SUPPLEMENTAL INFORMATION

Materials

Anti-p62 rabbit polyclonal antibody (BIOMOL Research Laboratories, Plymouth Meeting, PA), anti-TfR mouse monoclonal antibody (ZYMED Laboratories, San Francisco, CA), anti-β-actin mouse monoclonal antibody (Applied Biological Materials, Richmond, BC, Canada), anti-Lamp-1 rat monoclonal antibody (1D4b; BD Biosciences, San Jose, CA), anti-EGFR sheep polyclonal antibody (Fitzgerald Industries International, Concord, MA), anti-Rab11 mouse monoclonal antibody, anti-Akt mouse monoclonal antibody (BD Transduction Laboratories, Lexington, KY), and anti-Myc rabbit polyclonal antibody (Sigma-Aldrich, St. Louis, MO) were obtained commercially. Anti-mTOR antibody, anti-phospho p70S6K1 antibody, anti-p70S6K antibody, and anti-phospho Akt antibody were purchased from Cell Signaling Technology (Danvers, MA). HRP (horseradish peroxidase)-conjugated anti-HA tag mouse monoclonal antibody was from Roche Applied Science (Basel, Switzerland). HRP-conjugated anti-FLAG tag (M2) mouse monoclonal antibody was from Sigma-Aldrich. Alexa 488- and Alexa 594-conjugated secondary antibodies were from Invitrogen (Carlsbad, CA). E64-d and pepstatin A were purchased from Peptide Institute (Osaka, Japan). Bafilomycin A1 was purchased from Merck KGaA (Darmstadt, Germany). Phosphatase inhibitor cocktail 2 was purchased from Sigma-Aldrich. L-amino acids were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Plasmid construction

cDNAs encoding mouse PAT1 and PAT4 were amplified from Marathon-Ready adult mouse brain, liver, and testis cDNA (Clontech-Takara Bio, Shiga, Japan) by PCR with the following pairs of oligonucleotides with a *Bgl*II (or *Bam*HI) linker (underlined) or a stop codon (bold) as described previously [1]: 5'-AGATCTATGTCCACACAGAGGCTT-3' (Met primer, sense) and 5'-**TTA**TATGAAGGCACTGGT-3' (stop primer, antisense) for PAT1; and 5'-GGATCCATGGAAGCGCCGGCGCCG-3' (Met primer, sense) and 5'-**TTA**CAAACCACTTGTTAT-3' (stop primer, antisense) for PAT4. The cDNAs obtained were subcloned into the pCMV Myc-C1 vector or pMRX-IRES-puro-HA vector. The pMRX-IRES-puro-HA vector is a variant of the pMRX-IRES-puro vector (donated by Shoji Yamaoka, Tokyo Medical and Dental University, Tokyo, Japan) [2].

Cell culture, transfection, and infection

MEFs prepared from 13.5-day mouse embryos were transformed with pEF321-T, an SV40 large T antigen expression vector. The resulting immortalized MEF cell line (simply referred to as MEFs throughout this paper) was a generous gift of Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan). MEFs and COS-7 cells were maintained at 37°C in DMEM (Wako Pure Chemical Industries) containing 10% fetal bovine serum and antibiotics under 5% CO_2. Starved conditions were achieved by washing the cells once with HBSS (Sigma-Aldrich) and transferring them to HBSS for 2 hr for siRNA screening or for 1 hr for other experiments. Plat-E cells

were donated by Toshio Kitamura (University of Tokyo, Tokyo, Japan). Plat-E cell culture and retrovirus infection were performed essentially as described previously [3]. The cells were transfected with plasmid DNAs and siRNAs by using Lipofectamine 2000 and RNAiMAX (Invitrogen), respectively, each according to the manufacturer's instructions. Lysosomal proteases were inhibited by exposing cells to 100 nM E64-d and 100 μg/ml pepstatin A or 100 μM bafilomycin A1 for the times indicated in the legend to Supplementary Fig S6 online before the immunofluorescence analyses.

Immunoblotting

Cells were rinsed with ice-cold PBS (phosphate-buffered saline), scraped, and collected by centrifugation at 4°C. The cells were then lysed in 50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 1% Triton X-100, a phosphatase inhibitor cocktail (Sigma-Aldrich), and a protease inhibitor cocktail (Roche Applied Science), and total cell lysates were obtained by centrifugation at 15,000 ×*g* for 10 min at 4°C. The cell lysates were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The blots were blocked with 1% skim milk in PBS containing 0.1% Tween 20, and then incubated with the primary antibodies. Immunoreactive bands were detected by using horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich) and enhanced chemiluminescence (ECL). The intensity of the immunoreactive bands was quantified with the ImageJ software. The blots shown in this study are representative of three independent experiments.

Amino acid treatment

FLAG-Rab12-QL-overexpressing cells were treated with 0.8 mM Leu, 4 mM Gln, 0.4 mM Arg, 0.08 mM Trp, 0.1 mM Pro, or 0.1 mM Ala in the culture medium for 30 min, and cell lysates were immediately prepared and analyzed by immunoblotting as described above.

