Supplementary Information for

Coordination of the leucine-sensing Rag GTPase cycle by leucyl-tRNA synthetase in the mTORC1 signaling pathway

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SI Materials and Methods

Cell culture. WiDr, HCT-15, SW620, SW480, DLD-1, HCT-116, HT-29, LS1034, LoVo, NCI-H508, COLO205, LS174T (Human colon cancer cells), FHC (Normal colon epithelial cells), HCC1569, HC70, HCC1395, MDM-MB-231, HCC1937 (breast cancer cells) NIH-OVCAR3R, UWB1.289, SKOV-3, SNU49, OAOV-3 (ovarian cancer cells), T98G, U343, U8754, A172, SNB19 (*glioblastoma multiforme*), AsPc-1, BxPC-3, Panc10.05, Panc-1, MIA-Paca-2 (pancreatic cancer cells) and HEK 293 cells were purchased from American Type Culture Collection (ATCC). FHC cells, colon cancer cells, breast cancer cells, ovarian cancer cells, glioblastoma multiforme and pancreatic cancer cells were cultured in RPMI and HEK293T cells were cultured in DMEM. All cells were cultured in appropriated medium supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin-streptomycin at 37°C in a 5% CO_2. Cells were stably expressing Myc-LRS or LRS shRNA were generated by Tet-on inducible lentiviral system. Cells were selected with puromycin (1 μg/ml). Cells were transfected using either Turbofect (Invitrogene) or Lipofectamine (Invitrogene).

Lentiviral infection for experimental model. The SMART choice inducible lentivirus, which encodes shRNA against LRS (VSH6367-220936786, 5'-CTGGACATCACTTGTTTCT-3') or scrambled control, were purchased from Thermo Scientific. SW620 cells were infected with these lentiviruses in the presence of 8 μg/ml polybrene (Sigma-Aldrich). Infection was performed within serum free RPMI, and then complete medium was replaced in the presence of 1 μg/ml puromycin. To generate the Tet-on inducible LRS expression system, Myc-LRS clone was subcloned to pLVX-TetOne vector. HEK-293T cells were transfected with myc-LRS plasmid and lenti-X HTX packing mix with Xfect Polymer. After transfection, cellular debris was removed through a 0.45 μm filter. SW620 cells were infected with collected lentivirus in the presence of 8 μg/ml polybrene. After 5 hr, SW620 cells were selected with complete RPMI medium in the presence of 1 μg/ml puromycin.

Amino acids or leucine starvation and stimulation of cells. For amino acids or leucine starvation, cells were incubated in all amino acid-free or leucine-free RPMI for indicated time after cells were rinsed with amino acid-free RPMI. For re-stimulation, cells were incubated with all amino acids or leucine-containing RPMI for indicated time.

***In vivo* GTPase assay.** *In vivo* GTPase assay was done as previously described (13). Briefly, cells were washed with phosphate-free DMEM two times and further incubated with phosphate-free DMEM for 60 min. After labeling with 100 μCi of [^{32}P]phosphate/ml for 8 hr, cells were lysed with lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.5% NP-40, protease inhibitor cocktail) for 30 min on ice. Cell lysates were centrifuged at 12,000 X g for 15 min at 4°C. To quench GAP activity, the supernatant (160 μl) was mixed with 500 mM NaCl (16 μl). HA-RagD, HA-RagB or HA-ARF1 was immunoprecipitated with anti-HA antibody for 1 hr at 4°C. HA-RagD was also immunoprecipitated with anti-HA antibody after the transfection of si-Con, si-LRS or si-Folliculin or treatment of BC-LI-0186 for 1 hr at 4°C. The beads were washed with buffer 1 (50 mM Tris, pH 8.0, 500 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5% Triton X-100) three times at 4°C and then washed with buffer 2 (50 mM Tris, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100) three times at 4°C. The HA-RagD- or HA-ARF1-bound nucleotides were eluted with 20 μl of buffer 3 (2 mM EDTA, 0.2% sodium dodecyl sulfate, 1 mM GDP, 1 mM GTP) at 85°C for 3 min. The eluted nucleotides were separated onto polyethyleneimine (PEI) cellulose plates (Baker-flex) in 0.75 M KH_2PO_4[pH 3.4] solution. GTP and GDP was visualized and quantified by a phosphoimager.

GTP-agarose bead pull down assay. After cells were rinsed in ice-cold PBS, cells were collected in GTP-binding buffer (20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 2 mM PMSF, 20 μg/mL leupeptin, 10 μg/mL aprotinin, 150 mM NaCl and 0.1% Triton-X100). Cells were lysed by sonication for 15 sec. The lysates were then centrifuged at 13,000 x g for 10 min at 4°C, and the supernatant was collected. Protein extracts were incubated with 100 μl GTP-agarose beads (Sigma-Aldrich, cat no. G9768) in a

total of 500 µL of GTP-binding buffer for 30 min at 4°C. Beads were then washed with GTP binding buffer, and the supernatant was retained. The retained supernatant was incubated with washed beads for another 30 min. The beads were washed again, then incubated with the retained supernatant overnight at 4°C. After washing five times with GTP binding buffer, GTP-bounded protein extracts were eluted with 2x sample buffer. GTP bounded protein was visualized by performing immunoblot analysis

Immunoblot analysis. After boiling proteins at 95°C for 5 min in Laemmli sample buffer for denaturization, proteins were separated by sodium dodecyl sulfatepolyarylamide. The acquired gel was then transferred to nitrocellulose membrane. The membranes were incubated with antibodies in 1% TTBS buffer (10 mM Tris-HCl, pH7.5, 150 mM NaCl, 0.05%, Tween20) after blocking in 5% skimmed milk solution, followed by incubation of the membranes with secondary antibody, which was coupled to horseradish peroxidase. Finally, membranes were processed and scanned using chemiluminescence kit (ATTO).

