Supplementary Materials for

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

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Materials and Methods

Materials

Reagents were obtained from the following sources: antibody to Nprl3 from Atlas Antibodies; HRP-labeled anti-mouse and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, S6K1, RagA and RagC, phospho-T398 dS6K, mTOR, LC3, and FLAG epitope from Cell Signaling Technology; the antibody to Mios and DEPDC5 was produced in collaboration with Cell Signaling Technology; antibodies to the HA epitope from Bethyl laboratories. RPMI, FLAG M2 affinity gel, ATP, GDP, and amino acids from Sigma Aldrich; DMEM from SAFC Biosciences; Xtremegene 9, Fugene 6 and Complete Protease Cocktail from Roche; Alexa 488 and 568-conjugated secondary antibodies; Schneider's media, Express Five-SFM, and Inactivated Fetal Calf Serum (IFS) from Invitrogen; amino acid-free RPMI, and amino acid free Schneider's media from US Biological; Cellulose PEI TLC plates from Sorbent Technologies; [^{3}H]GDP, [α-^{32}P]GTP, and [γ-^{32}P]GTP from Perkin Elmer; GTP, XTP and XDP from Jena Biosciences; siRNAs targeting indicated genes and siRNA transfection reagent from Dharmacon; nitrocellulose membrane filters from Advantec; DSP from Pierce. The dS6K antibody was a generous gift from Mary Stewart (North Dakota State University).

Cell lines and tissue culture

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

Cell lysis and immunoprecipitation

HEK-293T cells transiently transfected with cDNA expression vectors (see below) were rinsed once with ice-cold PBS and lysed with Triton lysis buffer (1% Triton X-100, 10 mM β-glycerol phosphate, 10 mM pyrophosphate, 40 mM HEPES pH 7.4, 2.5 mM $MgCl_2$ and 1 tablet of EDTA-free protease inhibitor [Roche] (per 25 ml buffer)). Lysis of HEK-293T cells stably expressing FLAG-tagged DEPDC5, WDR24 or Metap2, was identical to the procedure described in *(7)*. The soluble fractions of cell lysates were isolated by centrifugation at 13,000 rpm in a microcentrifuge for 10 minutes. For anti-FLAG-immunoprecipitations, the FLAG-M2 affinity gel was washed with lysis buffer 3 times. 20 μl of a 50% slurry of the affinity gel was then added to cleared cell lysates and incubated with rotation for 2 hours at 4°C. The beads were washed 3 times with lysis

buffer containing 150 mM NaCl. Immunoprecipitated proteins were denatured by the addition of 50 µl of sample buffer and boiling for 5 minutes as described (*1*), resolved by 8%–16% SDS-PAGE, and analyzed by immunoblotting.

For co-transfection experiments, 2,000,000 HEK-293T cells were plated in 10 cm culture dishes. Twenty-four hours later, cells were transfected using Xtremegene 9 transection reagent with the pRK5-based cDNA expression plasmids indicated in the Figures in the following amounts: 100 ng HA-RagB; 100 ng HA- or HA-GST-RagC; 300 ng HA-GST-RagB^{Q99L} or 300 ng HA-GST-RagB^{T54N}; 300 ng HA-GST-RagC^{S75N} or 300 ng HA-GST-RagC^{Q120L}; 500 ng HA- or 100-1000 ng Flag-Metap2; 2000 ng Flag- or 100 ng HA-Mios; 300 ng Flag- or 100 ng HA-WDR24; 200 ng Flag- or 100-300 ng HA-Nprl2; 100 ng HA-WDR59; 100-300 ng HA-Nprl3; 100 ng HA-Seh1L; 100 ng HA-Sec13, 3 ng Flag-S6K. The total amount of plasmid DNA in each transfection was normalized to 2 µg with empty pRK5. Thirty-six hours after transfection, cells were lysed as described above.

Mass spectrometric analyses

HEK-293T cells stably expressing FLAG-tagged Metap2, RagB, Mios, DEPDC5, WDR24, or Nprl2 were chemically crosslinked with DSP prior to cell lysis with Triton lysis buffer as described in (*2*). Cell lysates and FLAG-immunoprecipitations were prepared as described above. Proteins were eluted with the FLAG peptide (sequence DYKDDDDK) from the FLAG-M2 affinity gel, resolved on 4-12% NuPage gels (Invitrogen), and stained with simply blue stain (Invitrogen). Each gel lane was sliced into 10-12 pieces and the proteins in each gel slice digested overnight with trypsin. The resulting digests were analyzed by mass spectrometry as described (*3*). Peptides corresponding to GATOR members, Ragulator, v-ATPase or Rags were detected in the FLAG-RagB, FLAG-Mios, FLAG-DEPDC5, FLAG-Nprl2 and FLAG-WDR24 immunoprecipitates, while no peptides were detected in negative control immunoprecipitates of FLAG-Metap2.

Amino acid starvation and stimulation and concanamycin A treatment of cell lines

HEK-293T cells in culture dishes or coated glass cover slips were rinsed with and incubated in amino acid-free RPMI for either 50 minutes or 2 hours and stimulated with a 10X mixture of amino acids for 10-30 minutes. After stimulation, the final concentration of amino acids in the media was the same as in RPMI. The 10X mixture was prepared from individual powders of amino acids. Amino acid starvation for other cancer cell lines cells was identical to the procedure described above, with the addition of 10% dialyzed IFS to amino acid-free RPMI. When concanamycin A (ConA) was used, cells were incubated with 2.5 µM of ConA during the 4 hr amino acid starvation and 15 min amino acid stimulation periods.

RNAi in Drosophila S2 cells

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

Mio (CG7074)
Forward primer CG7074_1F:

GAATTAATACGACTCACTATAGGGAGATGCCTTATATATCCGTGAACTACCT
Reverse primer CG7047_1R:
GAATTAATACGACTCACTATAGGGAGACTCAATGTCCCAGATGGTGAT
Forward primer CG7074_2F:
GAATTAATACGACTCACTATAGGGAGAAGATGATAAAGCTGTTCGATCTGAG
Reverse primer CG7047_2R:
GAATTAATACGACTCACTATAGGGAGACAATTAACAAACGAAAACTTTCCAC
Forward primer CG7074_3F:
GAATTAATACGACTCACTATAGGGAGAATCGCTTTATAGACCAGTTGTATGC
Reverse primer CG7047_3R:
GAATTAATACGACTCACTATAGGGAGAAGCTTGGTCTCCGATAGATATTTG

dSeh1L (CG8722)
Forward primer CG8722_1F:
GAATTAATACGACTCACTATAGGGAGAGAAAGTGCGTTAAAATCGATACTGT
Reverse primer CG8722_1R:
GAATTAATACGACTCACTATAGGGAGACAATTGTGCTCGCTAAACTTAATG

dWDR59 (CG4705)
Forward primer CG4705_1F:
GAATTAATACGACTCACTATAGGGAGAAGGCAGAGCAACAAATACTATGAC
Reverse primer CG4705_1R:
GAATTAATACGACTCACTATAGGGAGAAGTCCCAAATATGAGAAAATGTGTC
Forward primer CG4705_2F:
GAATTAATACGACTCACTATAGGGAGACATTATGGAGAAGACAGTTCGACTT
Reverse primer CG4705_2R:
GAATTAATACGACTCACTATAGGGAGAATTGATCAAACAGTGGCATCTTAGT