Preparation of total RNA and RT-PCR

Total RNA was prepared from MEFs with TRI reagent (Sigma-Aldrich), and reverse transcription was performed by using a ReverTra Ace-kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The following primers were used for GAPDH (forward primer, 5'-ATGGTGAAGGTCGGAGTCAA-3' and reverse primer, 5'-GCCATGTAGACCATGAGGTC-3'); for Rab12 (forward primer, 5'-GTCATCATCATCGGCTCCCG-3' and reverse primer, 5'-TCAACAGCATCGGACGTGTG-3'); and for PAT4 (forward primer, 5'-TGGCTGCCAGTCTTGTGATAATTTACCAGT-3' and reverse primer, 5'-TTACAAACCACTTGTTAT-3'). cDNAs were amplified by PCR using KOD DNA polymerase (Toyobo) with 25 cycles or 30 cycles (for PAT4) of denaturation at 98°C for 10 sec, annealing at 55°C for 0.5 min, and extension at 68°C for 1 min.

Cell surface biotinylation assays

Biotinylation assays were performed essentially as described previously [4]. In brief, MEFs stably expressing HA-PAT4 grown on 10-cm dishes were washed three times with

ice-cold PBS (+) (containing 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$, pH7.4) and incubated with 0.5 mg/ml Sulfo-NHS-LC-biotin (Pierce, Rockford, IL) in PBS (+) for 30 min on ice to biotinylate cell surface proteins. The reaction was quenched by washing the dishes twice with ice-cold PBS (+) containing 100 mM glycine and 0.3% (w/v) BSA, and the dishes were subsequently washed twice with PBS (+). The cells were then lysed in 1 ml of the lysis buffer described above, and the lysates were centrifuged at 15,000 ×*g* for 10 min at 4°C in a microcentrifuge to remove cellular debris and insoluble materials. The supernatant was incubated with streptavidin-agarose beads (Pierce) overnight at 4°C with constant rotation. The beads were then washed three times with the lysis buffer, twice with a high-salt wash buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 1% Triton X-100), and, finally, once with 50 mM Tris-HCl, pH 7.5. The proteins were eluted from the beads in 30 μl of the SDS sample buffer, separated by SDS-PAGE, and analyzed by immunoblotting with HRP-conjugated anti-HA tag antibody, anti-EGFR antibody, anti-TfR antibody, and anti-Rab11 antibody.

Supplementary references

1. Fukuda M, Kanno E, Mikoshiba K (1999) Conserved N-terminal cysteine motif is essential for homo- and heterodimer formation of synaptotagmins III, V, VI, and X. *J Biol Chem* **274:** 31421-31427

2. Saitoh T, Nakayama M, Nakano H, Yagita H, Yamamoto N, Yamaoka S (2003) TWEAK Induces NF-κB2 p100 processing and long lasting NF-κB activation. *J Biol Chem* **278:** 36005-36012

3. Morita S, Kojima T, Kitamura T (2000) Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther* **7:** 1063-1066

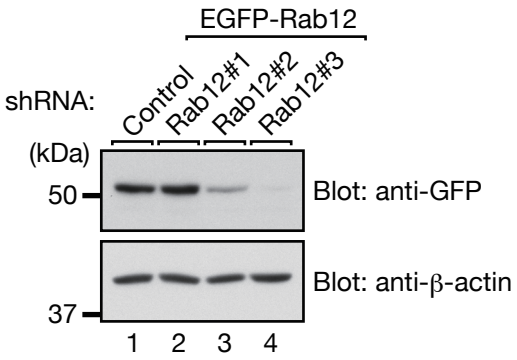
4. Shin HW, Kobayashi H, Kitamura M, Waguri S, Suganuma T, Uchiyama Y, Nakayama K (2005) Roles of ARFRP1 (ADP-ribosylation factor-related protein 1) in post-Golgi membrane trafficking. *J Cell Sci* **118:** 4039-4048

Supplementary Table S1.

The target sequences for each mouse *Rab* siRNA used in this study.