Flow Cytometry. To measure cell size, the BD FACSAria^{TM} III cytometer with software was used. Cells were washed once with PBS, transferred to 15 mL conical tubes, centrifuged for 5 min at 1,000 rpm, and then cell pellets were fixed by adding 70% ethanol. For analysis on the flow cytometer, the fixed cells were centrifuged at 1000 rpm for 5 min, washed with PBS and then incubated at 37°C for 1 hr in 5 ng/ml propidium iodide in the presence of RNase. The mean of FSC of G1 phase cells was determined by performing fluorescence-activated cell sorting (FACS) analysis.

Cell growth and viability assays. SW620 cells stably expressing red fluorescence were designed by using CellPlayer^{TM} NucLight Red (Essen BioScience, Cat no. 4476). Red SW620 cells were seeded in 96 well plates, incubated for 24 hr. Phase and red fluorescence images were acquired every 2 hr

using IncuCyte™ Zoom (Essen BioScience). Red fluorescence was counted to monitor cell viability. Quantitative analysis was performed using IncuCyte™ Zoom basic analyzer. Red object count/mm^2 over time was used to quantify number of cell viability and analyzed by GraphPad Prism tools.

Human CRC tissues and immunohistochemistry. This study was carried out according to the provisions of the Helsinki Declaration of 1975 and was reviewed and approved by the Institutional Review Board of Severance Hospital (IRB-3-2014-0287) with a waiver of informed consent. TMA blocks were made with formalin fixed paraffin embedded tissues of 116 patients who underwent curative resection for stage II colorectal cancer between June 2004 and December 2010. For each patient, TMA cores were obtained from malignant infiltrating tumor with paired normal colonic mucosa (cutting edge of surgical excision beyond 5 cm from the cancer areas) after precise review of their corresponding haematoxylin and eosin stained slides of donor blocks. The criteria for inclusion were stage II patients after radical resection, and patients who had undergone 5-FU based adjuvant chemotherapy. The patients with distant metastases at preoperative staging, microscopic cancer invasion on the surgical margins (including radial resection margin), any preoperative anti-cancer treatments (including preoperative radiotherapy), and patients treated with adjuvant chemotherapy including oxaliplatin or irinotecan-based regimen or radiotherapy were excluded. All tumors resected from mice were fixed with 4% formaldehyde in PBS and embedded in paraffin. Paraffin-embedded specimens were deparaffinized in xylene, subjected to heat-mediated antigen-retrieval in 10 mM sodium citrate citrate (pH 6.0), permeabilized in 0.2% Triton X-100 and blocked in 5% donkey sera. The primary antibodies used in this study were the same as those used in the western blotting analysis (1:50), followed by incubation with peroxidase-coupled anti-mouse IgG, or anti-rabbit IgG, amplified with AB reagent and detected using DAB reagent. Images were acquired using a Zeiss microscope. To score a tumor cell as positive, both cytoplasmic and nuclear staining was counted. For the quantitative analysis, a Histo score (H score) was calculated based on the staining intensity and percentage of stained cells using the Aperio ScanScope systems. The intensity score was defined as follows: 0, no appreciable staining in cells; 1, weak staining in cells comparable with stromal cells; 2,

intermediate staining; 3, strong staining. The fraction of positive cells was scored as 0%–100%. The H score was calculated by multiplying the intensity score and the fraction score, producing a total range of 0–300. Tissue sections were examined and scored separately by two independent investigators blinded to the clinicopathologic data.

Oncomine database. The expression level of *LARS* in cancer and normal cells was analyzed using Oncomine database (https://www.oncomine.org/resource/login.html). Briefly, the *P* value for statistical significance was set up as 1E-4, while the fold change was defined as 2 and the gene rank was defined as top 10%. The reporter ID and platform used in the current study were as follows: Skrzypczak Colorectal 2, reporter: 222428_s_at, platform: Human Genome U133 Plus 2.0 Array/ Skrzypczak Colorectal 2, reporter: 222427_s_at, platform: Human Genome U133 Plus 2.0 Array/ TCGA Colorectal, reporter: A_24_P51360, platform: not pre-defined/ Pyeon Multi-cancer, reporter: 222427_s_at, platform: Human Genome U133 Plus 2.0 Array/ Riker Melanoma, reporter: 223888_s_at, platform: Human Genome U133 Plus 2.0 Array.

TCGA database analysis. The number of samples included in this TCGA dataset at the time of this analysis was 433. Log2 normalized counts were imported into GeneSpring GX V12.1 (Agilent Technologies). Baseline transformation was set as the mean for all samples. Only genes that expressed higher than median in at least one sample were selected for downstream analysis. The GeneSpring Volcano Plot function was used for statistical test of genes between primary tumor and normal samples. Statistical test parameters were as following: selected test, moderated *t*-test; p-value computation, Asymptotic; multiple testing correction, Benjamini-Hochberg. The GeneSpring heatmap function was used to generate the heatmap of *LARS* and *MTOR* pathway genes.

Figure S1.