dWDR24 (CG7609)
Forward primer CG7609_1F:
GAATTAATACGACTCACTATAGGGAGATGTACAAATTCATGGTAAACGACAC
Reverse primer CG7609_1R:
GAATTAATACGACTCACTATAGGGAGAGTGAGTTCATTGGATTCTTTTGATT
Forward primer CG7609_2F:
GAATTAATACGACTCACTATAGGGAGAAATCAAAAGAATCCAATGAACTCAC
Reverse primer CG7609_2R:
GAATTAATACGACTCACTATAGGGAGAAAGAGCTCAAAGTTGTCAAAGGTAA

dDEPDC5 (CG12090)
Forward primer CG12090_1F:
GAATTAATACGACTCACTATAGGGAGAGGACTTGGTGATGAATCTAAAGGAT
Reverse primer CG12090_1R:
GAATTAATACGACTCACTATAGGGAGATGAAGGTAATCTCTATCGAGTCCAG
Forward primer CG12090_2F:
GAATTAATACGACTCACTATAGGGAGATCGAAAAGCATTACTTGGATAGAAC
Reverse primer CG12090_2R:

GAATTAATACGACTCACTATAGGGAGAATCAAAGAGCGAGTTGTGCTTATAC

dNprl2 (CG9104)
Forward primer CG9104_1F:
GAATTAATACGACTCACTATAGGGAGAATGTGTTCGATGCTATCAATGTTTA
Reverse primer CG9104_1R:
GAATTAATACGACTCACTATAGGGAGATATATAAGGCAGGATCTGTTGTGTG
Forward primer CG9104_2F:
GAATTAATACGACTCACTATAGGGAGATGCATACAGAATCTGGTCTACTACG
Reverse primer CG9104_2R:
GAATTAATACGACTCACTATAGGGAGACACTTCCAGATCACAGTCACATTC

dNprl3 (CG8783)
Forward primer CG8783_1F:
GAATTAATACGACTCACTATAGGGAGACTGTACAGGTATCCGTACCAGACTC
Reverse primer CG8783_1R:
GAATTAATACGACTCACTATAGGGAGAATAGCTGTGGTTTAACAGCAAACAG
Forward primer CG8783_2F:
GAATTAATACGACTCACTATAGGGAGAATCTTCCATGATCTATGCACCAC
Reverse primer CG8783_2R:
GAATTAATACGACTCACTATAGGGAGAACGAGCTTATAAACATGCTCGATAC

dsRNAs targeting GFP and dRagC were used as negative and positive controls, respectively. On day one, 4,000,000 S2 cells were plated in 6 cm culture dishes in 5 ml of Express Five SFM media. Cells were transfected with 1 μg of dsRNA per million cells using Fugene 6 (Roche). Two days later, a second round of dsRNA transfection was performed. On day five, cells were rinsed once with amino acid-free Schneider's medium, and starved for amino acids by replacing the media with amino acid-free Schneider's medium for 1.5 hours. To stimulate with amino acids, the amino acid-free medium was replaced with complete Schneider's medium for 30 minutes. Cells were then washed with ice cold PBS, lysed, and subjected to immunoblotting for phospho-T398 dS6K and total dS6K.

Mammalian RNAi

On day one, 200,000 HEK-293T cells were plated in a 6 well plate. Twenty-fours hours later, the cells were transfected with 250 nM of a pool of siRNAs [Dharmacon] targeting Mios, WDR24, WDR59 or Seh1L or a non-targeting pool. On day four, the cells were transfected again but this time with double the amount of siRNAs. On day five, the cells were either split onto coated glass cover slips or rinsed with ice-cold PBS, lysed and subjected to immunobloting as described above.
Lentiviral shRNAs targeting Mios, WDR24, WDR59, Seh1L, DEPDC5, Nprl2, Nprl3 and GFP were obtained from the TRC. The TRC number for each shRNA is as follows:
Human Mios shRNA_1: TRCN0000303645
Human Mios shRNA_2: TRCN0000370186
Human WDR24 shRNA_1: TRCN0000130142
Human WDR24 shRNA_2: TRCN0000416122

Human WDR24 shRNA_3: TRCN0000445462
Human WDR59 shRNA_1: TRCN0000156940
Human WDR59 shRNA_2: TRCN0000156869
Human Seh1L shRNA_1: TRCN0000330510
Human Seh1L shRNA_2: TRCN0000330507
Human Nprl2 shRNA_1: TRCN0000234677
Human Nprl2 shRNA_2: TRCN0000234673
Human Nprl3 shRNA_2: TRCN0000135594
Human DEPDC5 shRNA_1: TRCN0000137523

The following shRNAs targeting Nprl3 and DEPDC5 were made in the lab and cloned into pLKO.1 vector as described (*4*).
The target sequence for the Nprl3 shRNA:
Human Nprl3 shRNA_1: GATGTTATTCTGGCAACAATT

The target sequence for the DEPDC5 shRNA:

Human DEPDC5 shRNA_2: CAGGTATTTGAAGAGTTTATT

shRNA-encoding plasmids were co-transfected with the ΔVPR envelope and CMV VSV-G packaging plasmids into actively growing HEK-293T cells using Xtremegene 9 transfection reagent as previously described (*4*). Virus-containing supernatants were collected 48 hours after transfection, filtered to eliminate cells and target cells were infected in the presence of 8 μg/ml polybrene. 24 hours later, cells were selected with puromycin and analyzed on the 2nd or 3rd day after infection.

Immunofluorescence assays
Immunofluorescence assays were performed as described in (*2*). Briefly, 200,000 HEK-293T (infected with lentiviral shRNAs) cells or 75,000 cells for other cell lines used (HeLa, SW780, HCC1500) were plated on fibronectin-coated glass coverslips in 12-well tissue culture plates. Twenty-four hours later, the slides were rinsed with PBS once and fixed for 15 min with 4% paraformaldehyde in PBS at room temperature. The slides were rinsed twice with PBS and cells were permeabilized with 0.05% Triton X-100 in PBS for 5 min. In assays requiring the LC3 primary antibody, cells were fixed and permeabilized in ice-cold MeOH for 10 min. After rinsing twice with PBS, the slides were incubated with primary antibody in 5% normal donkey serum for 1 hr at room temperature, rinsed four times with PBS, and incubated with secondary antibodies produced in donkey (diluted 1:1000 in 5% normal donkey serum) for 45 min at room temperature in the dark and washed four times with PBS. Slides were mounted on glass coverslips using Vectashield (Vector Laboratories) and imaged on a spinning disk confocal system (Perkin Elmer) or a Zeiss Laser Scanning Microscope (LSM) 710.

S2 cell size determinations
For measurements of cell size, S2 cells treated with dsRNAs as described above, were harvested in a 4 ml volume and diluted 1:20 with counting solution (Isoton II Diluent,

Beckman Coulter). Cell diameters were determined with a particle size counter (Coulter Z2, Beckman Coulter) running Coulter Z2 AccuComp software.