Name of Rab	Target sequence	Name of Rab	Target sequence
Rab1A	CAAGTTGTTGGTAGGGAAC	Rab19	CTTGGTCATCATGCTGATC
Rab1B	TTTGCAGACTCTCTGGGTG	Rab20	GATCCTGAAGTACAAGATG
Rab2A	GTACATCATCATCGGCGAC	Rab21	TTTACTACCGAGATTCGAA
Rab2B	GTCATGTCTCCTCCTTCAG	Rab22A	GACTGTCCAGTACCAAAAT
Rab3A	GGACAACATTAATGTCAAG	Rab22B	GGAGTACGCTGAATCCATA
Rab3B/D	CTTTGACTACATGTTCAAA	Rab23	CCTTAGACCTAACAAACAA
Rab3C	GTTGCTGATCATTGGCAAT	Rab24	AGTGGCTGAGGATTACGTC
Rab4A	ATTCAAAGATGACTCAAAT	Rab25	CAAAAGTGACCTCAGCCAG
Rab4B	CAAGATCGACTCAGGTGAA	Rab26	CAAAGATTCCTTCGACAAC
Rab5A	GCACAGTCCTATGCAGATG	Rab27A	AAGAGAGTGGTGTACAGAG
Rab5B	GAGCCAGTGTTGTAGCAAC	Rab27B	ACGTGTGGTTTATGACACA
Rab5C	GTTTGAGATCTGGGACACA	Rab28	GGCAGATATTGTAAACTAC
Rab6A	CACCTATCAGGCAACAATT	Rab29	TGCCACTACTTTCAGCAAC
Rab6B	TTCAAGTTGGTTTTCCTAG	Rab30	TGCCTTGATCCTTACCTAT
Rab7	GAAAGTGTTGCTGAAGGTC	Rab32	CTGGGACAGCAGGACGCTC
Rab8A	TCACGACAGCCTACTACAG	Rab33A	ATCCCTCTTGTACCGTGAT
Rab8B	ATCCTTTGACAATATTAAA	Rab33B	GAATTCTGGTGGGAAATA
Rab9A	CAAGACTGACATAAAAGAA	Rab34	CCTGAATGACGTGGCATCC
Rab9B	GTGCCAAAGATGATACTAA	Rab35	GCGATGGCTTCATGAAATC
Rab10	GTGGCTTAGAAACATAGAT	Rab36	AGACTAGCCTCATTCACAG
Rab11A	TCTGGAAAGCAAGAGTACC	Rab37	AGGGTGATCCGTTCTGAAG
Rab11B	GCATTCAAGAACATCCTCA	Rab38	CAATGGACTCAAGATGGAC
Rab12#3	TAGCATCCTTTCTCTACAA	Rab39A	CATGTGAAAGATTGGCTAG
Rab12#6	TGTGGACGAGATCTTTCTG	Rab39B	ATCAGAGAGGAGATGTTTG
Rab13	GAACGATTCAAGACAATAA	Rab40B	CAGCTGCAAAATTTCTTAG
Rab14	ACGGGAGAGAATGTAGAAG	Rab40C	TCCTTCTGGTGTACGACAT
Rab15	GTGCCTGCACCAACCTTAA	Rab41	GCTGGTGTTAGTGGGCGAC
Rab17	TGCTGCGCTCCTGGTTTAT	Rab42	GTTAGTGCGAAGAATGACA
Rab18	AGTGAGAACCAGAACAAAG	Rab43	AAGCAACTGCAATGTCGAC

A



Design of shRNAs (at leaset three independent sites)

↓

Evaluation of the efficiency of shRNAs in COS-7 cells

↓

Selection of the most effective shRNA sequence
(e.g., Rab12#3 sequence)

↓

Production of the corresponding siRNA

B

siRNAs for 58 mouse Rabs

↓ 1st screening:
Amount of LC3-II under starved conditions

Amount of LC3-II is...

up-regulated: Rab1A,1B, 6A, 11A

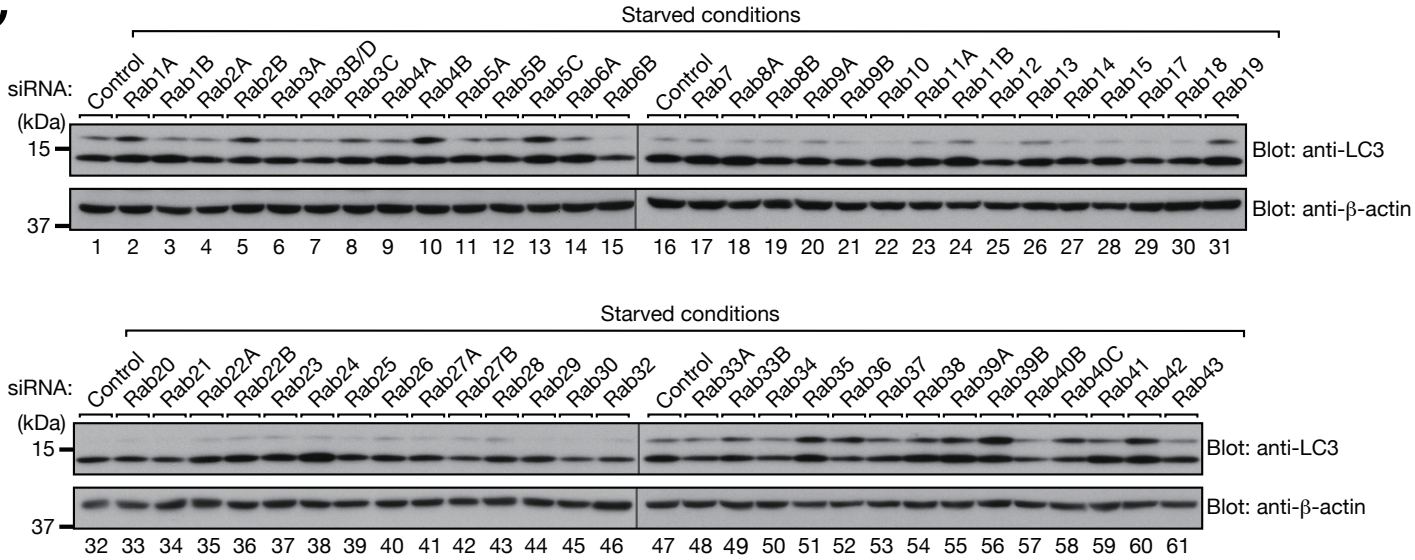
down-regulated: Rab9B, 12, 17, 18, 32, 40B, 40C

↓ 2nd screening:
Amount of p62 under starved conditions.