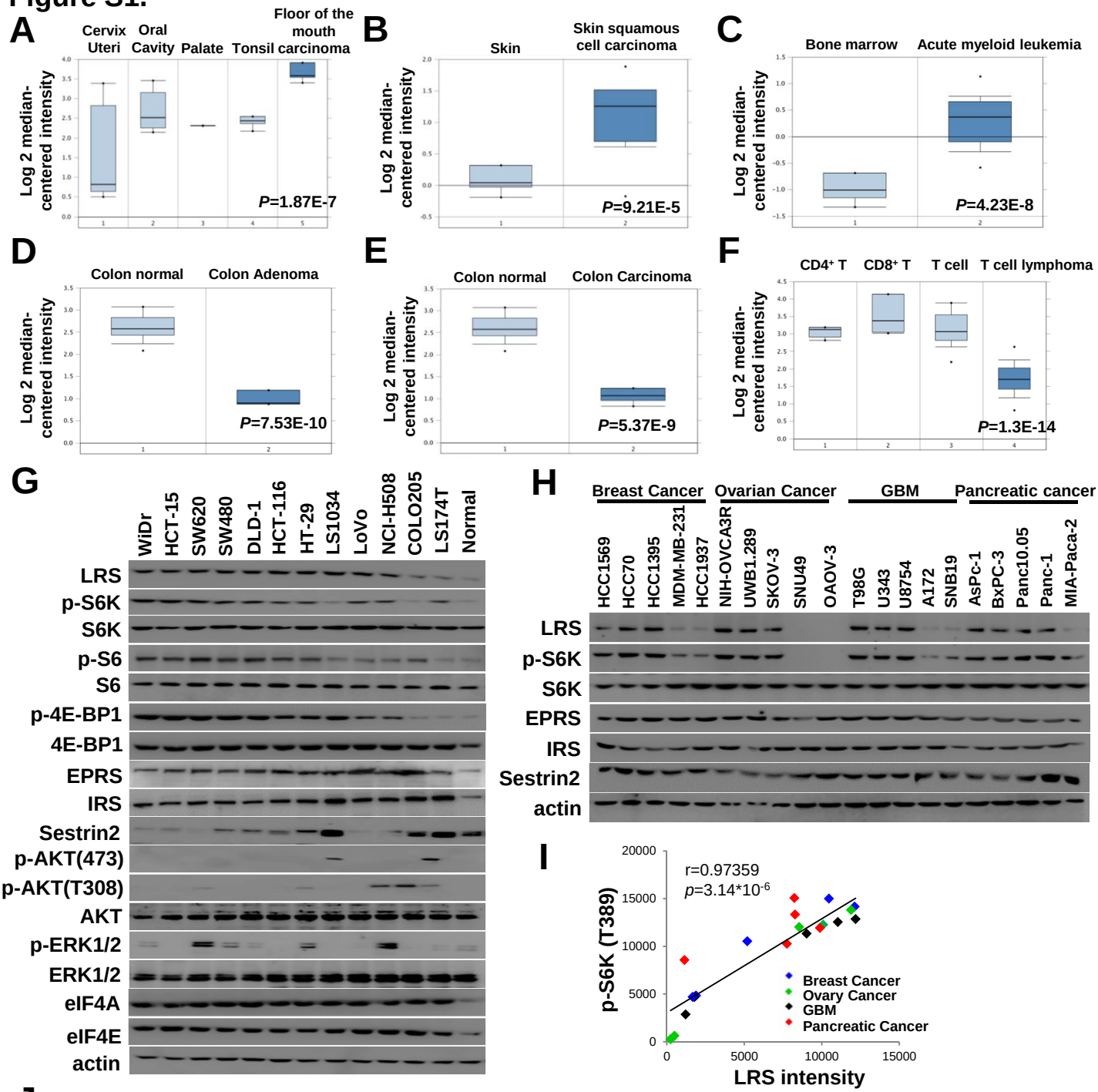

A
Cervix Uteri
Oral Cavity
Palate
Tonsil
Floor of the mouth carcinoma
Log 2 median-centered intensity
P=1.87E-7
B
Skin
Skin squamous cell carcinoma
Log 2 median-centered intensity
P=9.21E-5
C
Bone marrow
Acute myeloid leukemia
Log 2 median-centered intensity
P=4.23E-8
D
Colon normal
Colon Adenoma
Log 2 median-centered intensity
P=7.53E-10
E
Colon normal
Colon Carcinoma
Log 2 median-centered intensity
P=5.37E-9
F
CD4+ T
CD8+ T
T cell
T cell lymphoma
Log 2 median-centered intensity
P=1.3E-14
G
WiDr
HCT-15
SW620
SW480
DLD-1
HCT-116
HT-29
LS1034
LoVo
NCI-H508
COLO205
LS174T
Normal
LRS
p-S6K
S6K
p-S6
S6
p-4E-BP1
4E-BP1
EPRS
IRS
Sestrin2
p-AKT(473)
p-AKT(T308)
AKT
p-ERK1/2
ERK1/2
eIF4A
eIF4E
actin
H
Breast Cancer
Ovarian Cancer
GBM
Pancreatic cancer
HCC1569
HCC70
HCC1395
MDM-MB-231
HCC1937
NIH-OVCA3R
UWB1.289
SKOV-3
SNU49
OAOV-3
T98G
U343
U8754
A172
SNB19
AsPc-1
BxPC-3
Panc10.05
Panc-1
MIA-Paca-2
LRS
p-S6K
S6K
EPRS
IRS
Sestrin2
actin
I
r=0.97359
p=3.14*10-6
p-S6K (T389)
LRS intensity
Breast Cancer
Ovary Cancer
GBM
Pancreatic Cancer
J
Cancer patients: 10 9 2 11 12 3 6 7 4 5 8 1 13
N T N T N T N T N T N T N T N T N T N T N T N T N T
RagD
RagB
GTP-agarose pull down
LRS
p-S6K
S6K
RagD
RagB
Sestrin2
actin
Cell lysate

Fig. S1. Correlation of overexpressed LRS with hyperactive mTORC1 in several types of cancer. *(A-C)* The box plots show that the expression level of *LARS* is up-regulated in floor of the mouth carcinoma (*A*, P=1.87E^{-7}), skin squamous cell carcinoma (*B*, P=9.21E^{-5}), and acute myeloid leukemia (*C*, P=4.23E^{-8}). *LARS* data extracted from oncomine database and expressed as log2 median-centered intensity. *(D-F)* The box plots show that the expression level of *SESN2* is down-regulated in colon adenoma (*D*, P=7.53E^{-10}), colon carcinoma (*E*, P=5.37E^{-9}), and T cell lymphoma (*F*, P=*1.30*E^{-14}). *SESN2* data extracted from oncomine database and expressed as log2 median-centered intensity. *(G)* Cellular levels of LRS and S6K phosphorylation were determined by immunoblotting with their specific antibodies in 12 colon cancer cells and normal colon epithelial cell. *(H)* Cellular levels of LRS and S6K phosphorylation were determined by immunoblotting with their specific antibodies in several cancer cells including breast cancer, ovarian cancer, *glioblastoma multiforme (GBM), and pancreatic cancer cells. (I)* Correlation between cellular levels of LRS and S6K phosphorylation is displayed as a scattered plot and evaluated by a Pearson correlation coefficient. *(J)* Normal or tumor tissues from human CRC patients were analyzed by GTP pull down assay or immunoblotting with their specific antibodies.

