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Supplemental Data

Bidirectional Transport of Amino Acids Regulates mTOR and Autophagy

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Supplemental Experimental Procedures

Mammalian cell culture, treatments and extract preparation.

HeLa cells were maintained in Dulbecco's minimal essential medium (DMEM) containing 4.5 g/L D-glucose, 4 mM L-glutamine and 110 mg/mL sodium pyruvate supplemented with 10% heat-inactivated FBS. MCF7 cells were cultured in MEM alpha medium containing 10 % heat-inactivated FBS. Extraction of cellular proteins for western and cap binding assays was performed by incubating cell monolayers with ice-cold 10 mM KPO_4, 1 mM EDTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 5 mM EGTA, 0.5% NP-40, 0.1% Brij-35, 1 mM sodium orthovanadate, 40 µg/mL PMSF, 10 µg/mL leupeptin and 5 µg/mL pepstatin, pH7.2.

Mesoscale phospho-S6K1(Thr389) assay.

Cell extracts were prepared using cell lysis buffer (Cell Signaling Technologies, Danvers, MA) and assayed for phosphorylated S6K1(Thr389) levels according to manufacturers guidelines (Meso Scale Discovery, #K110DND-1).

Antibodies used in western assays.

Anti-phospho-S6K1(Thr389), anti-phospho-dS6K, anti-phospho-AKT(Ser473), anti-mTOR, anti-eIF4E, anti-4EBP1, anti-eIF4G, anti-S6K1 (Cell Signaling Technologies, #9205, #9209, #9271, #2972, #9742, #9452, #2498, #9202) were used at a dilution of 1:1000. Anti-tubulin antibody (Sigma, St. Louis, MO, #T-6199) was used at 1:5000, anti-SLC7A5 (Cosmo Bio Company, Tokyo, Japan, #KAL-KE026) was used at 1:1000, anti-SLC3A2 (Santa Cruz Biotechnology, Santa Cruz, CA, #SC-9160) was used at 1:3000.

In-cell western phospho-S6 assay.

HeLa, HaCaT (non-transformed human keratinocyte), SW480 (colon carcinoma) or MCF7 cells were seeded on to 96-well plates (5000 cells/well), cultured for 72 h prior to serum deprivation for 20 h, in a humidified environment of 5% CO_2 at 37°C. Thereafter, they were incubated at 37 ^{o}C in 200 µL of starve-medium for 3 h prior to a 1 h incubation with 200 µL of test-medium. At the end of treatments cells were fixed by the addition of 50 µL of a 5X Mirsky's solution (National Diagnostics, Atlanta, GA) for 60 min, before washing in Tris-buffered saline, pH 7.4, (TBS, 8 x 200 µL). After incubation in TBS containing 0.1% Triton-X100 and 0.1% bovine serum albumin (blocking buffer) for 2 h, fixed cells were incubated overnight at 4°C with anti-phospho-S6(Ser235/236) or S6(Ser240/244) primary antibodies (Cell Signaling Technologies #2211 or # 2215; 50 µL at 1:300 in blocking buffer), washed (8 x 200 µL TBS) before 100 µL of europium-labeled anti-rabbit secondary antibody (Perkin Elmer, #AD0106) was added for 90 min at room temperature in the dark. After a final wash (8 x 200 µL TBS), 100 µL of Delphia™

Enhancement-solution was added to each well and the fluorescence quantified 120 min later using an Envision 2101 multilabel reader (Perkin Elmer, Waltham, MA).

eIF4E-containing 5' mRNA cap complex analysis.