Amount of p62 is...

up-regualted: Rab12>>1A, 1B, 11A, 18, 40B

C



D

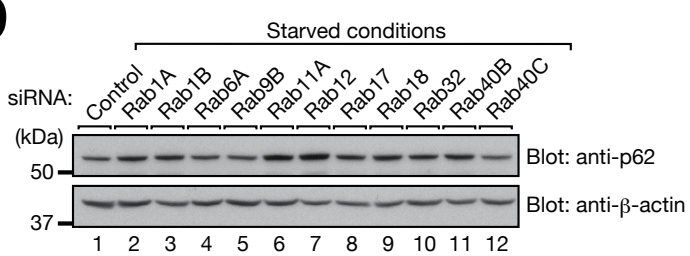


Figure S1. Screening strategy for Rabs involved in autophagy. (A) An example of production of an effective siRNA against mouse Rabs. Lysates of COS-7 cells that had been transfected with pEGFP-C1-Rab12 and pSilencer-Neo-Rab12#1, #2, or #3 were analyzed by immunoblotting with anti-GFP antibody and anti-β-actin antibody. (B) Summary of the screenings and their results. (C) Representative examples of the effect of knockdown of each mouse Rab on the amount of LC3-II under starved conditions. After knockdown of each Rab with a specific siRNA, MEFs were starved by incubation in HBSS for 2 hr. Cell lysates were analyzed by immunoblotting with anti-LC3 antibody and anti-β-actin antibody. (D) Representative examples of the effect of knockdown of candidate Rabs on the amount of p62 under starved conditions. After knockdown of the candidate Rabs by specific siRNAs, cells were cultured as in (C) and lysates were analyzed by immunoblotting with anti-p62 antibody and anti-β-actin antibody.

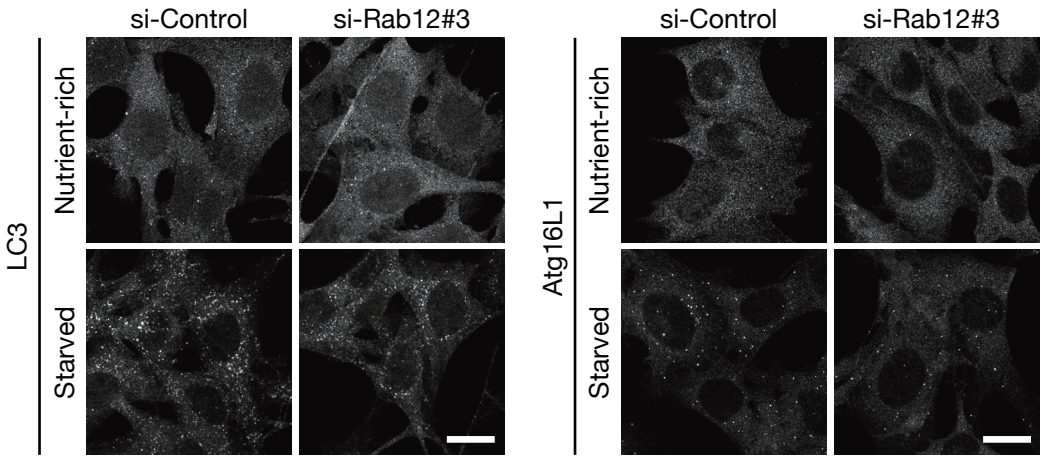


Figure S2. Representative images of the data shown in Fig 1C and 1D. Control and Rab12-knockdown MEFs were cultured under nutrient-rich or starved conditions, fixed, and then immunostained with the antibodies indicated. Scale bars, 20 μm.

