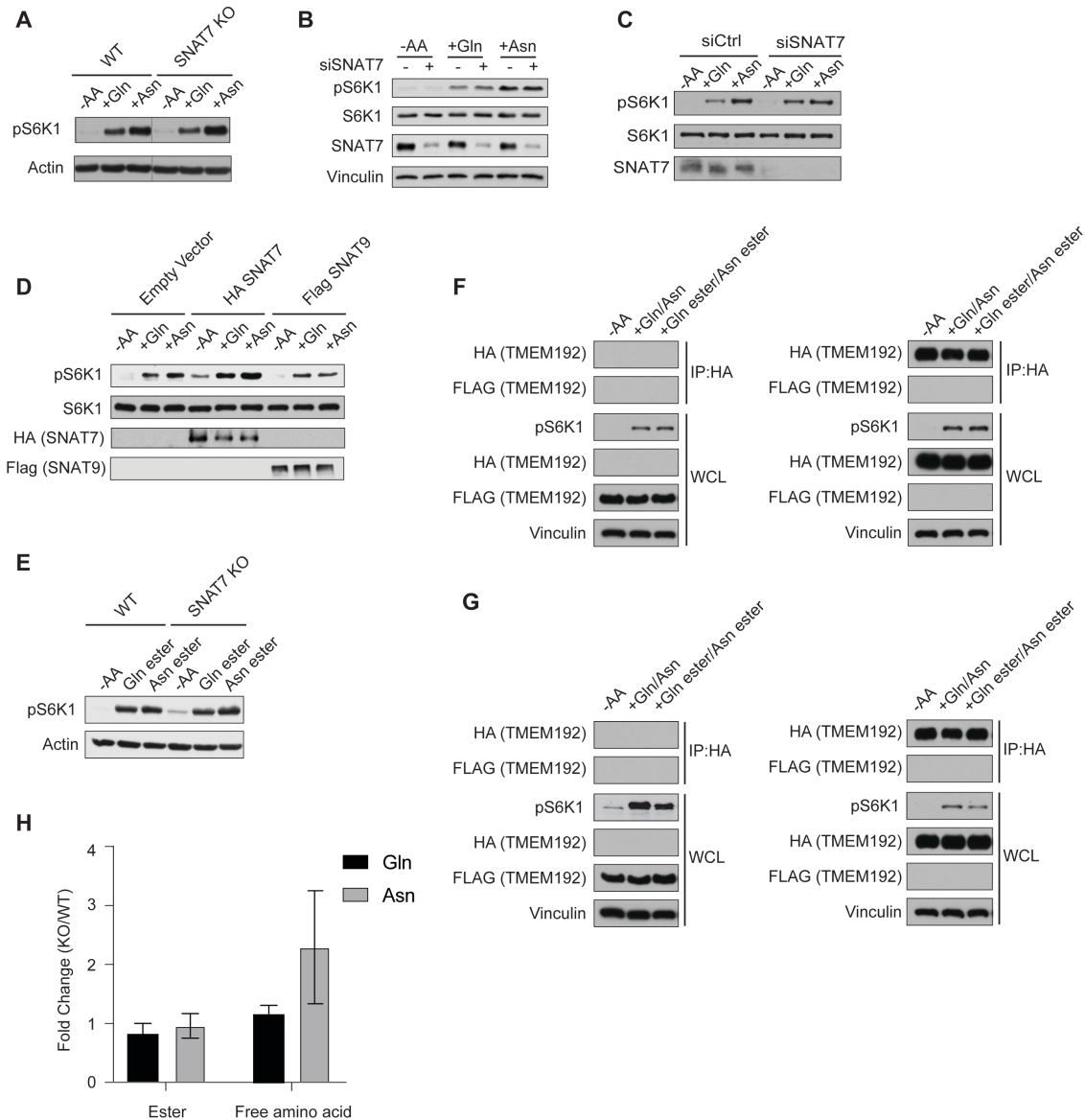


Fig. S6: SNAT7 does not block cytosolic glutamine- or asparagine-induced mTORC1 activation (A) SNAT7 is not required for cytosolic glutamine (Gln) and asparagine (Asn) to signal to mTORC1. Wildtype (WT) or SNAT7 knockout (KO) MIA PaCa-2 cells were starved of amino acids (-AA) and stimulated with 4mM Gln (+Gln) or Asn (+Asn) for 2 h. mTORC1 activity was analyzed by immunoblotting for the phosphorylation status of S6K1 (pS6K1) at threonine 389. Actin was used as a loading control. (B) Knockdown of SNAT7 doesn't alter Gln- and Asn-induced mTORC1 activation. Wildtype (WT) human embryonic kidney 293A (HEK293A) cells were transfected with control small interfering RNA (siRNA) (-) or siRNA against SNAT7 (+) for 48 h, starved for amino acids (-AA) for 1 h, and Gln (+Gln) or Asn (+Asn) was added for 2 h at 4 mM. mTORC1 activity was analyzed by immunoblotting for the phosphorylation status of S6K1 (pS6K1) at threonine 389. SNAT7 levels were confirmed by Western blotting and Vinculin was used as a loading control. (C) RagA/B knockout (KO) HEK293A cells were transfected with control siRNA (siCtrl) or siRNA against SNAT7 (siSNAT7) for 48 h, starved of amino

acids for 1 h and then stimulated with or without Gln or Asn for 1 h. mTORC1 activity was analyzed as described in (A). SNAT7 immunoblot shows expression, and S6K1 is a loading control. **(D)** SNAT7 enhances Gln and Asn signaling to mTORC1. RagA/B KO HEK293A cells were transfected with HA-tagged SNAT7, FLAG-tagged SNAT9 or empty vector, followed by amino acids starvation (-AA) for 1 h and 1mM Gln (+Gln) or Asn (+Asn) stimulation for 2 h. mTORC1 activity was analyzed as described in (A). HA-tagged SNAT7 and FLAG-tagged SNAT9 expression were confirmed by Western blotting