HeLa cells were plated in 10 cm dishes (0.75 x 10^6 cells) and maintained for 72 h prior to serum deprivation. siRNA transfections were performed 24 h after cell plating (1 x 10^6 cells/well). Approximately 500 μg of total protein/condition was incubated with 7-methyl GTP Sepharose 4B beads (GE Healthcare, Piscataway, NJ, #27-5025-01)) for 2 h at 4 ^oC. Pelleted beads were washed twice with lysis buffer and suspended in 1X NuPage LDS sample buffer (Invitrogen, Carlsbad, CA) containing 12.5 % v/v β-mercaptoethanol and eIF4E, eIF4G and 4EBP1 levels contained in the eluates analyzed by western analysis.

Drosophila cell culture and stimulations

S2 and S2-R cells were maintained in complete Schneider's medium (United States Biological, Swampscott, MA) containing 10% FBS and penicillin/streptomycin. Amino acid starvation was performed by washing cells three times in amino acid free Schneider's medium and incubation in the same medium for 1.5 h. Medium was then removed and cells treated with the same medium or Schneider's medium with or without 1.8 g/L L-glutamine. After 20 minutes cell extracts were prepared using lysis buffer.

LC/MS/MS measurement of L-glutamine and stable-isotope labeled amino acid analogs.

The LC system was run in the hydrophilic interaction chromatography (HILIC) mode using a Thermo Betasil Diol-100 column (2.1x100 mm, 5μm particle size). The LC mobile phases contained 50 mM ammonium formate in water (mobile A) and acetonitrile (mobile B). The LC gradient elution was from 95% B to 50 % B over 7.5 minutes, at a flow rate of 0.8 mL/min. MS data was acquired under positive electrospray ionization at an ion voltage of 4.5 kV. All stable-isotope labeled amino acids were purchased from Cambridge Isotope Inc (Andover, MA). The MS MRM (multiple reaction monitoring) transitions for L-glutamine, $^{13}C_5$,$^{15}N_2$-labeled L-glutamine (#CLM-1822), $^{13}C_5$, ^{15}N-labeled L-glutamic acid (#CNLM-554), and isopropyl-D_7-L-leucine (#DLM-4212) were *m/z* 147→84, 152→88, 153→89, and 139→93, respectively, with a 150 ms dwell time for each transition. $^{13}C_5$-labeled L-glutamine (#CNLM-1275), 2,3,3,4,4-D_5-L-glutamic acid (#DLM-556) and D_{10}-L-leucine (#DLM-567) were used as internal standards for the calibration and normalization of the sample preparation and quantification. Data were acquired and processed for calibration and quantification of all analytes using the Analyst software 1.4.2.

siRNA design and RNAi complementation

All siRNA sequences were designed using PredSi (Huesken et al., 2005) and custom synthesized (Qiagen, Valencia, CA). The following siRNA sequences were used: SLC1A5_1 (CCGGTCCTGTACCGTCCTCAA), SLC1A5_2 (TCGCTCATACTCTACCACCTA), SLC3A2_1 (CAGATCCTGAGCCTACTCGAA),

SLC3A2_2 (TCCGTGTCATTCTGGACCTTA), SLC7A5_1 (TGCTAACGTCTTACTAATTTA), SLC7A5_2 (GAGGATGGAATTACTTGAATT), mTOR (CGCATTGTCTCTATCAAGTTA), TSC2 (AAGGATTACCCTTCCAACGAA) and non-silencing or scramble (AATTCTCCGAACGTGTCACGT). Cells were collected for cell size quantitation after a total of 96 h.

The human SLC1A5 cDNA was from ATCC (#MGC-1387) and sequenced verified against NM_ 005628. PCR mutagenesis was used to change amino acid 512 (from L-leucine to L-valine) in accordance with NM_005628 and to change nucleotide 1524 (from T to C) to destroy an internal BamHI site for subsequent cloning. The cDNA was cloned into the BamHI and HindIII sites in pLNCX (Clontech, Mountain View, CA) containing a 5' eGFP sequence. Quick-change PCR was used to generate the RNAi-resistant GFP-SLC1A5 cDNA containing 11 mismatches in the SLC1A5_1 target sequence (TAGAAGTTGCACTGTGTTAAA, where underlined nucleotides are silent mutations). HeLa clones expressing the modified GFP-SLC1A5 allele were obtained by retroviral transduction.