Figure S2.

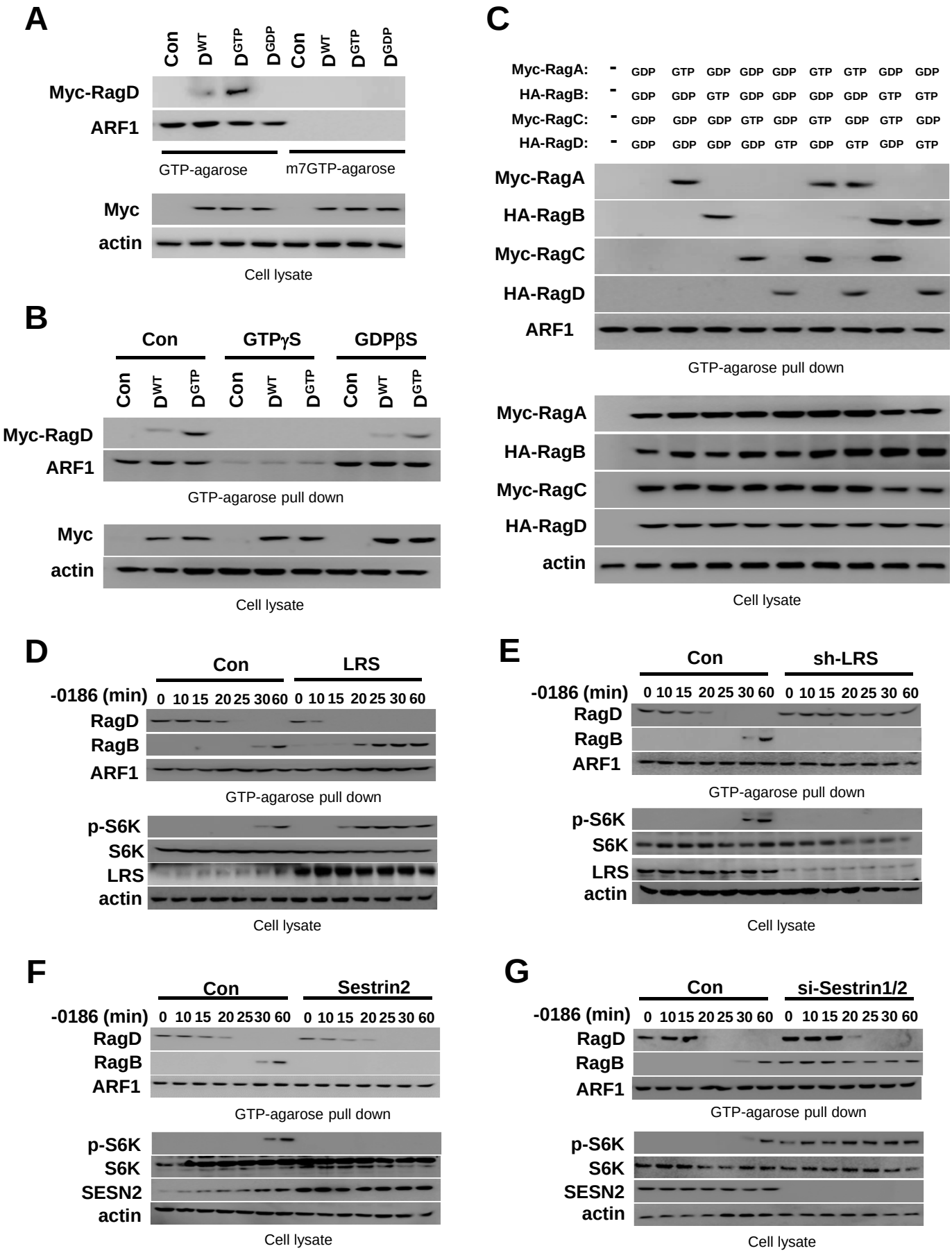

A
Con
D^WT
D^GTP
D^GDP
Con
D^WT
D^GTP
D^GDP
Myc-RagD
ARF1
GTP-agarose
m7GTP-agarose
Myc
actin
Cell lysate
B
Con
GTPγS
GDPβS
Con
D^WT
D^GTP
Con
D^WT
D^GTP
Con
D^WT
D^GTP
Myc-RagD
ARF1
GTP-agarose pull down
Myc
actin
Cell lysate
C
Myc-RagA: - GDP GTP GDP GDP GDP GTP GTP GDP GDP
HA-RagB: - GDP GDP GTP GDP GDP GDP GDP GTP GTP
Myc-RagC: - GDP GDP GDP GTP GDP GTP GDP GTP GDP
HA-RagD: - GDP GDP GDP GDP GTP GDP GTP GDP GTP
Myc-RagA
HA-RagB
Myc-RagC
HA-RagD
ARF1
GTP-agarose pull down
Myc-RagA
HA-RagB
Myc-RagC
HA-RagD
actin
Cell lysate
D
Con
LRS
-0186 (min)
0 10 15 20 25 30 60 0 10 15 20 25 30 60
RagD
RagB
ARF1
GTP-agarose pull down
p-S6K
S6K
LRS
actin
Cell lysate
E
Con
sh-LRS
-0186 (min)
0 10 15 20 25 30 60 0 10 15 20 25 30 60
RagD
RagB
ARF1
GTP-agarose pull down
p-S6K
S6K
LRS
actin
Cell lysate
F
Con
Sestrin2
-0186 (min)
0 10 15 20 25 30 60 0 10 15 20 25 30 60
RagD
RagB
ARF1
GTP-agarose pull down
p-S6K
S6K
SESN2
actin
Cell lysate
G
Con
si-Sestrin1/2
-0186 (min)
0 10 15 20 25 30 60 0 10 15 20 25 30 60
RagD
RagB
ARF1
GTP-agarose pull down
p-S6K
S6K
SESN2
actin
Cell lysate