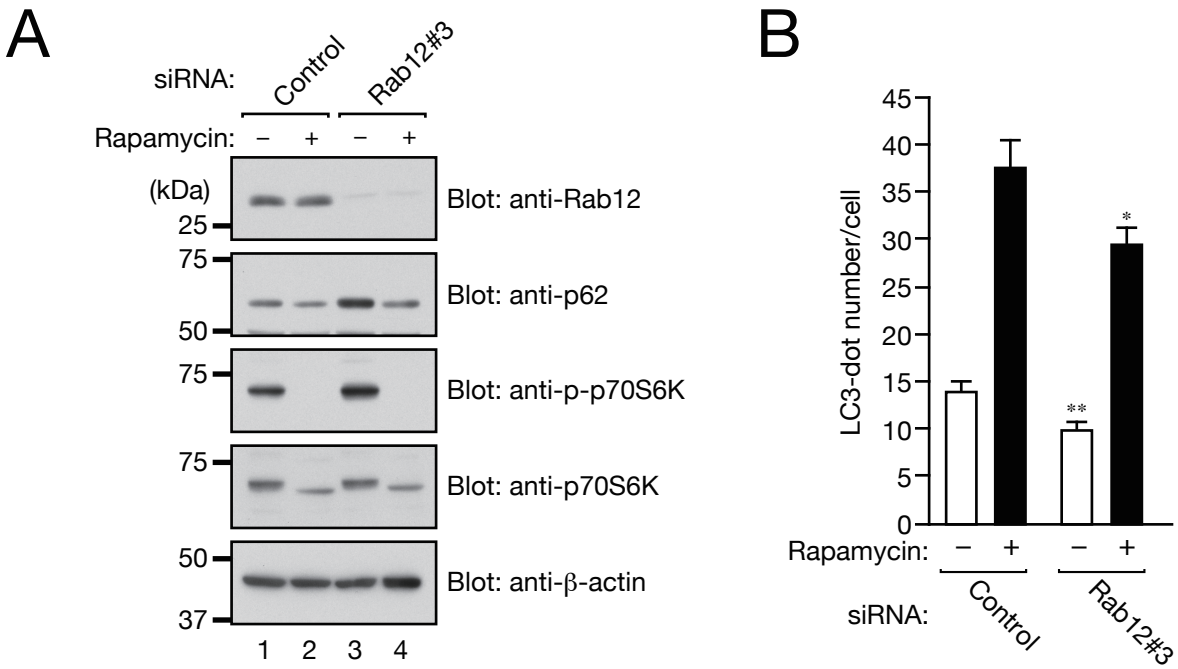


Figure S3. Effect of rapamycin on autophagy in Rab12-knockdown MEFs. (A) Control and Rab12-knockdown MEFs were treated with 100 nM rapamycin for 9 hr. Lysates were analyzed by immunoblotting with the antibodies indicated. (B) Control and Rab12-knockdown MEFs were cultured as described in (A), fixed, and then immunostained with anti-LC3 antibody. The mean numbers of LC3-positive dots per cell are shown. Error bars represent the means and SEM of representative data (n ≥80) from three independent experiments. Note that rapamycin reduced mTORC1 activity and induced autophagy in the Rab12-depleted cells, the same as in the control cells.