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

To produce protein complexes used for GAP or GEF assays, 4,000,000 HEK-293T cells were plated in 15 cm culture dishes. Forty-eight hours later, cells were transfected with the following combination of constructs (all cDNAs were expressed from pRK5 expression plasmid). For FLAG-GATOR1: 4 µg FLAG-DEPDC5, 8 µg HA-Nprl2 and 8 µg HA-Nprl3. For FLAG-Ragulator: 4 µg Flag-p14, 8 µg HA-MP1, 8 µg HA-p18^{G2A} (a lipidation defective mutant), 8 µg HA-HBXIP, and 8 µg HA-C7orf59. For RagA/B-RagC^{X}: 16 µg HA-RagB or HA-RagA and 8 µg Flag-RagC^{D181N}; for RagB^{X}-RagC: 8 µg FLAG-RagB^{D163N} and 16 µg HA-RagB. For Rags used in in-vitro binding assays: 8 µg HA-GST-RagB^{T54N} and 16 µg HA-RagC^{Q120L}; 4 µg HA-GST-RagB^{Q99L} and 8 µg HA-RagC^{S75N}. For individual proteins: 10 µg Flag- or HA-GST-Rap2a, 15 µg of FLAG-Leucyl tRNA synthetase (LRS), or 10 µg Flag-Metap2.

Thirty-six hours post transfection cell lysates were prepared as described above, with the exception that for all FLAG-GATOR1 and FLAG-LRS purifications, 1 mM ATP was added to the lysis buffer. 200 µl of a 50% slurry of FLAG-M2 affinity gel or immobilized glutathione beads were added to lysates from cells expressing FLAG-tagged proteins or HA-GST tagged proteins, respectively. Recombinant proteins were immunoprecipitated for 3 hours at 4°C. Each sample was washed once with Triton lysis buffer, followed by 3 washes with Triton lysis buffer supplemented with 500 mM NaCl. For FLAG-LRS and FLAG-GATOR1 an additional wash was preformed and samples were incubated in Triton lysis buffer supplemented with 500mM NaCl for 30 min. Finally samples were washed 4 times with CHAPS buffer (40 mM Hepes pH 7.4, 0.3% CHAPS) supplemented with 2.5 mM $MgCl_2$ for GTPase purifications. FLAG-tagged proteins were eluted from the FLAG-M2 affinity gel with a competing FLAG peptide for 1 hour as described above. Proteins were subsequently purified on a HiLoad 16/60 Superdex 200 FPLC column (GE) pre-equilibrated with CHAPS buffer supplemented with 150 mM NaCl. The peak corresponding to the desired complex was concentrated with 10,000 MW CO columns (Amicon), snap frozen in CHAPS buffer supplemented with 10% glycerol and stored at -80°C. In some Rag GAP assays, FLAG-GATOR1 and FLAG-LRS were not further purified by FPLC and instead stored in CHAPS buffer supplemented with 10% glycerol immediately following elution with FLAG peptide. The FPLC-purified and -non-purified GATOR1 had very similar levels of GAP activity towards RagB.

In Vitro Binding Assays

For the binding reactions, 20 µl of a 50% slurry containing immobilized HA-GST-tagged proteins were incubated in binding buffer (1% Triton X-100, 2.5 mM $MgCl_2$, 40 mM HEPES [pH 7.4], 2 mM DTT, and 1 mg/ml BSA) with 2 µg of FLAG-GATOR1 in a total volume of 50 µl for 1 hr and 30 min at 4°C. To terminate binding assays, samples were washed three times with 1 ml of ice-cold binding buffer supplemented with 300 mM NaCl followed by the addition of 50 µl of sample buffer.

Rag GTP hydrolysis assays

14 μg of the indicated Rag heterodimers or Rap2a were incubated for 2 hours at 4°C with 20 μl of FLAG-M2 affinity gel prewashed in CHAPS loading buffer (4 mM HEPES pH 7.4, 30 mM NaCl, 0.3% CHAPS). The resin was then washed 3 times with CHAPS loading buffer to remove unbound protein. The GTPases were loaded in 100 μl CHAPS loading buffer containing 0.1 μM XDP or 0.1 μM XTP, 70 pmols of the specified radioactive GTP species ([α-^{32}P]GTP for TLC assays or [γ-^{32}P]GTP for phosphate capture assays), 2 mM DTT, 0.01 μg/μl BSA, and 5 mM EDTA at 25°C for 10 minutes. Following nucleotide loading, $MgCl_2$ was added to a final concentration of 20 mM and the GTPases were incubated overnight at 4°C. The GTPases were washed 6 times with GTPase wash buffer (4 mM HEPES pH 7.4, 5 mM $MgCl_2$, 20 mM NaCl, 0.3% CHAPS, 2 mM DTT, 0.01 μg/μl BSA) to remove unbound nucleotide. 30 μl of competing FLAG-peptide was then added and the GTPases were eluted from the affinity gel for 2 hours. Protein concentrations were determined prior to use.

For the TLC-based GTP hydrolysis assay, 5 pmols of the indicated Rag heterodimer or Rap2a loaded with xanthosine nucleotides and [α-^{32}P]GTP were added to 20 pmols Flag-LRS or Flag-GATOR1 in 45 μl of GTPase wash buffer. The reaction was incubated at 25°C for the indicated time. The assay was terminated upon addition of 5 μl of 6X Elution Buffer (6.7 mM GTP, 6.7 mM GDP, 100 mM EDTA, 2% SDS) followed by further incubation for 5 minutes at 65°C at 1,400 rpm. Chloroform was added to separate the nucleotides from denatured proteins and the sample was spun at 13,200 rpm in a microcentrifuge for 1 min to separate the aqueous and organic phases. 30 μl of the aqueous layer was removed. Samples were spotted on a PEI Cellulose TLC plates and developed for 2.5 hours in 0.5 M KH_2PO_4 pH 3.4. Plates were exposed to film and spot densities were quantified with Multi Gauge V2.2 (Fujifilm).

For the phosphate capture GTP hydrolysis assay, Flag-RagC^{D181N}-HA-RagB was loaded with [γ-^{32}P]GTP and XDP as described above. A total of 48 pmols of loaded Flag-RagC^{D181N}-HA-RagB was added to GTPase wash buffer containing Flag-LRS or FLAG-GATOR1 in a total volume of 140 μl and incubated at 25°C. At the indicated time points three aliquots of 10 μl were taken and quenched by addition of 500 μl of activated charcoal mixture (5% activated charcoal, Norit® (Sigma) in 50 mM NaH_2PO_4). This mixture was then vortexed and spun at 13200 rpm in a microcentrifuge for 10 minutes at 4°C. 375 μl of the supernatant was added to 3.5 ml of Optifluor scintillation fluid and free $^{32}P_i$ was measured using a TriCarb scintillation counter (Perkin Elmer).

Nucleotide Exchange Assays

These assays were essentially performed as described in (*5*). Briefly, 40 pmols of FLAG-RagB^{X}-HA-RagC or FLAG-RagC^{X}-HA-RagB were loaded with 2 μM of [^{3}H]GDP (25-50 Ci/mmol). The GTPase-[^{3}H]GDP were stabilized by addition of 20 mM $MgCl_2$ followed by a further incubation at 4°C for 12 hours. To initiate the GEF assay, 40 pmols of the indicated proteins were added along with 200 μM GTPγS and incubated at 25°C. Samples were taken every 2 minutes and spotted on nitrocellulose filters, which were washed with 2 ml of wash buffer (40 mM HEPES pH 7.4, 150 mM NaCl and 5 mM $MgCl_2$). Filter-associated radioactivity was measured using a TriCarb scintillation counter (Perkin Elmer).