Fig. S2. Distinct roles of LRS and Sestrin2 in Rag GTPase cycle. *(A)* Human SW620 cells were transfected with control (Con), Myc-RagD^{WT}, -RagD^{GTP} or -RagD^{GDP}. After 24 hr, cell lysates were incubated with GTP- or m^{7}GTP-conjugated agarose beads. The proteins precipitated with the beads were visualized by immunoblotting. *(B)* SW620 cells were transfected with control (Con), Myc-RagD^{WT}, or -RagD^{GTP}. After 24 hr, cell lysates were incubated with GTP-conjugated agarose beads in the presence of 100 μM GTPγS or GDPβS. The proteins precipitated with the beads were analyzed by immunoblotting with anti-Myc or ARF1 antibody. *(C)* SW620 cells were transfected with control, RagX^{GTP} or RagX^{GDP} (X=A, B, C and D). After 24 hr, cell lysates were incubated with GTP-conjugated agarose beads. The proteins precipitated with the beads were analyzed by immunoblotting with anti-Myc and -HA antibody. *(D)* SW620 cells harboring DOX-inducible LRS were untreated (Con) or treated with DOX for 72 hr (LRS). Cells were incubated with 20 μM BC-LI-0186 for 90 min, and then deprived of BC-LI-0186 for the indicated times. Cell lysates were incubated with GTP-conjugated agarose beads and the proteins precipitated with the beads were analyzed by immunoblotting with the indicated antibodies. *(E)* SW620 cells harboring DOX-inducible sh-LRS were untreated (Con) or treated with DOX for 72 hr (sh-LRS). Cells were incubated with 20 μM BC-LI-0186 for 90 min, and then deprived of BC-LI-0186 for the indicated times. Cell lysates were incubated with GTP-conjugated agarose beads and the proteins precipitated with the beads were analyzed by immunoblotting with the indicated antibodies. *(F)* SW620 cells were transfected with control or Sestrin2 cDNA for 24 hr. Cells were incubated with 20 μM BC-LI-0186 for 90 min, and then deprived of BC-LI-0186 for the indicated times. Cell lysates were incubated with GTP agarose beads and the proteins precipitated with the beads were analyzed by immunoblotting with the indicated antibodies. *(G)* SW620 cells were transfected with si-control (Con) or si-Sestrin1/2 for 48 hr. Cells were incubated with 20 μM BC-LI-0186 for 90 min, and then deprived of BC-LI-0186 for the indicated times. Cell lysates were incubated with GTP agarose beads and the proteins precipitated with the beads were analyzed by immunoblotting with the indicated antibodies.

Figure S3.