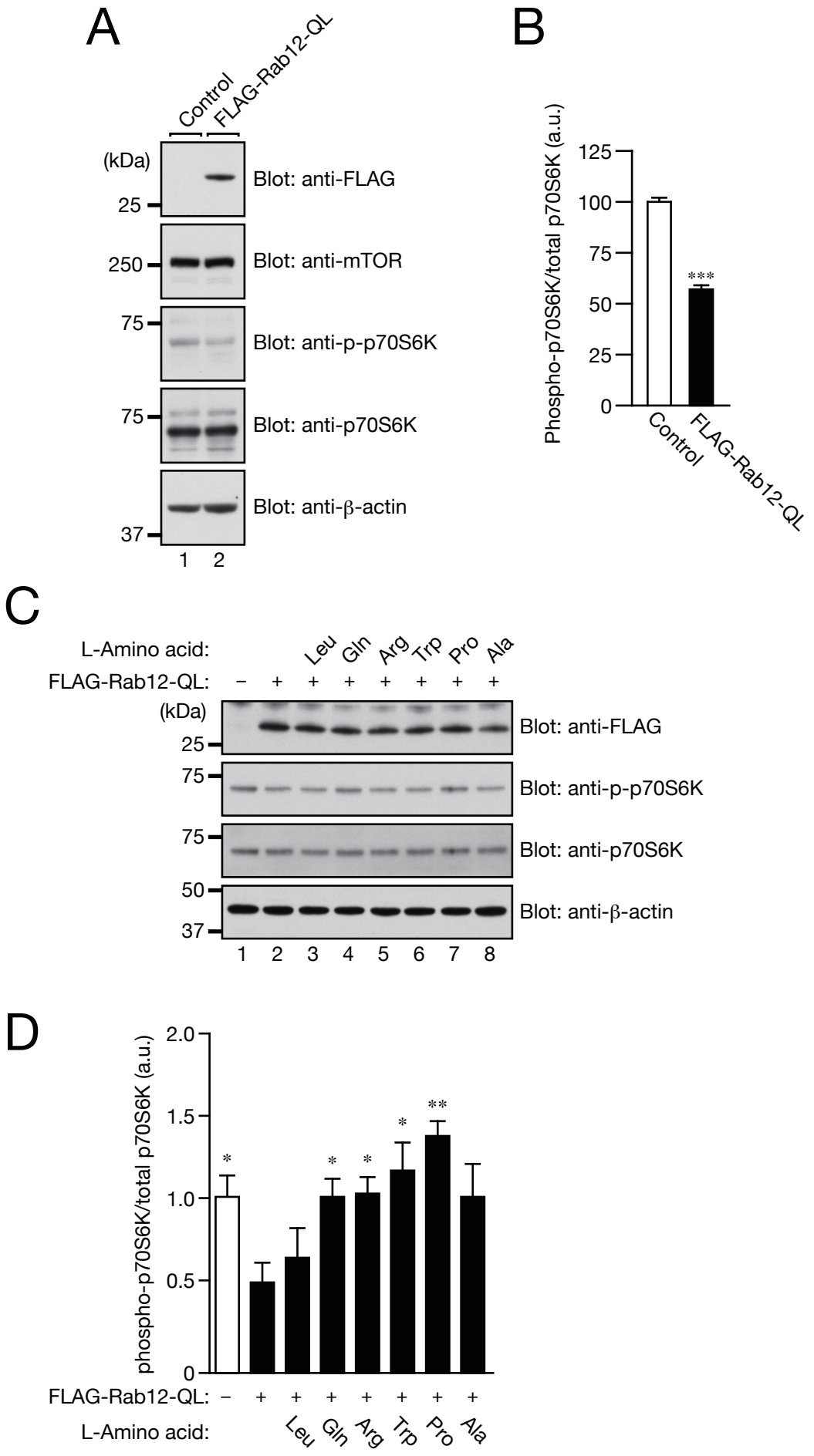


Figure S4. Effect of overexpression of the Rab12 constitutive active mutant (Rab12-QL) on mTORC1 activity. (A) Lysates of MEFs transiently expressing control or FLAG-Rab12-QL were analyzed by immunoblotting with the antibodies indicated. (B) Quantification of the phospho-S6K levels relative to the total amount of S6K is shown in (A). (C) Addition of L-amino acids partially restored the phospho-S6K level in Rab12-QL-overexpressing cells. Lysates of MEFs transiently expressing FLAG-Rab12-QL were analyzed as described in (A). (D) Quantification of the phospho-S6K levels shown in (C).