Identification of *DEPDC5* and *NPRL2* genomic alterations in glioblastoma and

ovarian tumors
Mutations and chromosome alterations in the TCGA dataset of Glioblastoma and Serous Ovarian tumors are available at *cancergenome.nih.gov (6, 7)*. DEDPC5 and NPRL2 mutation identification came from analysis of exome sequencing data and copy number alternations were based on Affiymatrix SNP 6.0 microarray data. Tumors chosen for analysis had point mutations or focal deletions encompassing all or a portion of either DEPDC5 or NPRL2. Loss of heterozygosity, biallelic inactivation, hemizygous and homozygous deletions in these tumors were determined using the ABSOLUTE algorithm *(8)*.

Identification of GATOR1-null cancer cell lines
To identify cancer cell lines null for GATOR1 components we searched the following publically available databases: Cancer Cell Line Encyclopedia (CCLE) (http://www.broadinstitute.org/ccle/home) and Cancer Genome Project (CPG) (http://www.sanger.ac.uk/cgi-bin/genetics/CGP/conan/search.cgi). GATOR1 null cell lines were identified in CCLE based on a value <-4 when sorted by deletion of the indicated GATOR1 genes. GATOR1-null cells were identified at CGP based on copy number analysis using CONAN. *NPRL3*-null cells were verified by immunoblotting for the Nprl3 protein. *DEPDC5*- and *NPRL2*-null cells were verified by genomic PCR as follows. Genomic DNA from cancer cells was extracted using the QiAmp DNA Mini kit Blood and Tissue (Qiagen) and used in PCR reactions with the gene-specific primers listed below. Runx2 is a positive control.
Runx2_exon6_fwd: CGCATTCCTCATCCCAGTATG
Runx2_exon6_rev: AAAGGACTTGGTGCAGAGTTCAG
DEPDC5_intron7_fwd: CCAAGCAACTAAAGCACAACCCAA
DEPDC5_intron7_rev: CAGGCTTCCTGACCCTGGATAC
DEPDC5_intron12_fwd: TGGGCCATCTGCTGTACTGAC
DEPDC5_intron12_rev: CAGAAGAGCTCTCATGGTTCCTGG
DEPDC5_intron24_fwd: AGTGACTTTCCTTTCAAGCCATCCT
DEPDC5_intron24_rev: CCTTAGCACAGTGCCTAGAGTTCA
DEPDC5_intron29_fwd: TGAAGCTCAGGGATGACGTGC
DEPDC5_intron29_rev: AATCAGGCGTCACAAAGCTACCA
NRPL2_intron1_fwd: GCTCCCAATGTGGCAGGGAA
NRPL2_intron1_rev: TCACCTTCTGTGGGACCTGGA
NRPL2_exon8_fwd: CTGATCCCTGGCACCCACAG
NRPL2_exon8_rev: CCAATGAGGTCTCGCACGGT
NRPL2_intron11_fwd: GAGCTGGATGAGCGGCTTGA
NRPL2_intron11_rev: AGGAGGGACTACCCACAGCA

Rapamycin sensitivity assays
The indicated cancer cell lines were seeded in 96 well plates (Corning). Rapamycin, (0.4 pM – 4 μM), was added 24 hours post-seeding, and DMSO was used as the control. Cell viability was measured 4 days after drug addition with CellTiter Glo luminescent viability assay (Promega). Readings were normalized to the untreated cells and IC_{50} values calculated with Prism 5 (Graphpad). All assays were performed in triplicate.

Cell proliferation assays

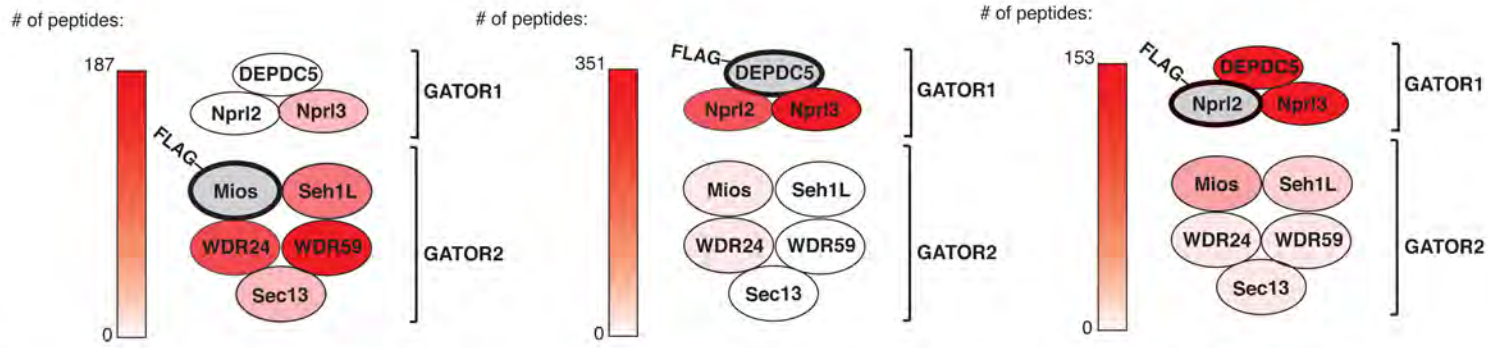
On day minus 1 of the assay, 1,000,000 MRKNU1 cells were electroporated using a Lonza Nucleofector II following the manufacturers recommendation (Buffer L, program L029) with pRK5-based cDNA expression plasmids in the following amounts: 2.5 µg of FLAG-Metap2 and 2.5 µg of Empty pRK5; 5 µg FLAG-DEPDC5. On day 0, 40,000 electroporated cells were seeded in a 12-well in 2 mL of the appropriate media. Cell numbers were counted on subsequent days. At each time point, assays were repeated 3-9 times.