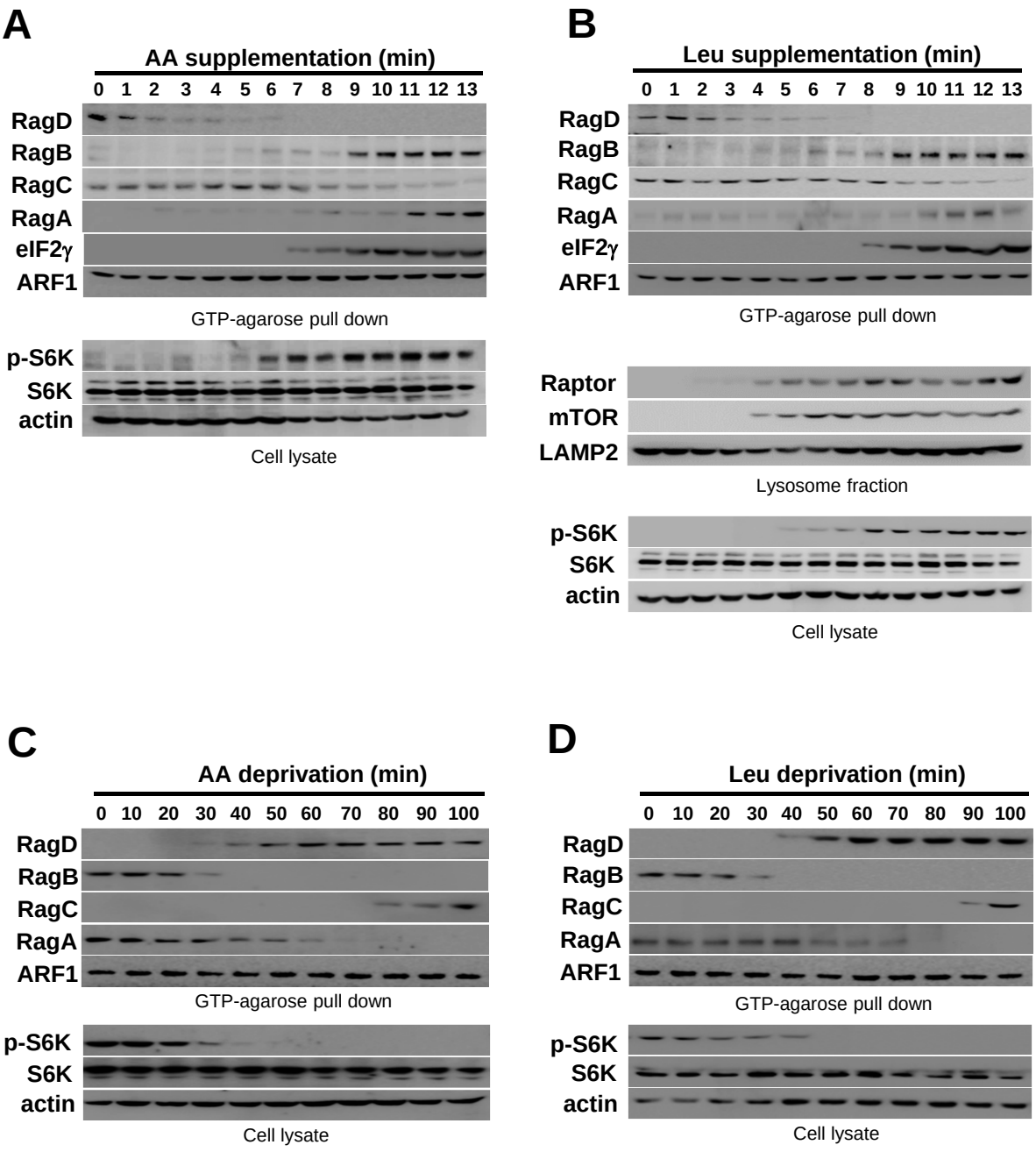
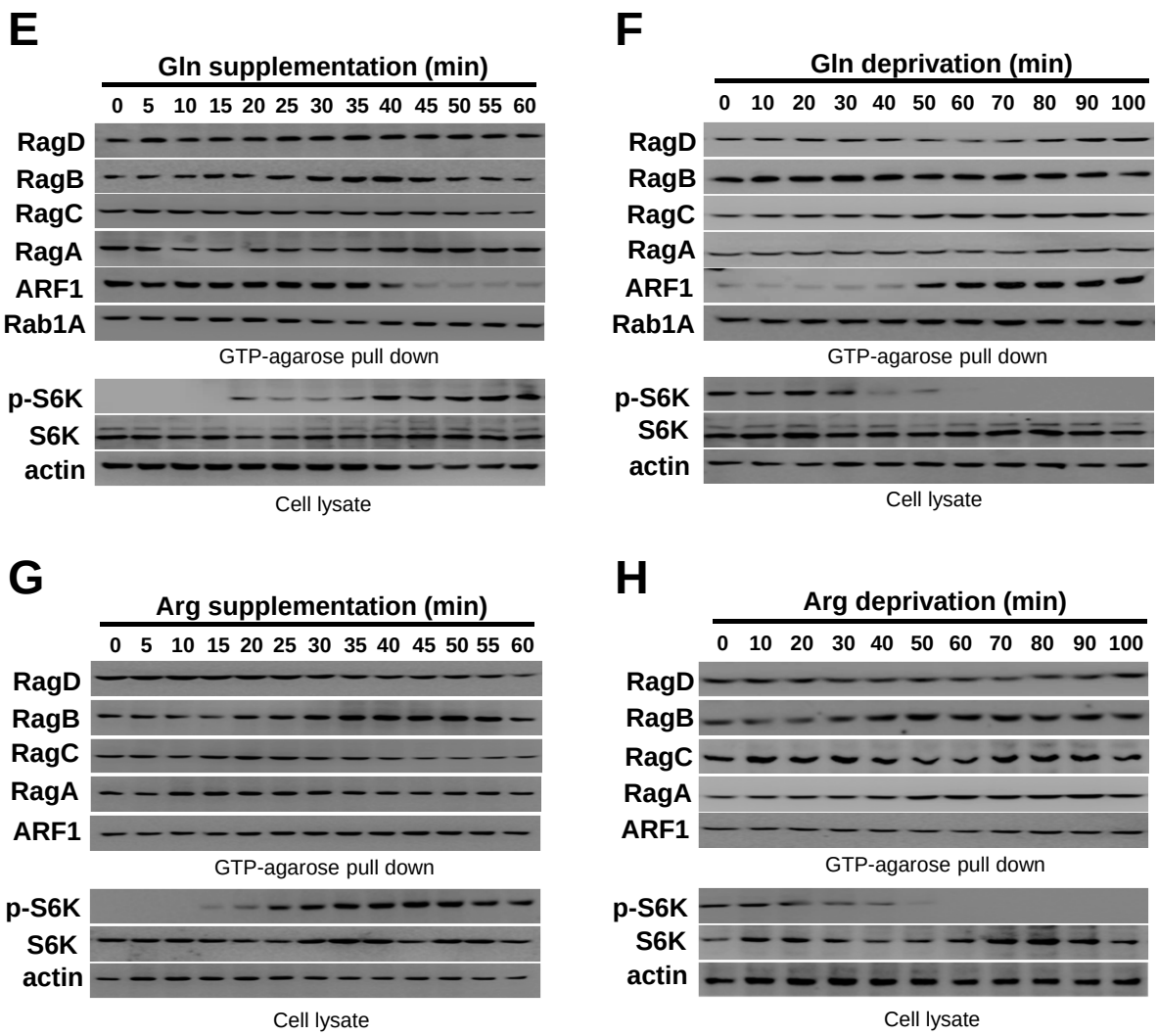

A
AA supplementation (min)
0 1 2 3 4 5 6 7 8 9 10 11 12 13
RagD
RagB
RagC
RagA
eIF2γ
ARF1
GTP-agarose pull down
p-S6K
S6K
actin
Cell lysate
B
Leu supplementation (min)
0 1 2 3 4 5 6 7 8 9 10 11 12 13
RagD
RagB
RagC
RagA
eIF2γ
ARF1
GTP-agarose pull down
Raptor
mTOR
LAMP2
Lysosome fraction
p-S6K
S6K
actin
Cell lysate
C
AA deprivation (min)
0 10 20 30 40 50 60 70 80 90 100
RagD
RagB
RagC
RagA
ARF1
GTP-agarose pull down
p-S6K
S6K
actin
Cell lysate
D
Leu deprivation (min)
0 10 20 30 40 50 60 70 80 90 100
RagD
RagB
RagC
RagA
ARF1
GTP-agarose pull down
p-S6K
S6K
actin
Cell lysate

Figure S3.


E
Gln supplementation (min)
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RagD
RagB
RagC
RagA
ARF1
Rab1A
GTP-agarose pull down
p-S6K
S6K
actin
Cell lysate
F
Gln deprivation (min)
0 10 20 30 40 50 60 70 80 90 100
RagD
RagB
RagC
RagA
ARF1
Rab1A
GTP-agarose pull down
p-S6K
S6K
actin
Cell lysate
G
Arg supplementation (min)
0 5 10 15 20 25 30 35 40 45 50 55 60
RagD
RagB
RagC
RagA
ARF1
GTP-agarose pull down
p-S6K
S6K
actin
Cell lysate
H
Arg deprivation (min)
0 10 20 30 40 50 60 70 80 90 100
RagD
RagB
RagC
RagA
ARF1
GTP-agarose pull down
p-S6K
S6K
actin
Cell lysate