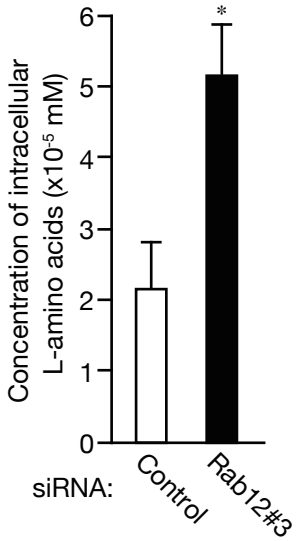


Figure S5. Effect of Rab12 knockdown on the concentration of intracellular L-amino acids. Control and Rab12-knockdown MEFs were analyzed with an L-Amino Acid Quantitation Kit (see METHODS). Note that Rab12 knockdown dramatically increased the amount of intracellular L-amino acids.

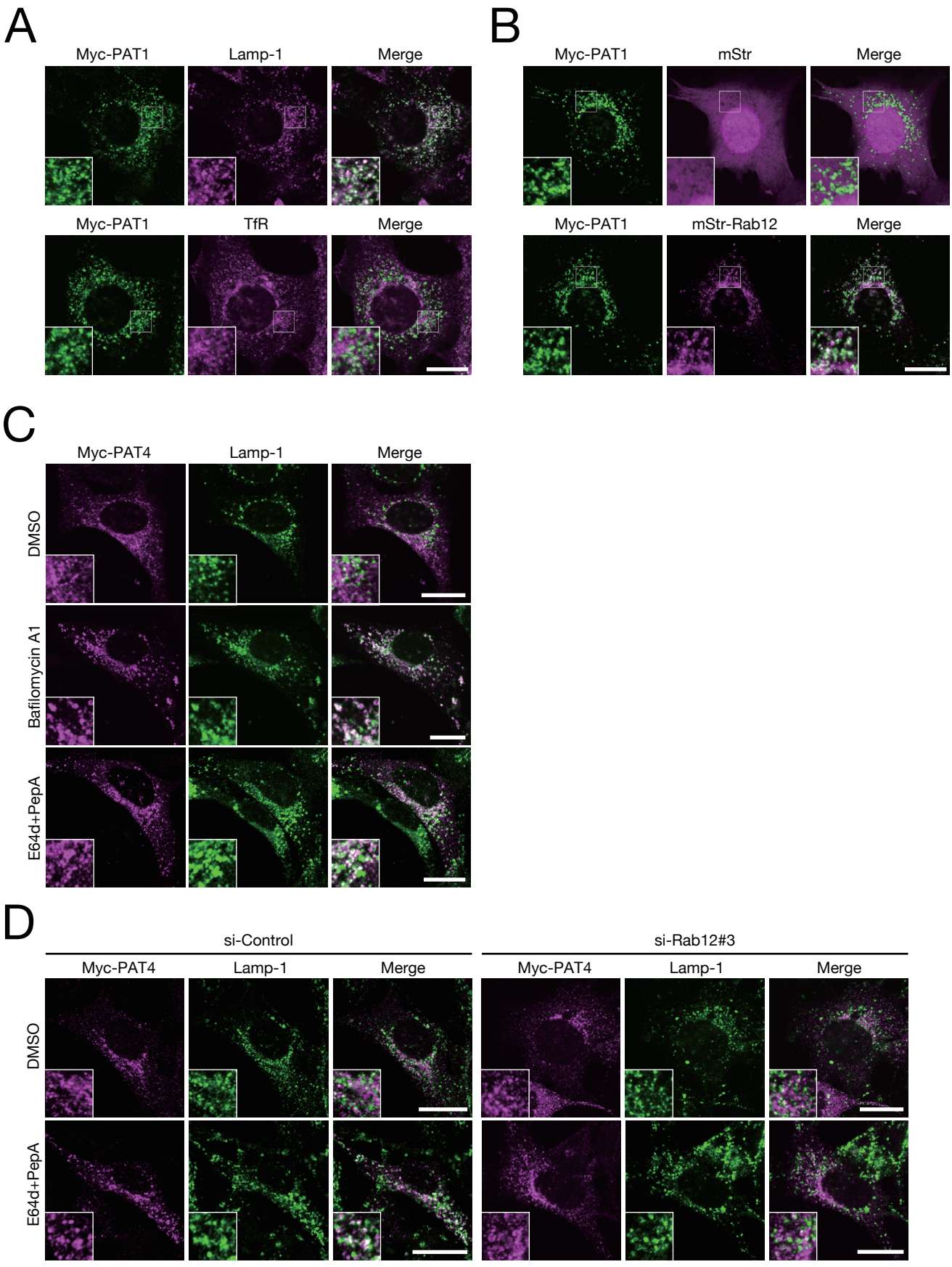


Figure S6. Subcellular localization of PAT1 and PAT4. (A) PAT1 was mainly localized at Lamp-1-positive lysosomes. MEFs transiently expressing Myc-PAT1 were immunostained with anti-Myc antibody and either anti-Lamp-1 antibody or anti-TfR antibody. (B) PAT1 partially co-localized with the Rab12-positive dots. MEFs transiently co-expressing Myc-PAT1 and mStr-Rab12 (or mStr alone) were immunostained with anti-Myc antibody. (C) MEFs transiently expressing Myc-PAT4 were treated with DMSO, 100 μM bafilomycin A1, or 100 nM E64d and 100 μg/ml PepstatinA (PepA) for 12 hr and immunostained with anti-Myc antibody and anti-Lamp-1 antibody. Note that Myc-PAT4 and Lamp-1 were co-localized only after lysosomal inhibitor treatment. (D) Control and Rab12-knockdown MEFs transiently expressing Myc-PAT4 were treated with DMSO or E64d and PepA for 8 hr and immunostained as in (C). Note that Rab12 knockdown reduced the co-localization between Myc-PAT4 and Lamp-1 induced by E64d/PepA treatment. Scale bars, 20 μm.

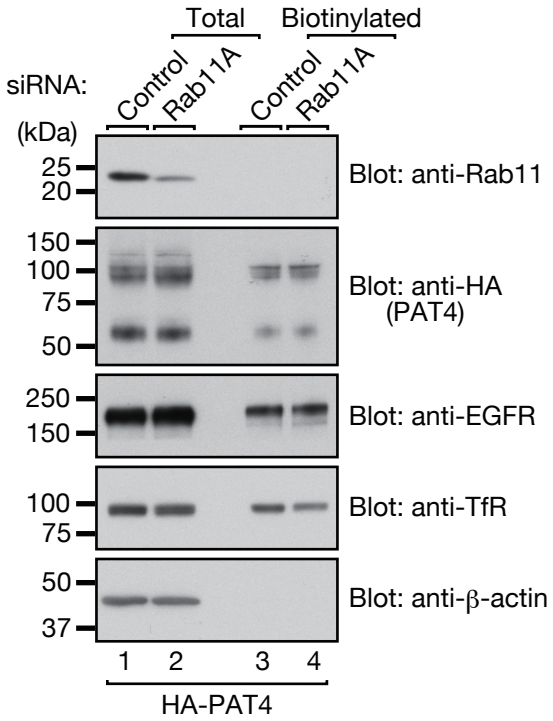


Figure S7. Effect of Rab11A knockdown on the amount of plasma membrane-localized PAT4. Total cell lysates and surface biotinylated proteins from MEFs stably expressing HA-PAT4 that had been transfected with control or *Rab11A* siRNA were analyzed by immunoblotting with the antibodies indicated. Note that Rab11A knockdown did not alter the amount of plasma membrane-localized HA-PAT4 protein but that the amount of plasma membrane-localized TfR protein decreased under the same experimental conditions.