Cell Size.

HeLa cells were seeded in 10 cm dishes (0.75×10^6) for 24 h and then transfected with siRNAs for 72 h. Cell monolayers were gently washed with D-PBS and harvested using D-PBS containing 2.5 mM EDTA (15 minutes, 37 ^{o}C). Pelleted cells were washed with D-PBS/1% FBS and resuspended in D-PBS/1% FBS, 10 μg/mL propidium iodide, 10 μg/mL Hoechst and incubated for 1 h at 37 ^{o}C prior to forward scatter analysis using an LSRII cytometer (Becton Dickinson, San Jose, CA).

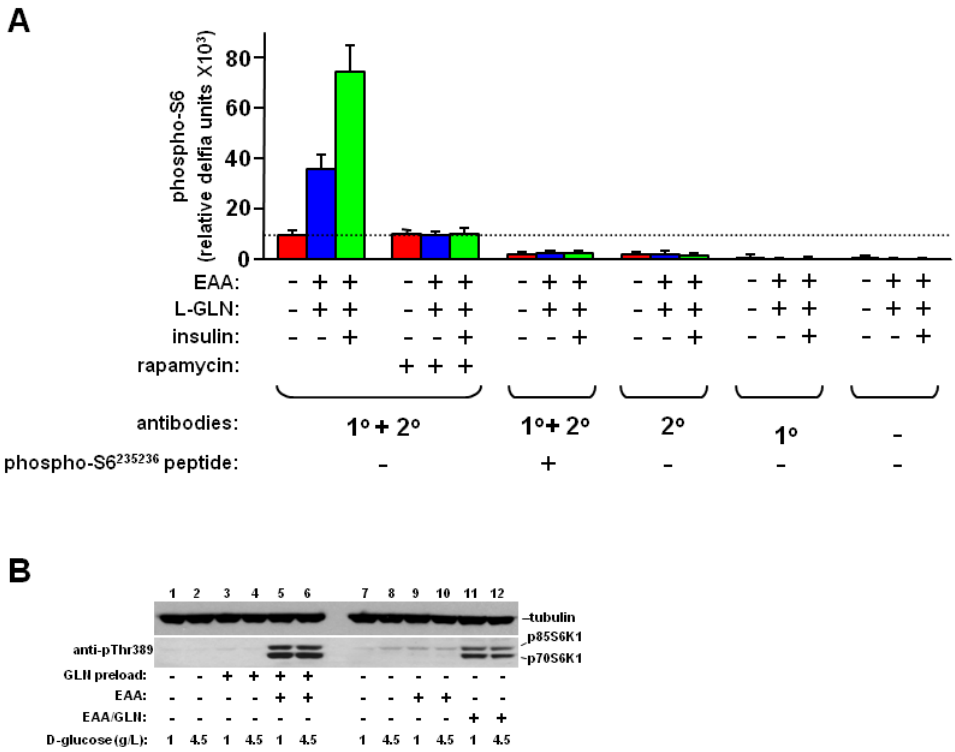

Figure S1. (A) HeLa cells were growth factor and amino acid starved and treated with either EAA/GLN, EAA/GLN/insulin or starve medium for 60 minutes. Rapamycin was added at the same time as these treatments at a final concentration of 30 nM. After fixation cells were blocked, incubated with the primary (1^{o}) or secondary (2^{o}) antibodies and Delfia signal measured. The phospho-$S6^{Ser235/236}$ peptide (Cell Signaling Technology) was added at the same time as primary antibody. (B) HeLa cells were nutrient starved, treated as indicated in the presence of either 1 or 4.5 g/L D-glucose. Cells pretreated with L-glutamine or starve medium (-) for 1 h were stimulated with EAA for 15 minutes prior to lysis (lanes 1-6). Cells treated with starve, EAA or EAA/GLN media (lanes 7-12) were lysed after 1 h.

