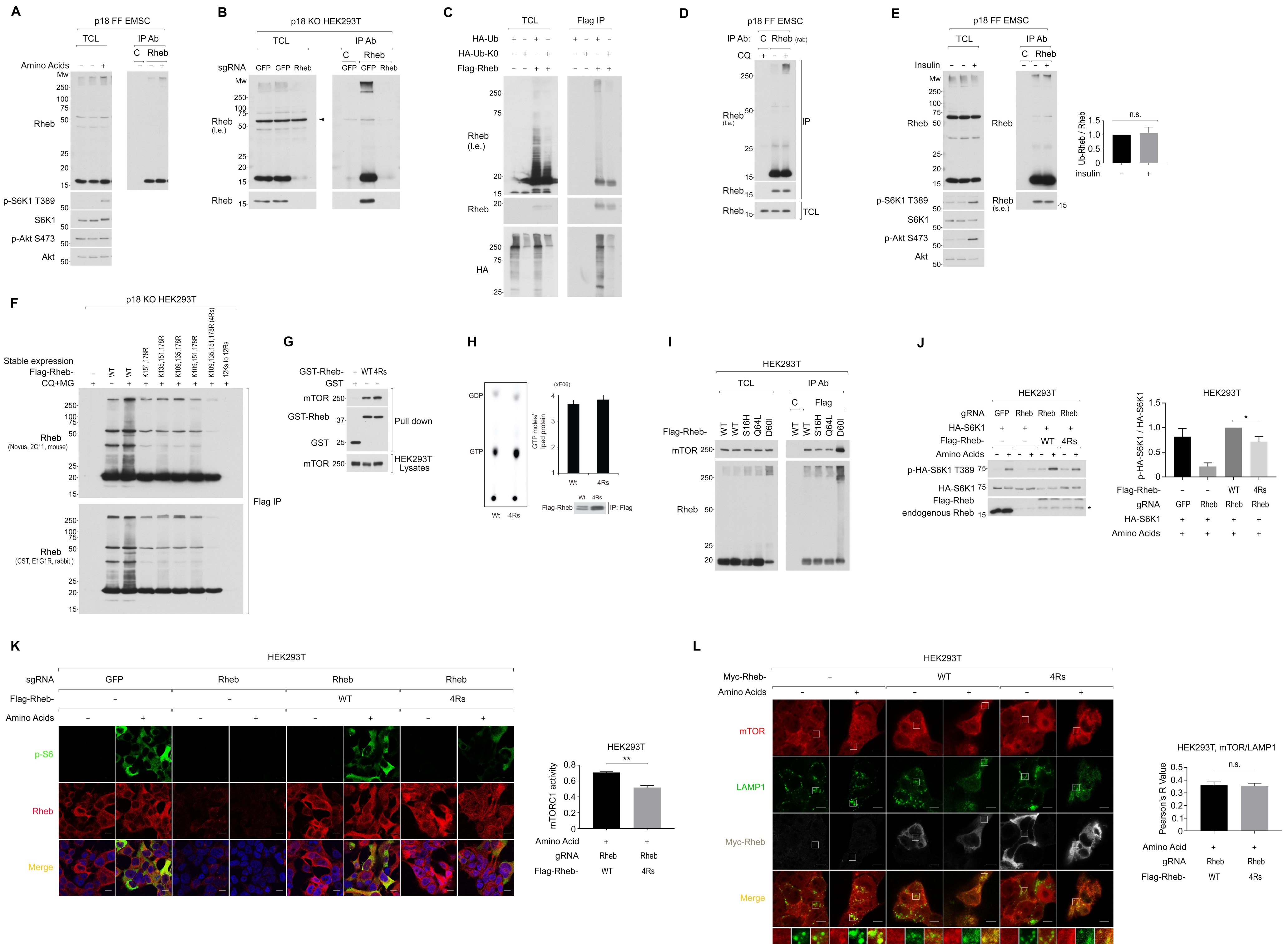
SUPPLEMENTAL INFORMATION

Amino acids enhance polyubiquitination of Rheb and its binding to mTORC1 by blocking lysosomal ATXN3 deubiquitinase activity

Yao Yao, Sungki Hong, Takayuki Ikeda, Hiroyuki Mori, Ormond A. MacDougald
Shigeyuki Nada, Masato Okada, and Ken Inoki

Supplemental Figure 1



Supplemental Figure 1. Amino acid-induced Ub-Rheb accumulation is important for Rheb-mTORC1 interaction and mTORC1 activation, related to Figure 1.

S1A. Amino acid replenishment increases levels of high molecular weight (HMW)-Rheb species in EMSC cells. Wild-type EMSC cells (p18 FF) were starved for amino acids in the amino acid-free DMEM/F12 containing 2% dialyzed-FBS for 50 min and stimulated with 1x amino acid mixture for 15 min. Endogenous Rheb was IPed with the rabbit monoclonal Rheb antibody and detected by the mouse monoclonal Rheb antibody.