Fig. S3. Kinetics of Rag GTPase cycle during amino acid signaling. *(A-B)* Human SW620 cells were starved of amino acids *(A)* or leucine *(B)* for 90 min and re-stimulated with amino acids or leucine for 13 min. At 1 min intervals, cells were harvested and cell lysates were incubated with GTP-conjugated agarose beads. The proteins precipitated with the beads were analyzed by immunoblotting with the indicated antibodies. *(C-D)* SW620 cells were starved of amino acids *(C)* and leucine *(D)* for 100 min. At 10 min intervals, cells were harvested and cell lysates were incubated with GTP-conjugated agarose beads and the proteins precipitated with the beads were analyzed by immunoblotting with the indicated antibodies. *(E-F)* SW620 cells were starved of glutamine for 100 min and re-stimulated with glutamine for 60 min *(E),* or starved of glutamine for 100 min *(F).* At the indicated intervals, cells were harvested and cell lysates were incubated with GTP-conjugated agarose beads and proteins precipitated with the beads were analyzed by immunoblotting with the indicated antibodies. *(G-H)* SW620 cells were starved of arginine for 100 min and re-stimulated with arginine for 60 min *(G),* or starved for arginine for 100 min *(H).* At the indicated intervals, cells were harvested and cell lysates were incubated with GTP-conjugated agarose beads and proteins precipitated with beads were analyzed by immunoblotting with the indicated antibodies.

Figure S4.

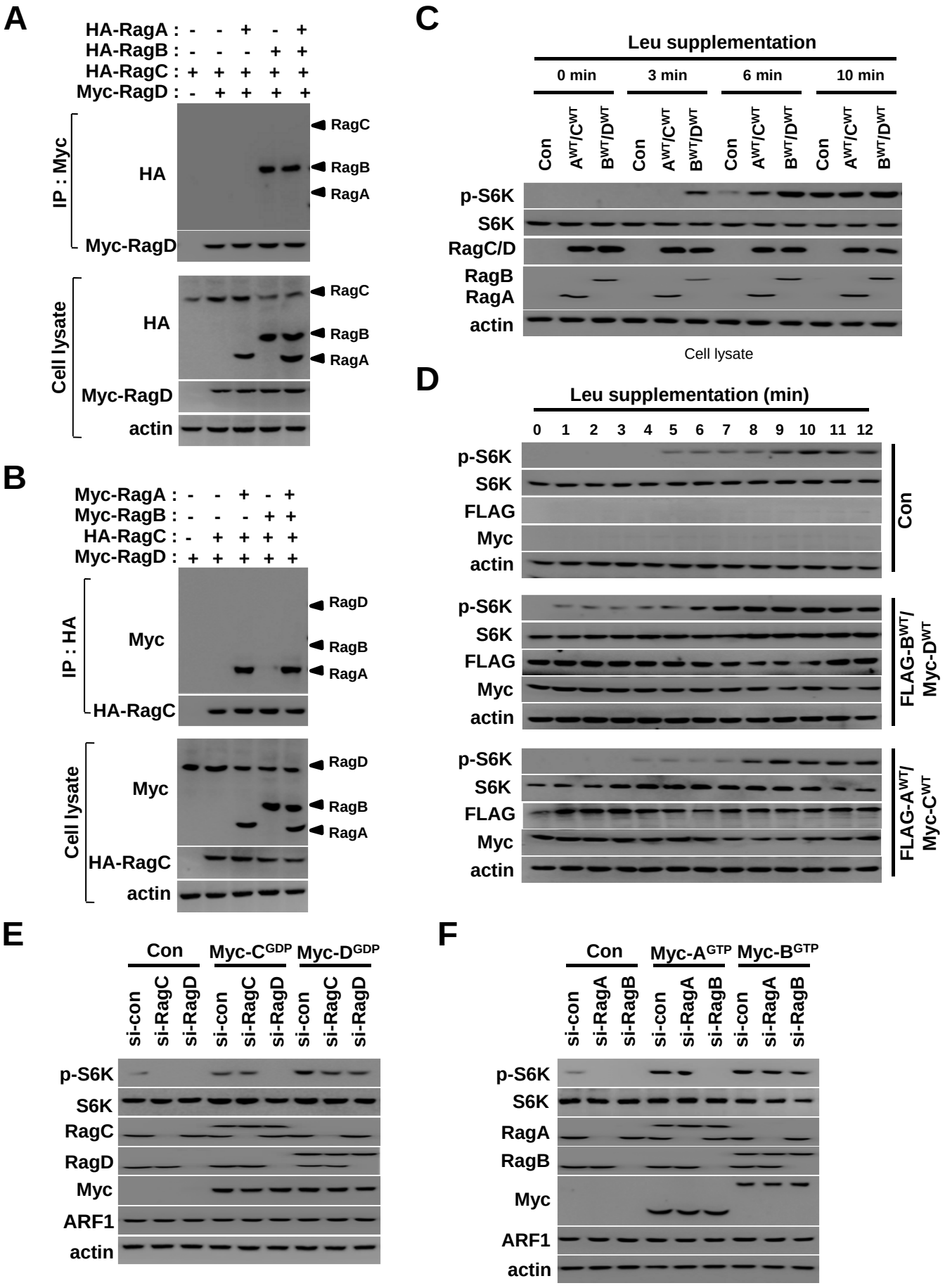
A
HA-RagA : - - + - +
HA-RagB : - - - + +
HA-RagC : + + + + +
Myc-RagD : - + + + +
IP : Myc
HA
Myc-RagD
RagC
RagB
RagA
Cell lysate
HA
Myc-RagD
actin
RagC
RagB
RagA
B
Myc-RagA : - - + - +
Myc-RagB : - - - + +
HA-RagC : - + + + +
Myc-RagD : + + + + +
IP : HA
Myc
HA-RagC
RagD
RagB
RagA
Cell lysate
Myc
HA-RagC
actin
RagD
RagB
RagA
C
Leu supplementation
0 min
3 min
6 min
10 min
Con
A^WT/C^WT
B^WT/D^WT
p-S6K
S6K
RagC/D
RagB
RagA
actin
Cell lysate
D
Leu supplementation (min)
0 1 2 3 4 5 6 7 8 9 10 11 12
p-S6K
S6K
FLAG
Myc
actin
Con
FLAG-B^WT/ Myc-D^WT
FLAG-A^WT/ Myc-C^WT
E
Con
Myc-C^GDP
Myc-D^GDP
si-con
si-RagC
si-RagD
p-S6K
S6K
RagC
RagD
Myc
ARF1
actin
F
Con
Myc-A^GTP
Myc-B^GTP
si-con
si-RagA
si-RagB
p-S6K
S6K
RagA
RagB
Myc
ARF1
actin