A

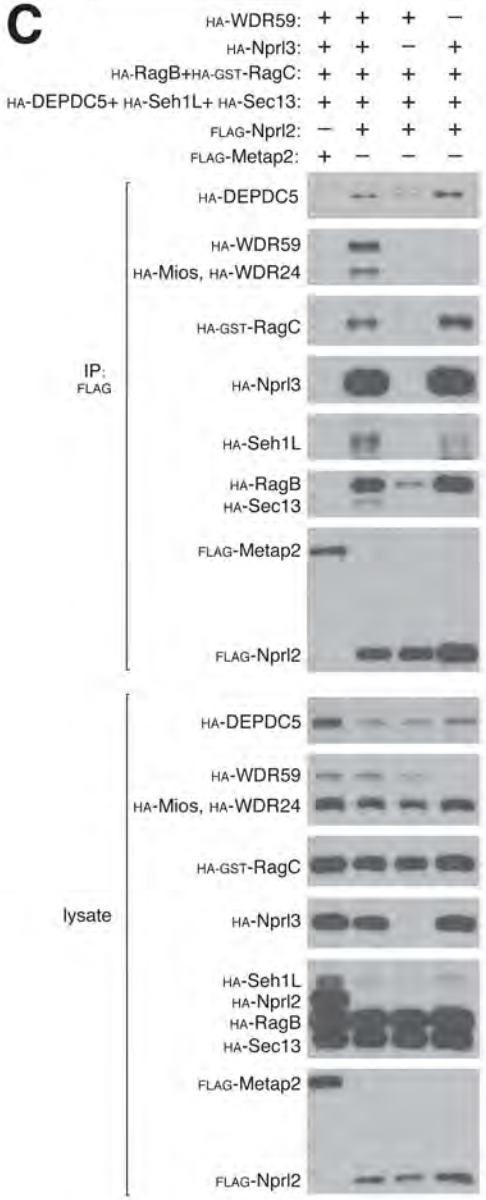
Proteins in FLAG-RagB IP

	Protein	Peptide count
Ragulator	p18	217
	MP1	38
	p14	86
	HBXIP	68
	C7orf59	16
v-ATPase	V-type proton ATPase subunit G1	9
	V-type proton ATPase subunit E1	18
	Mios	6

B



C



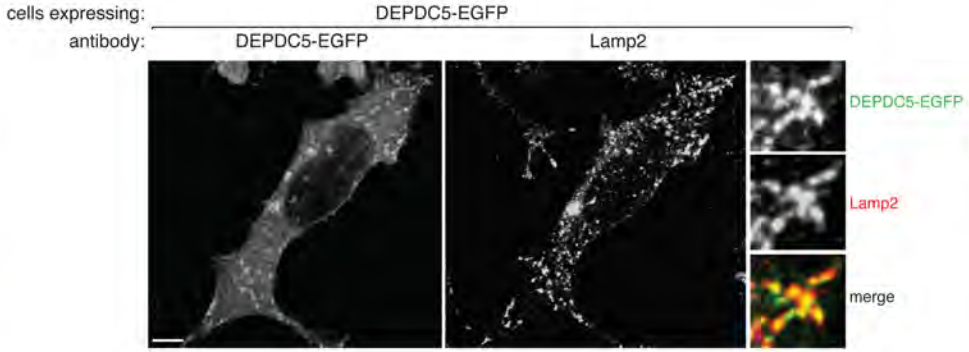
D

IP: FLAG-Mios

IP: FLAG-DEPDC5

		Peptide count	
		RagA	RagC
IP: FLAG	FLAG-DEPDC5	45	30
	FLAG-WDR24	2	0

E



F

IP: FLAG-Nprl2

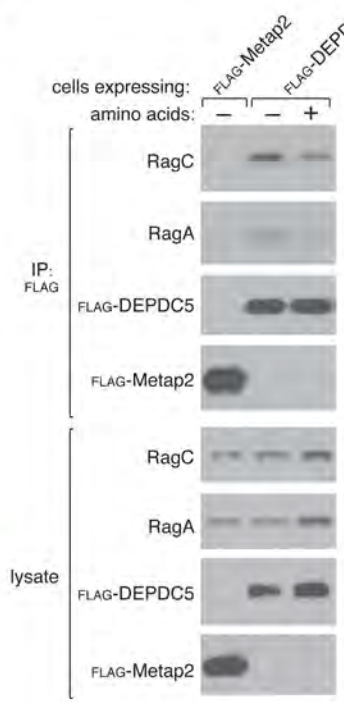
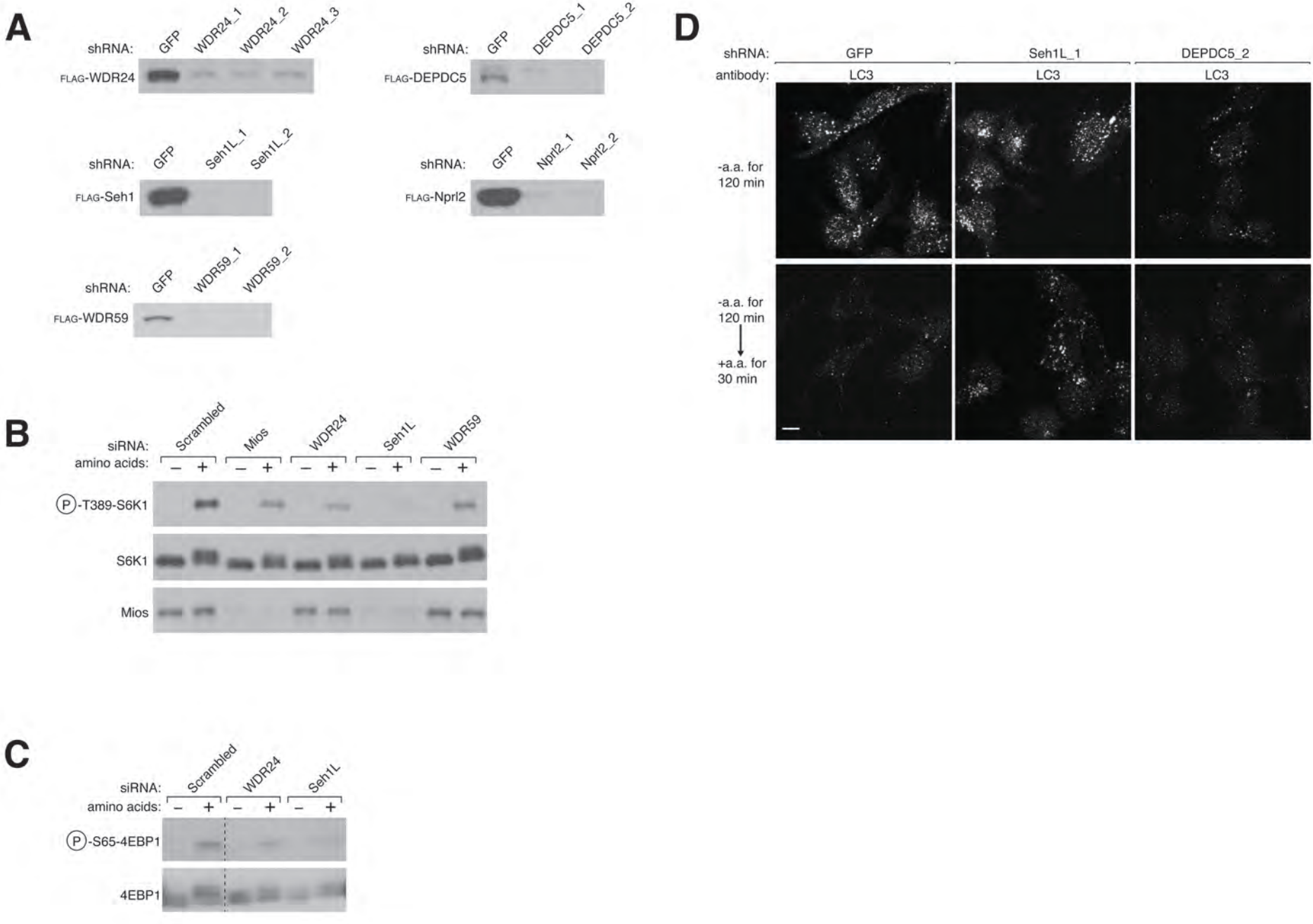