S1B. The ablation of Rheb eliminates the HMW Rheb species IPed by the Rheb antibody. Endogenous Rheb was ablated by the Rheb sgRNA in p18 KO HEK293T cells. Cell lysates were subjected to IP with the rabbit monoclonal Rheb antibody, and the IPed Rheb was blotted by the mouse monoclonal Rheb antibody. The arrowhead indicates a major non-specific band in HEK293T cells.

S1C. The overexpression of Ub-K0 mutant decreases levels of HMW Rheb species. Flag-Rheb was co-expressed with HA-Ub, or HA-Ub-K0 mutant in HEK293T cells. The Flag-Rheb was IPed, and levels of Ub-Rheb were monitored.

S1D. Blocking the lysosomal function also accumulates the Ub-Rheb. Wild-type EMSC cells were treated with chloroquine [50 μM] for 45 minutes. The endogenous Rheb was IPed and, levels of Ub-Rheb were monitored.

S1E. Growth factor replenishment has little effect on the accumulation of Ub-Rheb. Wild-type EMSC cells were starved for serum in the serum-free DMEM/F12 containing 1x amino acid mixture for 120 min and stimulated with insulin (172 nM) for 15 min. Endogenous Rheb was IPed with the rabbit monoclonal Rheb antibody and detected by the mouse monoclonal Rheb antibody. The intensity of HMW-Rheb species (>250 kD) was quantified and expressed as the ratio of HMW-Rheb/normal Rheb. mean±SEM, n.s., not significant, n=3.

S1F. Identification of the lysine residues responsible for Rheb poly-ubiquitination. p18 KO HEK293T cells expressing wild-type Flag-Rheb or the indicated Flag-Rheb KR mutants were treated with chloroquine and MG-132 before harvest. Flag-Rheb was IPed by Flag antibody-conjugated beads and eluted with Flag peptides. Levels of Ub-Rheb were monitored by the indicated Rheb antibodies. Among all fourteen lysine residues on Rheb, mutations of four lysine residues (K109, K135, K151, K178) to arginines (Rheb-4Rs) largely diminished poly-ubiquitination of Rheb. Note that the Rheb-12Rs mutant in which all the lysine residues except K19 and K120 were mutated to arginines, did not express detectable Rheb protein. Mutation of the K19, which locates in the P-loop of Rheb, and the mutation of K120R largely destabilized Rheb protein (data not shown).

S1G. Wild-type Rheb and Rheb-4Rs mutant purified from bacteria interact with mTOR with a similar affinity. The GST-Rheb, GST-Rheb-4Rs mutant, or GST protein (control) purified from E.coli. were subjected to GST-pulldown assays with HEK293T cell lysates. Levels of co-precipitated endogenous mTOR were monitored.

S1H. Levels of GTP bound to the Rheb-4Rs mutant are comparable to that of wild-type Rheb. HEK293T cells transiently expressing wild-type Flag-Rheb or Flag-Rheb-4Rs were washed once with phosphate-free DMEM then incubated with 25 µCi/ml of ^{32}P orthophosphate (ICN) for 6 hr. The Flag-Rheb or Flag-Rheb-4Rs was IPed, and the bound nucleotides were eluted and separated on the polyethyleneimine cellulose plate. GTP and GDP resolved by the thin-layer chromatography were visualized and quantified by a phosphor-imager. Levels of GTP bound to Rheb were expressed by the ratio of GTP/Flag-Rheb protein. mean±SEM, n=3.

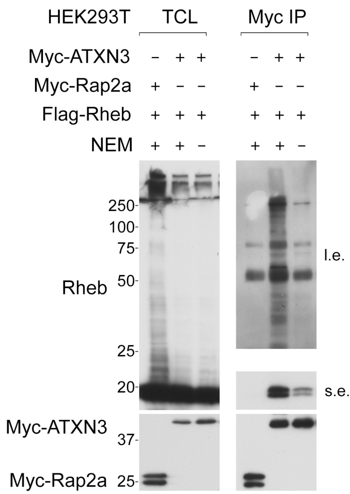
S1I. GDP-bound inactive Rheb D60I mutant displays a higher level of Ub-Rheb and a stronger binding preference for mTOR compared to wild-type Rheb or active GTP-bound Rheb mutants. The indicated Flag-Rheb active mutants (S16H and Q64L), inactive mutant (D60I), or wild-type Rheb was transiently expressed in HEK293T cells. Cell lysates were subjected to Flag IP, and levels of Ub-Rheb and co-IPed endogenous mTOR were monitored.