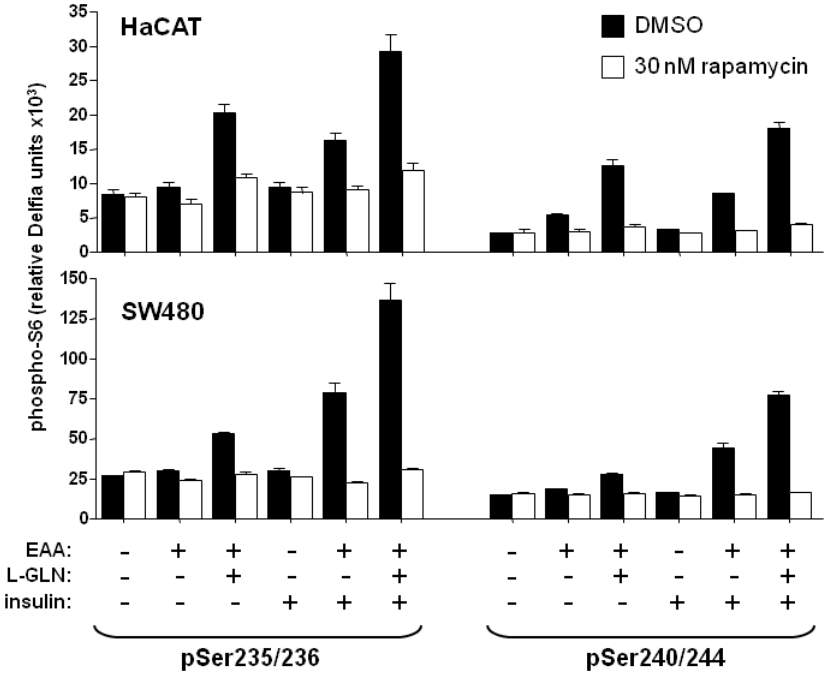

Figure S2. HaCAT or SW480 cells were growth factor and amino acid starved before being treated as indicated. 30 nM rapamycin was added at the same time as individual treatments. The phosphorylation of $S6^{Ser235/236}$ or $S6^{Ser240/244}$ was quantitated as described in the methods.