Fig. S1. The octomeric GATOR complex is composed of two distinct subcomplexes. (**A**) Table summarizing peptide counts of proteins that co-immunoprecipitate with FLAG-RagB. HEK-293T cells stably expressing FLAG-RagB were treated with a chemical cross-linker and cell lysates were subject to FLAG-immunoprecipitation followed by mass spectrometry analysis of co-immunoprecipitated proteins. (**B**) Cartoon summarizing peptide counts from mass spectrometric analyses of anti-FLAG immunoprecipitates from HEK-293T cells expressing FLAG-Mios (left), FLAG-DEPDC5 (center) and FLAG-Nprl2 (right). GATOR subunits are color-coded according to their peptide counts. (**C**) The GATOR-Rag interaction is primarily mediated by the GATOR1 subcomplex. HEK-293T cells were transfected with the indicated cDNAs in expression vectors. Cell lysates were prepared and subjected to FLAG immunoprecipitation (IP) followed by immunoblotting for indicated proteins. (**D**) Table of peptide counts for the RagA and RagC proteins that co-immunoprecipitate with FLAG-DEPDC5 or FLAG-WDR24. HEK-293T cells stably expressing FLAG-tagged proteins were processed as described in (**A**). (**E**) DEPDC5 localizes to the lysosomal surface. Images of HEK-293T cells stably expressing DEPDC5-EGFP (green) and immunostained for Lamp2 (red). In all images, insets show selected fields that were magnified five times and their overlays. Scale bar represents 10 μM. (**F**) The GATOR1-Rag interaction is regulated by amino acids. HEK-293T cells stably expressing FLAG-tagged DEPDC5 were starved of amino acids for 2 hours or starved and restimulated for 10 minutes. Cell lysates were prepared and subjected to an anti-FLAG immunoprecipitation, which was analyzed as in (**C**).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WDR24_3
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shRNA:
GFP
DEPDC5_1
DEPDC5_2
FLAG-DEPDC5
shRNA:
GFP
Seh1L_1
Seh1L_2
FLAG-Seh1
shRNA:
GFP
Nprl2_1
Nprl2_2
FLAG-Nprl2
shRNA:
GFP
WDR59_1
WDR59_2
FLAG-WDR59
B
siRNA:
Scrambled
Mios
WDR24
Seh1L
WDR59
amino acids:
– + – + – + – + – +
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S6K1
Mios
C
siRNA:
Scrambled
WDR24
Seh1L
amino acids:
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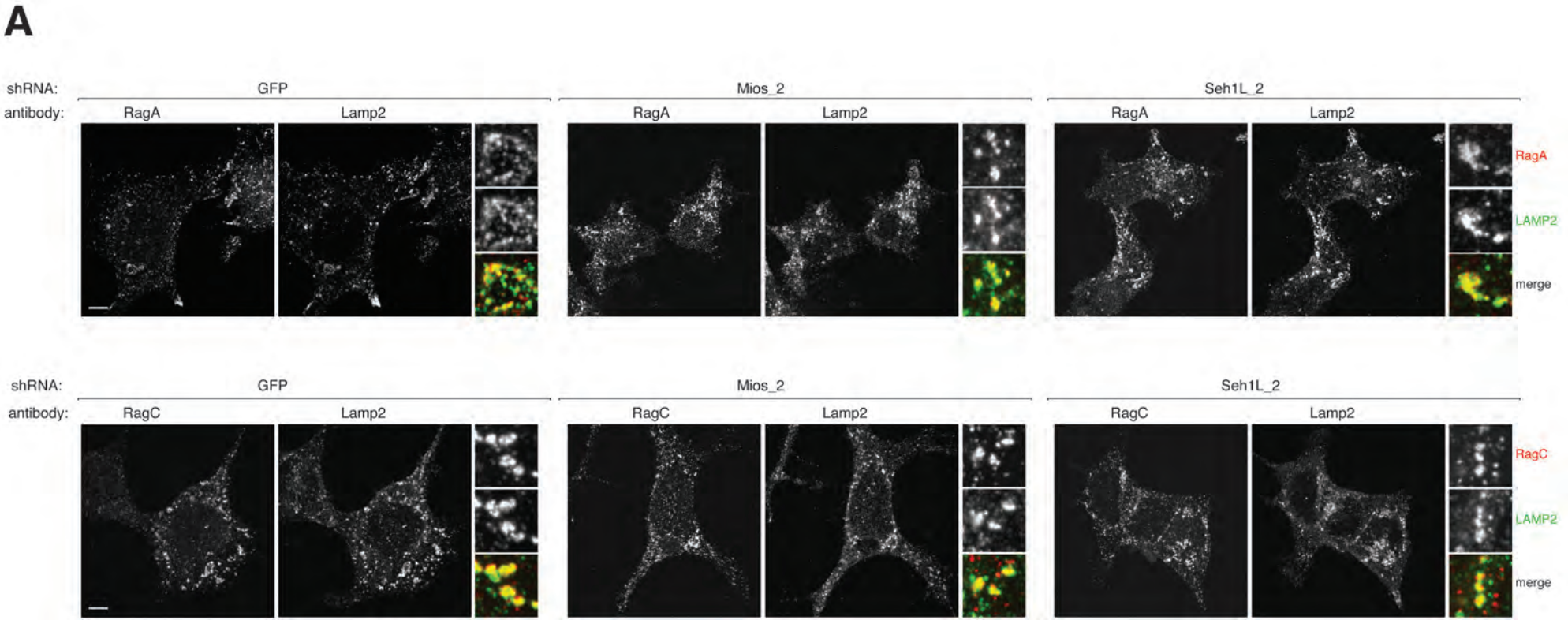
shRNA:
GFP
Seh1L_1
DEPDC5_2
antibody:
LC3
LC3
LC3
-a.a. for
120 min
-a.a. for
120 min
+a.a. for
30 min

Fig. S2. Depletion of GATOR components regulates multiple mTORC1-dependent outputs. (**A**) Validation of shRNAs targeting GATOR components. HEK-293T cells were transfected with indicated cDNAs and corresponding shRNAs in expression vectors. Cell lysates were analyzed by immunoblotting to determine the extent of recombinant protein depletion by the indicated shRNAs. (**B-C**) siRNA mediated depletion of GATOR2 components indicates that these proteins are necessary for amino-acid induced activation of mTORC1. HEK-293T cells treated with the indicated siRNAs were starved of amino acids for 50 min or starved and restimulated with amino acids for 10 min. Cell lysates were prepared followed by immunoblotting to detect the levels of the indicated proteins. (**D**) The GATOR complex regulates autophagy. Images of HEK-293T cells, expressing the indicated shRNAs targeting Seh1L or DEPDC5, starved for amino acids for 2 hours or starved and restimulated for 30 minutes, were analyzed for LC3 puncta formation. Scale bar represents 10 μM.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merge
shRNA:
GFP
Mios_2
Seh1L_2
antibody:
RagC
Lamp2
RagC
Lamp2
RagC
Lamp2
RagC
LAMP2
merge

Fig. S3 GATOR2 is not required for the localization of the Rag GTPases to the lysosomal surface. (**A**) Images of HEK-293T cells, expressing the indicated shRNAs targeting Mios or Seh1L, were co-immunostained for RagA or RagC (red) and Lamp2 (green). In all images, insets show selected fields that were magnified five times and their overlays. Scale bar represents 10 μM.