analysis. **(E)** SNAT7 is not required for Gln or Asn ester signaling to mTORC1. WT or SNAT7 KO MIA PaCa-2 cells were starved of amino acids (-AA) and stimulated with 4mM Gln (+Gln) ester or Asn (+Asn) ester for 2 h. pS6K1 was used to measure mTOR activity, and Actin was used for loading control. **(F)** Lyso-immunoprecipitations (Lyso-IPs) in WT or **(G)** SNAT7 KO MIA PaCa-2 cells. WT or SNAT7 KO MIA PaCa-2 cells with Flag-tagged or HA-tagged TMEM192 were starved of amino acids (-AA) for 2 h then stimulated with 2mM each of +Gln/Asn or + Gln/Asn esters for an additional 2 h. HA beads were utilized for immunoprecipitation experiments. Lysates were subjected to Western blot analysis. pS6K1 was used to measure mTORC1 activity, and Vinculin was used for loading control. **(H)** Gln and Asn esters don't appear to accumulate within the lysosome. Lysates from (F) and (G) were incubated with HA beads and processed for metabolite extraction and LC/MS analysis. Bar graph illustrates the ratio between WT and KO MiaPaCa-2 cells. Statistical significance: $P > 0.05$ Gln vs Gln ester, Asn vs Asn ester, measured using Student's t-test from two independent experiments.

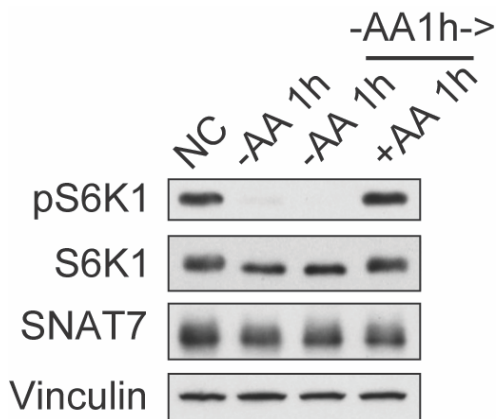


Fig. S7: SNAT7 protein levels are unchanged in different nutrient conditions. SNAT7 expression does not change under different nutrient conditions. WT HEK293A cells were starved of amino acids (-AA) for 1 h, and replaced of media containing amino acids (+AA) or maintained in starvation media (-AA) for 1 h. NC denotes normal culturing conditions. mTORC1 activity was analyzed by immunoblotting for the phosphorylation status of S6K1 (pS6K1) at threonine 389. S6K1 was used as loading control. SNAT7 levels were determined by Western blotting, and Vinculin was used as a loading control.