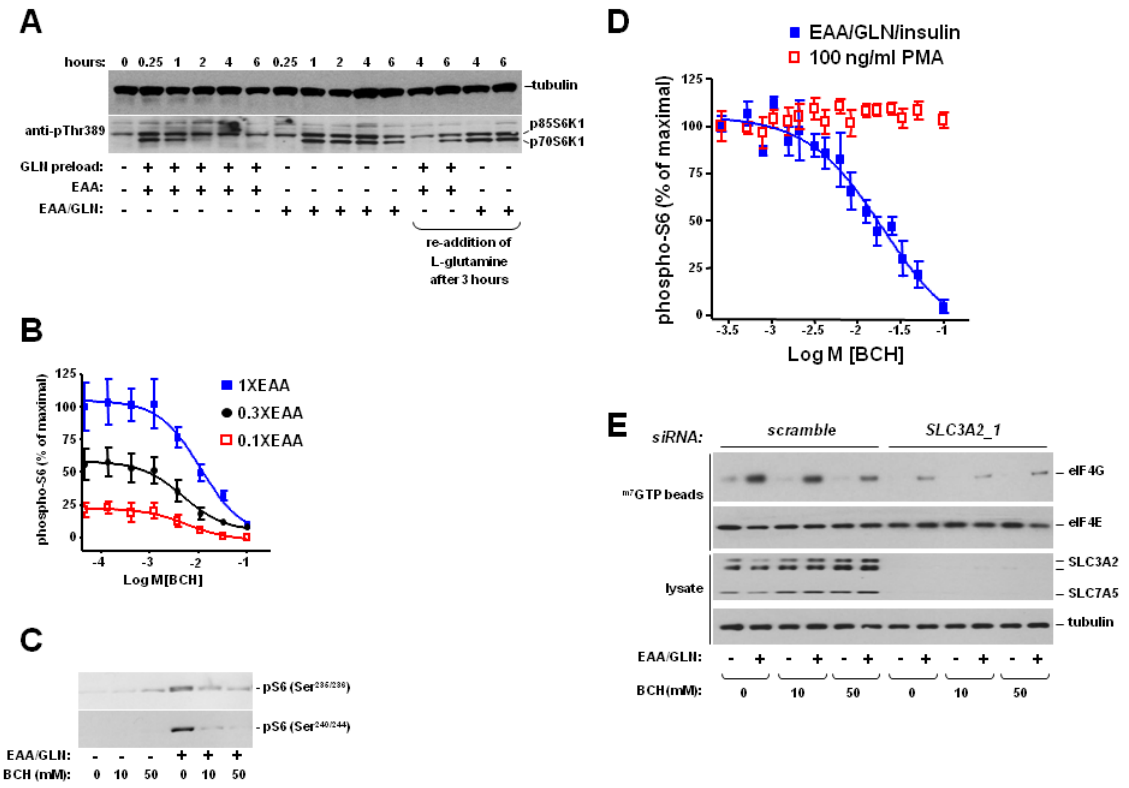

Figure S3. (A) Starved HeLa cells were preincubated with L-glutamine ("GLN preload") or starve medium (-) for 1 h prior to adding EAA or EAA/GLN for the times indicated. 10 mM L-glutamine was added back to some cells after 3 h. (B) The phosphorylation of $S6^{Ser235/236}$ was quantitated following treatment with the indicated concentrations (X) of EAA in the presence of 1 mM L-glutamine and BCH. (C) Starved HeLa cells (-) were treated with EAA containing 1 mM L-glutamine (EAA/GLN) in the absence or presence of BCH for 60 minutes. The phosphorylation state of S6 was analyzed using western blotting. (D) Starved HeLa cells were treated with PMA or EAA/GLN/insulin in the presence of the indicated concentrations of BCH for 60 minutes and the phosphorylation of $S6^{Ser235/236}$ was quantitated. (E) HeLa cells were transfected with scramble or SLC3A2_1 siRNA, starved and then treated with EAA/GLN for 60 minutes in the presence or absence of BCH.