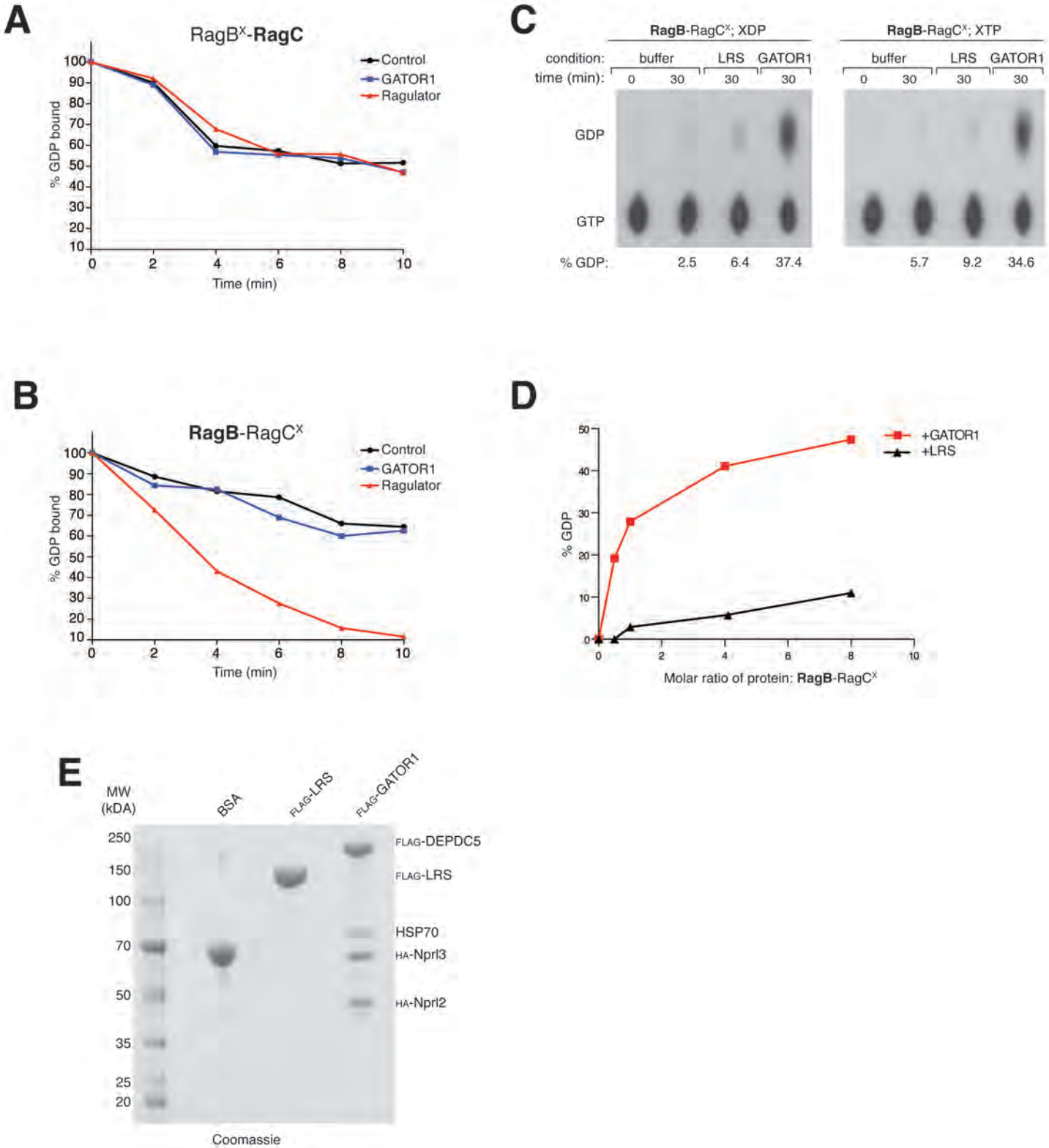
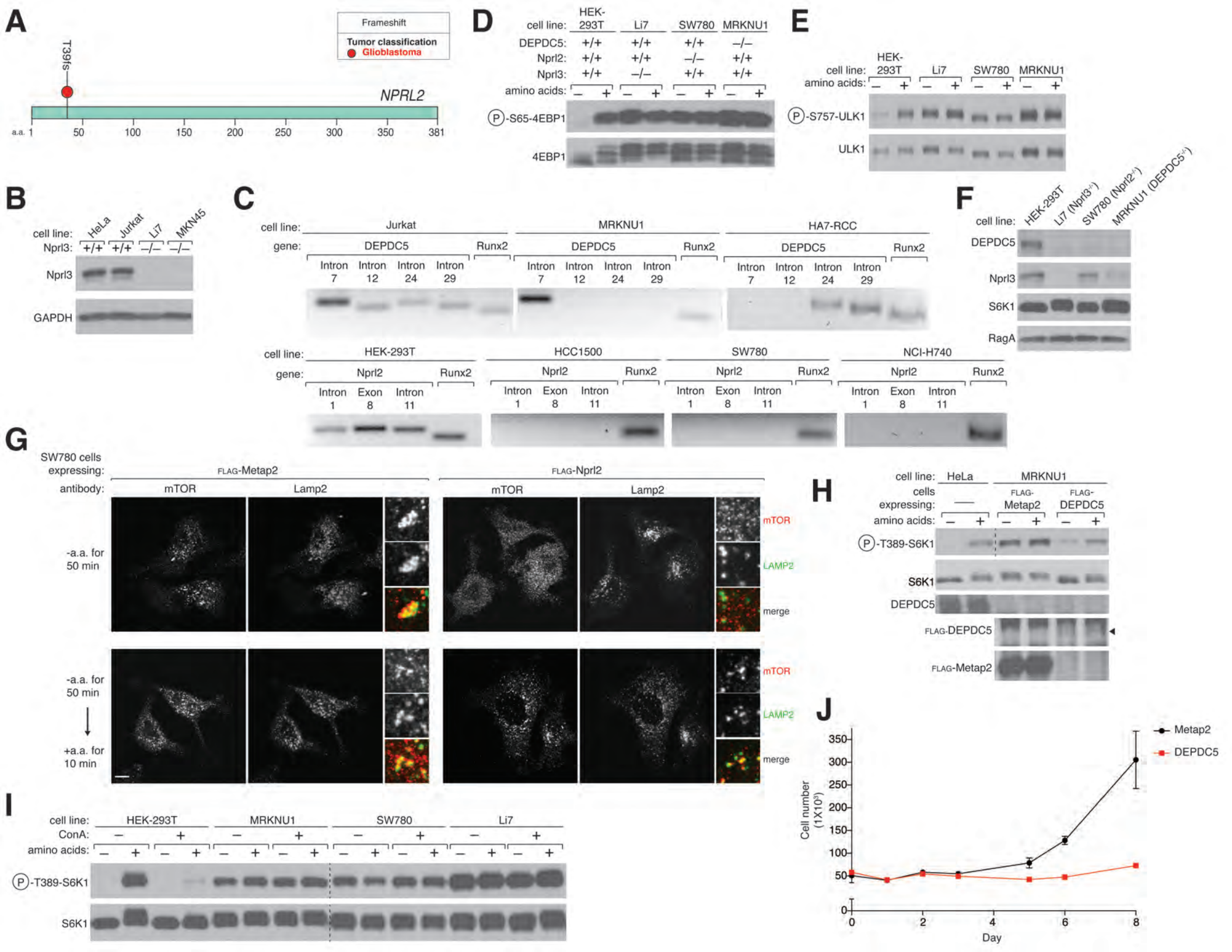