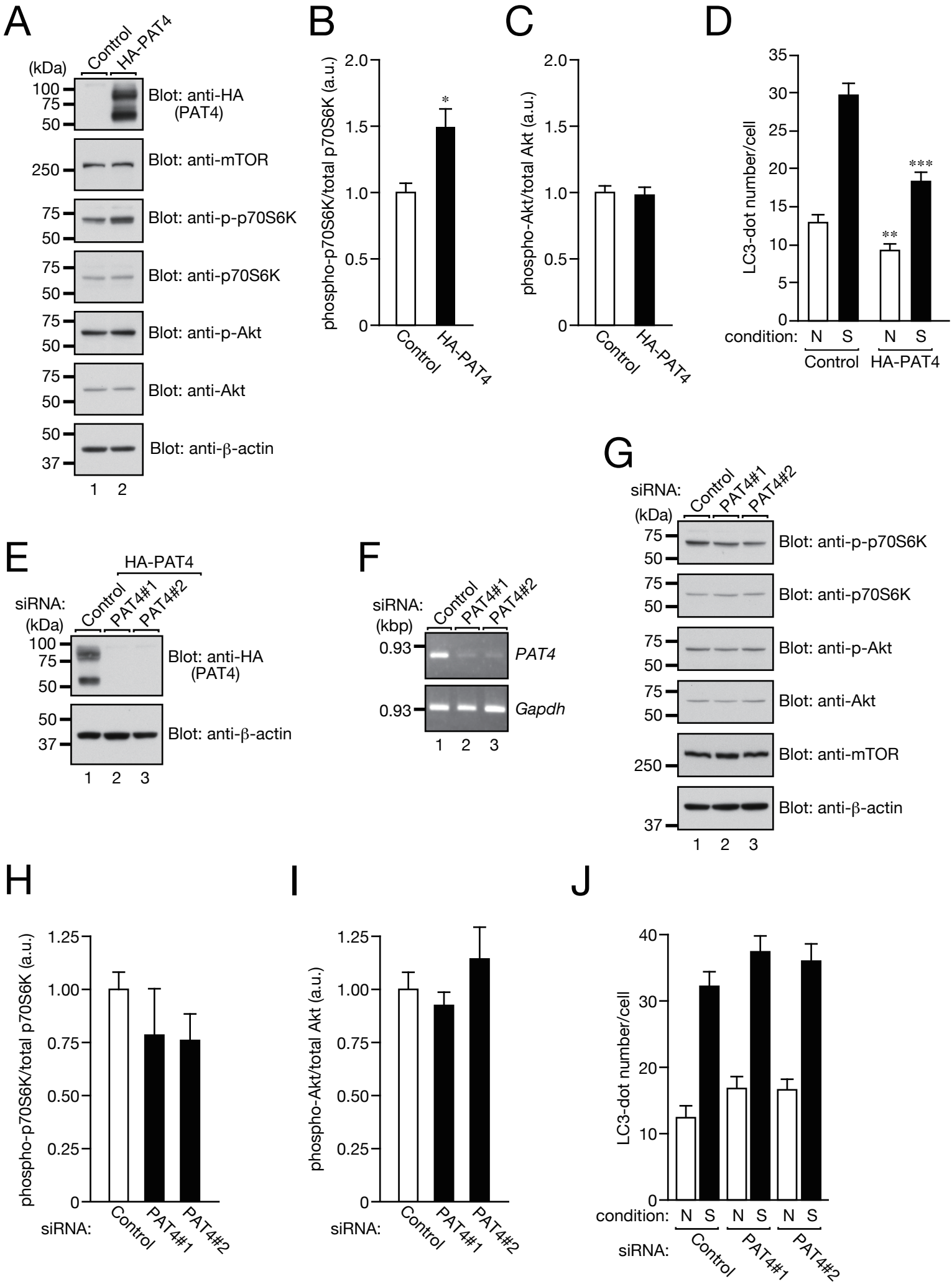

A
Control
HA-PAT4
(kDa)
100
75
50
Blot: anti-HA (PAT4)
250
Blot: anti-mTOR
75
50
Blot: anti-p-p70S6K
75
50
Blot: anti-p70S6K
75
50
Blot: anti-p-Akt
75
50
Blot: anti-Akt
50
37
Blot: anti-β-actin
1 2
B
phospho-p70S6K/total p70S6K (a.u.)
2.0
1.5
1.0
0.5
0
*
Control
HA-PAT4
C
phospho-Akt/total Akt (a.u.)
2.0
1.5
1.0
0.5
0
Control
HA-PAT4
D
LC3-dot number/cell
35
30
25
20
15
10
5
0
**
***
condition: N S N S
Control
HA-PAT4
E
HA-PAT4
siRNA:
Control
PAT4#1
PAT4#2
(kDa)
100
75
50
Blot: anti-HA (PAT4)
50
37
Blot: anti-β-actin
1 2 3
F
siRNA:
Control
PAT4#1
PAT4#2
(kbp)
0.93
PAT4
0.93
Gapdh
1 2 3
G
siRNA:
Control
PAT4#1
PAT4#2
(kDa)
75
50
Blot: anti-p-p70S6K
75
50
Blot: anti-p70S6K
75
50
Blot: anti-p-Akt
75
50
Blot: anti-Akt
250
Blot: anti-mTOR
37
Blot: anti-β-actin
1 2 3
H
phospho-p70S6K/total p70S6K (a.u.)
1.25
1.00
0.75
0.50
0.25
0
siRNA:
Control
PAT4#1
PAT4#2
I
phospho-Akt/total Akt (a.u.)
1.25
1.00
0.75
0.50
0.25
0
siRNA:
Control
PAT4#1
PAT4#2
J
LC3-dot number/cell
40
30
20
10
0
condition: N S N S N S
siRNA:
Control
PAT4#1
PAT4#2

Figure S8. Effect of overexpression or knockdown of PAT4 on mTORC1 activity and autophagy. (A) Lysates of MEFs stably expressing HA-PAT4 were analyzed by immunoblotting with the antibodies indicated. (B and C) Quantification of the phospho-S6K levels and phospho-Akt levels shown in (A). (D) MEFs stably expressing control or HA-PAT4 were cultured under nutrient-rich (N) or starved (S) conditions, fixed, and then immunostained with the antibodies indicated. The mean numbers of LC3-positive dots per cell are shown. Error bars represent the means and SEM of representative data (n ≥80) from three independent experiments. (E) The efficiency of siRNAs targeting PAT4 is shown. MEFs stably expressing HA-PAT4 were transfected with control or *PAT4* siRNA. Cell lysates were analyzed by immunoblotting with the antibodies indicated. (F) RNAi-mediated knockdown of PAT4 in wild type MEFs as revealed by RT-PCR analyses. *Gapdh* was used as an internal control. (G) Knockdown of PAT4 alone did not influence mTORC1 activity or Akt activity. Lysates of MEFs that had been transfected with control or PAT4 siRNA were analyzed by immunoblotting with the antibodies indicated. (H and I) Quantification of the phospho-S6K levels and phospho-Akt levels shown in (G). (J) Control and PAT4-knockdown MEFs were cultured as in (D), fixed, and then immunostained with anti-LC3 antibody. The mean numbers of LC3-positive dots per cell are shown. Error bars represent the means and SEM of representative data (n ≥80) from three independent experiments. Note that PAT4 knockdown slightly increased the numbers of LC3-positive dot, but the differences between the control and PAT4-knockdown cells were not significant.