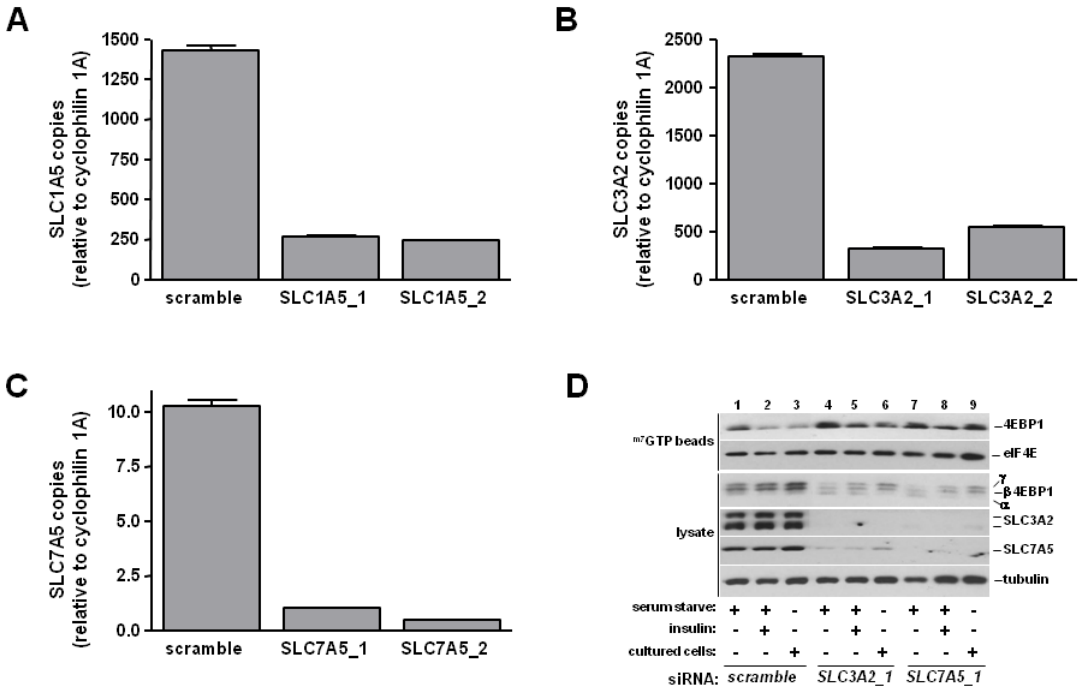

Figure S4. Quantitative RT-PCR analysis of SLC1A5, SLC3A2 and SLC7A5 siRNAs in HeLa cells. Cells were transfected with 25 nM each siRNA for 30 h and processed for Taqman analysis using probes specific for human SLC1A5 (A), SLC3A2 (B) or SLC7A5 (C). Expression levels of individual genes are normalized to cyclophilin A. (D) Following siRNA transfection, HeLa cells were either maintained in rich medium for a total of 72 h and then lysed (cultured cells), serum starved overnight and lysed (serum starve) or serum starved and treated with insulin for 60 minutes (insulin) before lysis. Data are representative of at least 3 individual experiments.

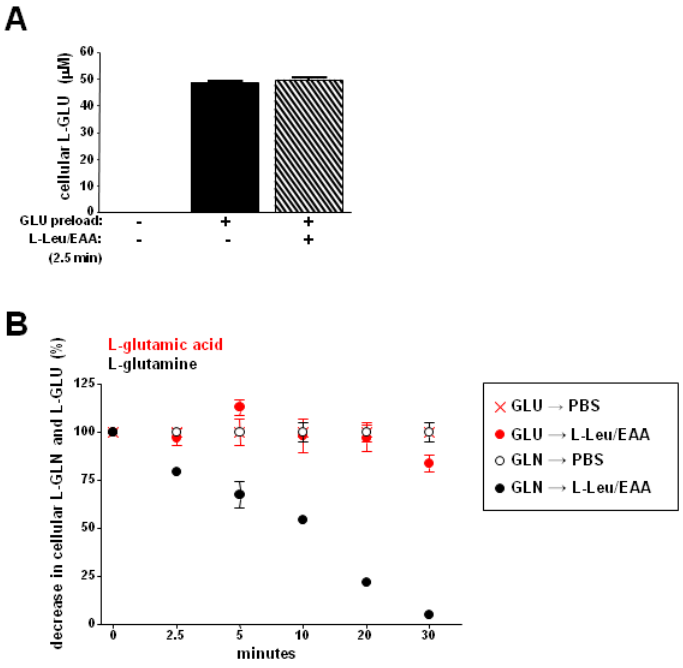

Figure S5. (A) Starved HeLa cells were preincubated with starve medium (-) or 10 mM labeled L-glutamic acid for 1 h, washed and then treated with labeled L-leucine/EAA for 2.5 minutes prior to cell lysis. Absolute quantitation of labeled L-glutamic acid in each cell extract is shown. (B) The time course of cellular labeled L-glutamine (black) and labeled L-glutamic acid (red) following treatment with labeled L-leucine/EAA. Data (mean ± SD) is expressed as a percentage of total cellular stable amino acid prior to L-leucine/EAA addition.