A
RagB^X-RagC
Control
GATOR1
Ragulator
% GDP bound
Time (min)
B
RagB-RagC^X
Control
GATOR1
Ragulator
% GDP bound
Time (min)
C
RagB-RagC^X; XDP
RagB-RagC^X; XTP
condition:
buffer
LRS
GATOR1
time (min):
GDP
GTP
% GDP:
2.5
6.4
37.4
5.7
9.2
34.6
D
+GATOR1
+LRS
% GDP
Molar ratio of protein: RagB-RagC^X
E
MW (kDA)
BSA
FLAG-LRS
FLAG-GATOR1
FLAG-DEPDC5
FLAG-LRS
HSP70
HA-Nprl3
HA-Nprl2
Coomassie

Fig. S4. GATOR1 does not promote nucleotide dissociation from RagB or RagC. (**A**) GATOR1 does not alter GDP dissociation by RagC. GDP dissociation assay, in which RagC-RagB^{X} was loaded with [^{3}H]GDP and incubated with GATOR1, Ragulator, or a control. Dissociation was monitored by a filter-binding assay. Each value represents the normalized mean for n=2. (**B**) GATOR1 does not alter GDP dissociation by RagB. RagB-RagC^{X} was loaded with [^{3}H]GDP, incubated with GATOR1, Ragulator, or a control protein and analyzed as in (**A**). Each value represents the normalized mean for n=2. (**C**) The nucleotide-bound state of RagC does not alter GATOR1-GAP activity for RagB. RagB-RagC^{X} was loaded with [a-^{32}P] GTP and XDP or XTP and incubated with GATOR1 or a control protein. GTP hydrolysis was determined by thin layer chromatography. (**D**) GATOR1 stimulates GTP hydrolysis of RagB in a dose dependent manner. RagB-RagC^{X} was loaded with [a-^{32}P]GTP and incubated with the indicated molar amount of GATOR1 or a control protein. GTP hydrolysis was determined as described in (**C**). (**E**) Coomassie blue stained SDS-PAGE of purified LRS and GATOR1. Identity of indicated proteins was validated by immunoblotting. HSP70, which co-purifies with GATOR1, does not stimulate RagB-mediated GTP hydrolysis.


A
Frameshift
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Glioblastoma
T39fs
NPRL2
a.a. 1
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100
150
200
250
300
350
381
B
cell line: HeLa Jurkat Li7 MKN45
Nprl3: +/+ +/+ –/– –/–
Nprl3
GAPDH
C
cell line: Jurkat MRKNU1 HA7-RCC
gene: DEPDC5 Runx2
Intron 7 Intron 12 Intron 24 Intron 29
cell line: HEK-293T HCC1500 SW780 NCI-H740
gene: Nprl2 Runx2
Intron 1 Exon 8 Intron 11
D
cell line: HEK-293T Li7 SW780 MRKNU1
DEPDC5: +/+ +/+ +/+ –/–
Nprl2: +/+ +/+ –/– +/+
Nprl3: +/+ –/– +/+ +/+
amino acids: – + – + – + – +
P-S65-4EBP1
4EBP1
E
cell line: HEK-293T Li7 SW780 MRKNU1
amino acids: – + – + – + – +
P-S757-ULK1
ULK1
F
cell line: HEK-293T Li7 (Nprl3-/-) SW780 (Nprl2-/-) MRKNU1 (DEPDC5-/-)
DEPDC5
Nprl3
S6K1
RagA
G
SW780 cells expressing: FLAG-Metap2 FLAG-Nprl2
antibody: mTOR Lamp2 mTOR Lamp2
-a.a. for 50 min
+a.a. for 10 min
mTOR
LAMP2
merge
H
cell line: HeLa MRKNU1
cells expressing: — FLAG-Metap2 FLAG-DEPDC5
amino acids: – + – + – +
P-T389-S6K1
S6K1
DEPDC5
FLAG-DEPDC5
FLAG-Metap2
I
cell line: HEK-293T MRKNU1 SW780 Li7
ConA: – + – + – + – +
amino acids: – + – + – + – + – + – + – + – +
P-T389-S6K1
S6K1
J
Cell number (1X10³)
350
300
250
200
150
100
50
0
0 2 4 6 8
Day
Metap2
DEPDC5

Fig. S5. GATOR1 re-expression in GATOR1-null cells restores amino acid-dependent regulation of mTORC1. (**A**) Mutation found in *NPRL2* in a glioblastoma tumor. (**B**) Immunoblot for the levels of Nprl3 in indicated cancer cell lines. (**C**) Genomic PCR for indicated *DEPDC5* and *NPRL2* regions from various cancer cell lines. Primers were designed to amplify the indicated intronic or exonic regions of the specified genes and PCR products were resolved by gel electrophoresis. Runx2 is a positive control. (**D-E**) mTORC1-dependent translation control and autophagy are insensitive to amino acid deprivation in GATOR1-null cells. The indicated cancer cell lines were starved of amino acids for 2 hours or starved and restimulated with amino acids for 15 min. Cell lysates were prepared and immunoblotting used to detect levels of the indicated proteins. (**F**) Cells missing a GATOR1 component have reduced expression of remaining GATOR1 proteins. Immunoblot shows levels of Nprl3 and DEPDC5 in the indicated cell lines. (**G**) Re-introduction of Nprl2 into SW780 (*NPRL2*$^{-/-}$) cells restores the amino acid-dependent localization of mTORC1. Images of SW780 cells stably expressing FLAG-Nprl2 or a control protein were starved of or starved of and restimulated with amino acids for the indicated times. Cells were co-immunostained for mTOR (red) and Lamp2 (green). In all images, insets show selected fields that were magnified five times and their overlays. Scale bar represents 10 μM. (**H**) Re-expression of DEPDC5 in MRKNU1 (*DEPDC5*$^{-/-}$) cells restores amino acid-dependent regulation of mTORC1. MRKNU1 cells stably expressing DEPDC5 or a control protein were treated and analyzed as in (**D**). (**I**) v-ATPase inhibition does not inhibit mTORC1 activity in GATOR1-null cells. The indicated cancer cell lines were deprived of amino acids for 4 hours in the presence or absence of 2.5 μM Concanamycin A (ConA) and then stimulated with amino acids for 15 minutes. Cell lysates were prepared and analyzed as described in (**D**). (**J**) Re-introduction of DEPDC5 into MRKNU1 (*DEPDC5*$^{-/-}$) cells inhibits their proliferation. Number of MRKNU1 cells expressing DEPDC5 or a control protein was determined on indicated days. Values are presented as mean ± SD (n = 3-9).

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