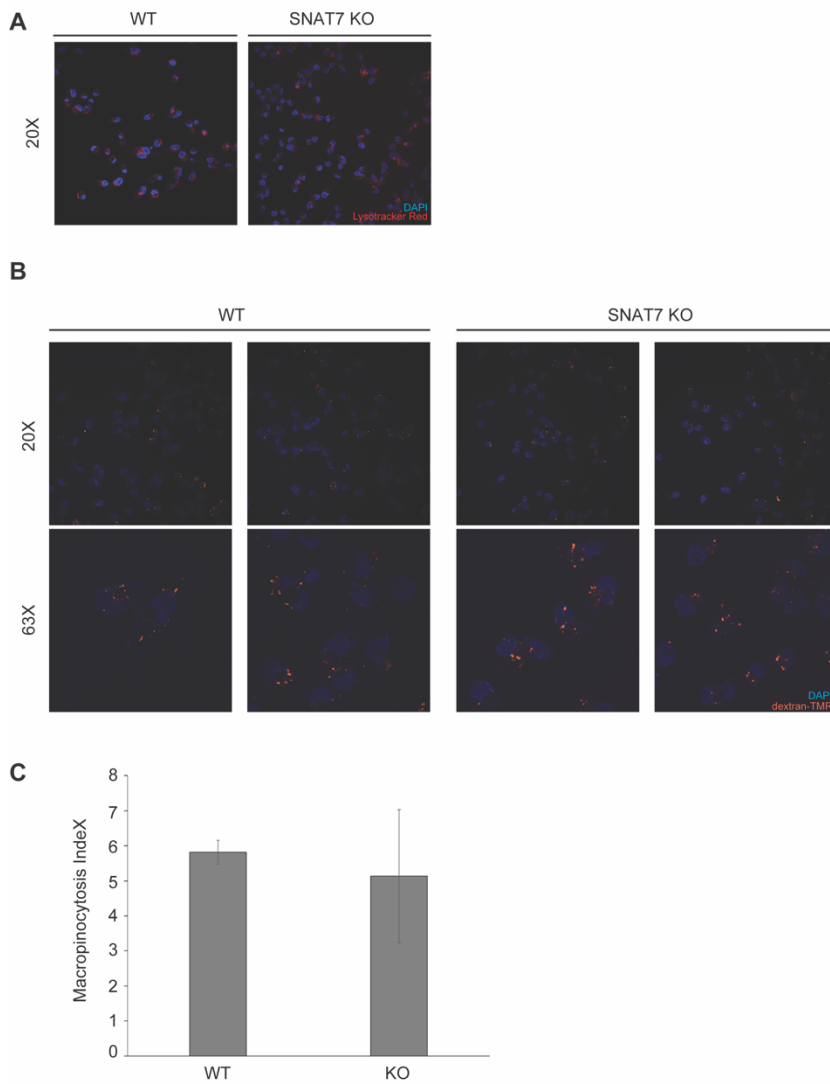


Fig. S8: Deletion of SNAT7 does not alter lysosomal function or macropinocytosis. **(A)** Depletion of SNAT7 does not alter lysosomal function. WT or SNAT7 KO MIA PaCa-2 cells were probed with 100 nM lysotracker (red) for 1 h and visualized by immunofluorescence microscopy. **(B)** Depletion of SNAT7 does not alter macropinocytosis. WT or SNAT7 KO MIA PaCa-2 cells were starved of amino acids for 2 h, treated with 1 mg/ml dextran-TMR (orange) for 30 mins, followed by immunofluorescence microscopy. **(C)** Macropinocytosis index was quantified for data (B) (9). Data show the mean \pm s.e.m. (N = 3 fields: around 50 cells per field). SNAT7 WT vs KO cells, $P = 0.69$.