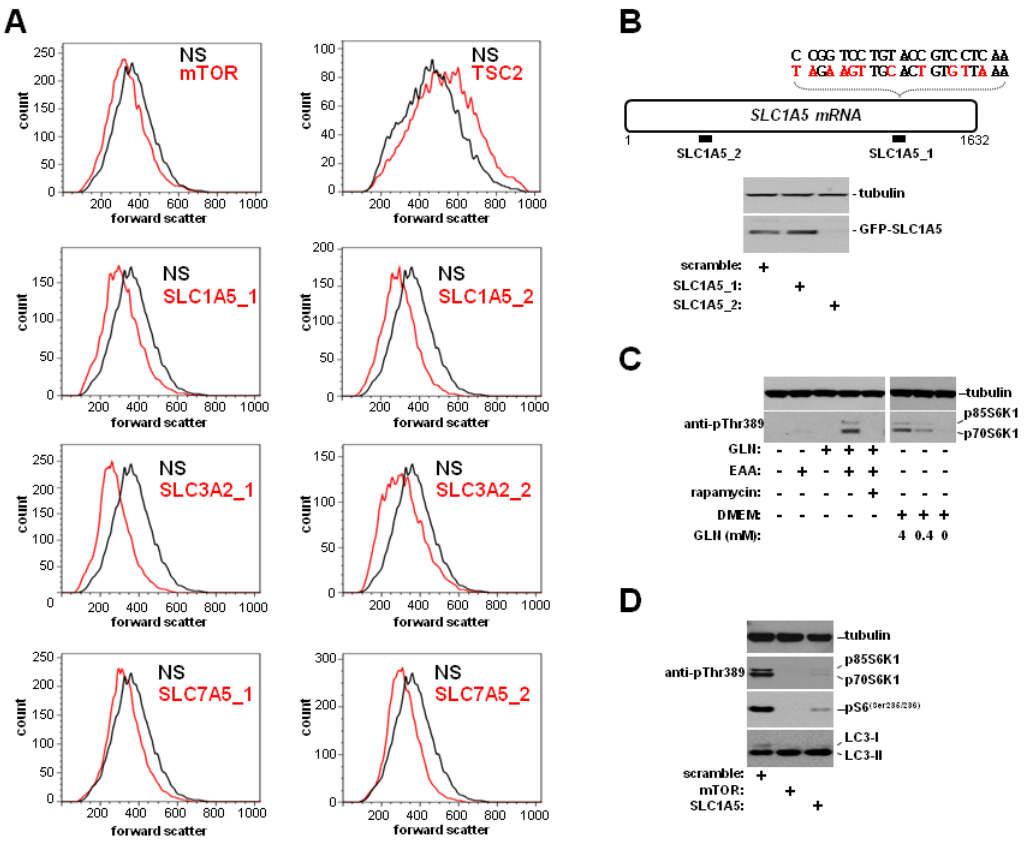

Figure S6. SLC1A5 and SLC7A5/SLC3A2 amino acid transporters regulate cell size. (A) HeLa cells were transfected with the indicated siRNAs (scrambled or non-silencing is "NS") for 72 h and then collected for cell size quantitation as described in the methods. (B) The locations of SLC1A5_1 and _2 siRNA target sites in the SLC1A5 mRNA is illustrated. The SLC1A5_1 site was mutated (red nucleotides) as described in methods. HeLa cells stably expressing the SLC1A5_1 siRNA-resistant GFP-SLC1A5 were transfected with the indicated siRNAs. As expected, the exogenous SLC1A5 is resistant to SLC1A5_1 but not SLC1A5_2. (C) RT112 mCherry-eGFP-LC3 expressing cells were nutrient starved and treated as indicated for 1 h. (D) HeLa cells were transfected with the indicated siRNAs. After 72 h cells were serum starved for 20 h and lysed. S6K1 and S6

phosphorylation as well as LC3 processing was analyzed using western blotting. Data shown are representative of four independent experiments.