Fig. S4. RagD/B heterodimer dominantly affects mTORC1 activity. *(A)* SW620 cells transfected with HA-RagA, -B, -C, and Myc-RagD were lysed and immunoprecipitated with anti-Myc antibody. The precipitated proteins were analyzed by immunoblotting with anti-HA antibody. *(B)* SW620 cells transfected with Myc-RagA, -B, -D, and HA-RagC were lysed and immunoprecipitated with anti-HA antibody. The precipitated proteins were analyzed by immunoblotting with anti-Myc antibody. *(C)* SW620 cells were transfected with FLAG-RagA^{WT}/Myc-RagC^{WT} or FLAG-RagB^{WT}/Myc-RagD^{WT}. After 24 hr, cells were starved of leucine for 90 min and re-stimulated with leucine for 10 min. At the indicated time intervals, cells were harvested and cell lysates were analyzed by immunoblotting with indicated antibodies. *(D)* SW620 cells were transfected with FLAG-RagB^{WT}/Myc-RagD^{WT} or FLAG-RagA^{WT}/Myc-RagC^{WT}. After 24 hr, cells were starved for leucine for 90 min and re-stimulated with leucine for 12 min. At 1 min intervals, cells were harvested and cell lysates were analyzed by immunoblotting with indicated antibodies. *(E)* Control (Con), Myc-RagC^{GDP} or -RagD^{GDP}-transfected SW620 cells were treated with siRNA against RagC or RagD. After 48 hr, cells were harvested and cell lysates were subjected to immunoblotting with the indicated antibodies. *(F)* Control (Con), Myc-RagA^{GTP} or -RagB^{GTP}-transfected SW620 cells were treated with siRNA against RagA or RagB. After 48 hr, cells were harvested and cell lysates were subjected to immunoblotting with the indicated antibodies.

Table S1. Antibody information for western blot

Antibody	Source	Cat. No.	Dilution
RagA (D8B5) Rabbit mAb	Cell signaling	#4357	1:1000
RagB (D18F3) Rabbit mAb	Cell signaling	#8150	1:1000
Rabbit polyclonal anti-RRAGC	Bethyl laboratories	#A304-300A	1:1000
Rabbit polyclonal anti-RRAGD	Bethyl laboratories	#A304-301A	1:1000
Phospho-p70 S6 Kinase (Thr389)	Cell signaling	#9205	1:1000
P70 S6 Kinase	Cell signaling	#9202	1:1000
ARF1(ARFS 1A/5)	Santa Cruz Biotechnology	#sc-53168	1:1000
DEPDC5 (C-12)	Santa Cruz Biotechnology	#sc-86115	1:1000
c-Myc (9E10)	Santa Cruz Biotechnology	#sc-40	1:1000
Actin (I-19)	Santa Cruz Biotechnology	#sc-1616	1:1000
LAMTOR2/ROBLD3 (D7C10)	Cell signaling	#8145	1:1000
WDR24 (D-20)	Santa Cruz Biotechnology	#sc-244614	1:1000
Sestrin2 Polyclonal antibody	Proteintech	#10795-1-AP	1:2000
HA-probe (F-7)	Santa Cruz Biotechnology	#sc-7392	1:1000
Leucyl-tRNA synthetase	Neomics	#NMS-01-0007	1:3000
Glutamyl-prolyl tRNA synthetase	Neomics	#NMS-01-0004	1:3000
Isoleucyl-tRNA synthetase	Neomics	#NMS-01-0006	1:3000
Anti-FLAG (9A3) Mouse mAb	Cell signaling	#8146	1:1000
Phospho-4E-BP1 (Thr37/46)	Cell signaling	#9459	1:1000
4E-BP1	Cell signaling	#9452	1:1000
eIF4A	Cell signaling	#2013	1:1000
eIF4E	BD Transduction Laboratories	#610269	1:500
EIF2S3 (eIF2γ)	Abnova	#H00001968-M01	1:1000

Table S2. siRNA information

Target	Source	Identifier
Leucyl-tRNA synthetase (LRS)	ThermoFisher Scientific	(5'-3') CCAGGGUCAUUGUCGUGGAUUUGCA
Glutamyl-prolyl tRNA synthetase (EPRS)	ThermoFisher Scientific	(5'-3') CCAGCACUACCAGGUUAACUUUAAA
RRAGA	ThermoFisher Scientific	Cat# 1299001 Prod# 116473
RRAGB	ThermoFisher Scientific	Cat# 1299001 Prod# 115795
RRAGC	ThermoFisher Scientific	Cat# 1299001 Prod# 184652
RRAGD	ThermoFisher Scientific	Cat# 1299001 Prod# 116923
Sestrin1	ThermoFisher Scientific	Cat# 1299001 Prod# HSS120529
Sestrin2	ThermoFisher Scientific	Cat# 1299001 Prod# HSS130295
DEPDC5	ThermoFisher Scientific	Cat# 1299001 Prod# HSS114496
WDR24	ThermoFisher Scientific	Cat#1299001 Prod# HSS130743
LAMTOR2	ThermoFisher Scientific	Cat#1299001 Prod# HSS120728

Table S3. Reagent information

Reagent	Source	Cat. No.
Guanosine 5′-triphosphate–Agarose	Sigma-aldrich	# 9768
7-methyl-GTP Sepharose 4B	GE Healthcare	# 27-5025-01
Protein A/G plus-agarose	Santa Cruz Biotechnology	# sc-2003
Protein G-agarose	ThermoFisher Scientific	# 20399
Propidium iodide	Sigma-Aldrich	# P4170