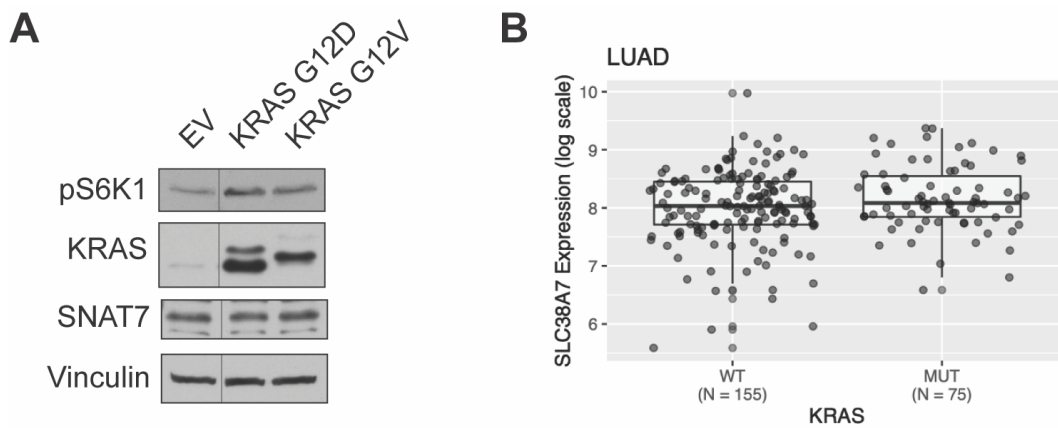


Fig. S9: KRAS mutations do not correlate with SNAT7 mRNA or protein levels. (A) KRAS mutations do not change SNAT7 (SLC38A7) protein or mRNA levels. HEK293A cells were transfected with KRAS G12D, KRAS G12V or empty vector (EV) for 72 h. Cell lysates were subjected to Western blot analysis. **(B)** KRAS mutations do not alter SNAT7 mRNA levels. mRNA levels of SNAT7 gene expression were analyzed for TCGA lung adenocarcinoma dataset (LUAD), comparing samples carrying or not carrying mutations in KRAS. Sample sizes are indicated.

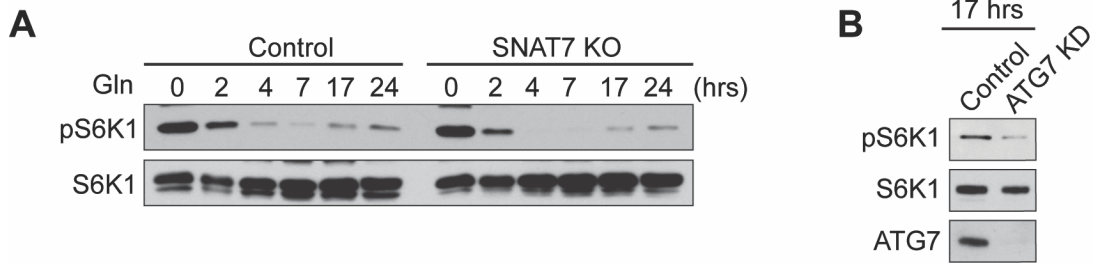


Fig. S10: SNAT7 does not alter glutamine starvation induced autophagy and mTORC1 reactivation. (A) Wildtype (WT, control) MIA PaCa-2 and SNAT7 knockout (KO) cells were starved of glutamine (Gln) for 0, 2, 4, 7, 17, and 24 h. mTORC1 activity in MIA PaCa-2 cells were analyzed by immunoblotting for phosphorylation of S6K1 at threonine 389 (pS6K1). S6K1 was used as a loading control. **(B)** MIA PaCa-2 cells were transfected with non-targeting control RNAi or RNAi against ATG7. Following transfection, cells were treated with Gln-deprived media for 17 h. mTORC1 activity was measured as stated in (A). ATG7 was immunoblotted to confirm knockdown and S6K was used as a loading control.

Supplementary References

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