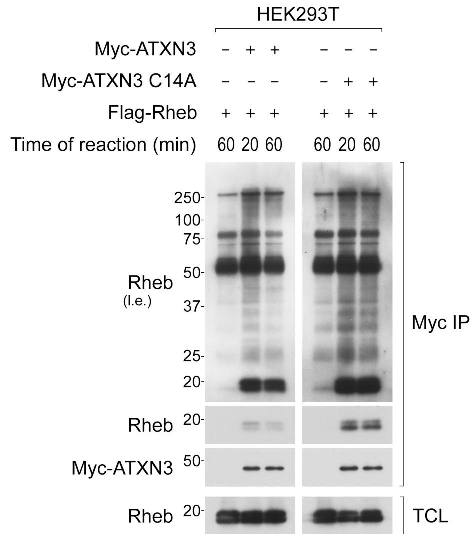
S1J. Amino acid-induced mTORC1 activation is compromised in cells expressing Rheb-4Rs mutant. Wild-type Rheb or Rheb-4Rs mutant was co-expressed with HA-S6K1 in HEK293T cells lacking endogenous Rheb. Cells were starved for amino acids for 50 min, then stimulated with a 1x amino acid mixture for 10 min. Levels of phosphorylated S6K1 were monitored and quantified. Data were expressed as the ratio of pS6K1/total S6K1. *$p<0.05$, mean±SEM, n=5. * denotes non-specific bands recognized by the Rheb antibody.

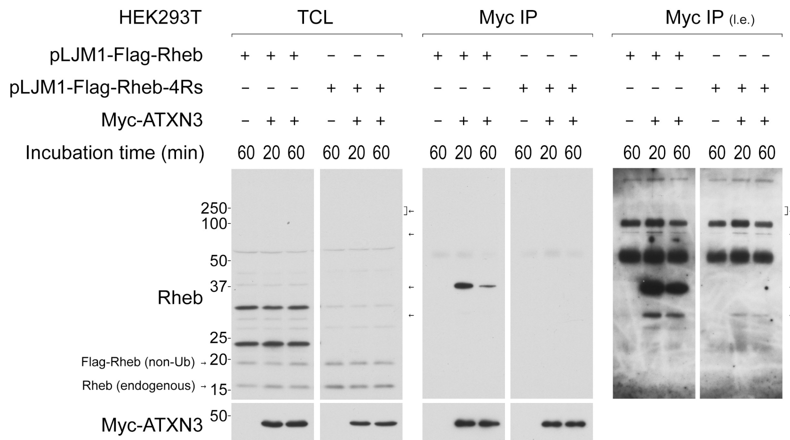
S1K. Ub-Rheb supports amino acid-induced mTORC1 activation. HEK293T cells lacking endogenous Rheb or exclusively expressing wild-type Rheb or Rheb-4Rs mutant were starved for amino acids for 50 min then replenished with 1x amino acid mixture for 10 min. Double immunofluorescence staining with pS6 and Rheb antibodies was performed. Among the cells expressing similar levels of exogenous Rheb, the number of p-S6 positive cells was counted. Data were expressed as the ratio (p-S6 positive cell/Rheb positive cell). **$p<0.01$, mean±SEM, n=137 (WT) and 151 (4Rs).

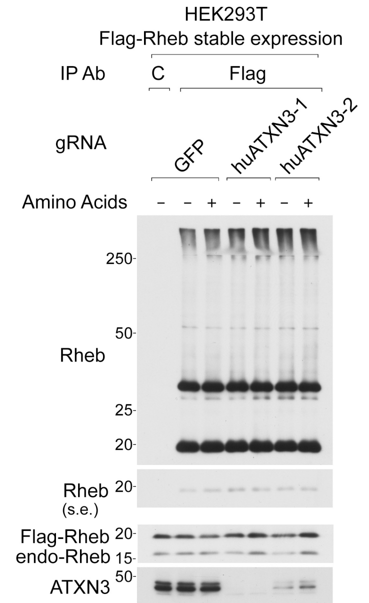
S1L. Amino acids induced equivalent levels of lysosomal mTOR localization in wild-type Rheb or Rheb-4Rs mutant expressing cells. HEK293T cells stably expressing LAMP1-RFP were transiently expressed with wild-type Rheb or Rheb-4Rs mutant. Cells were starved for amino acids for 50 min then replenished with a 1x amino acid mixture for 10 min. Double-immunofluorescent staining with mTOR and Myc antibodies was shown. LAMP1-RFP, a marker for the lysosomal membrane, was visualized using pseudo-green color. Pearson’s correlation coefficient, R values, were determined for quantifying colocalization of Myc-Rheb and LAMP1 and expressed as the mean ± SEM. n.s. denotes not significant, n=24 (WT) and 26 (4Rs).

Supplemental Figure 2









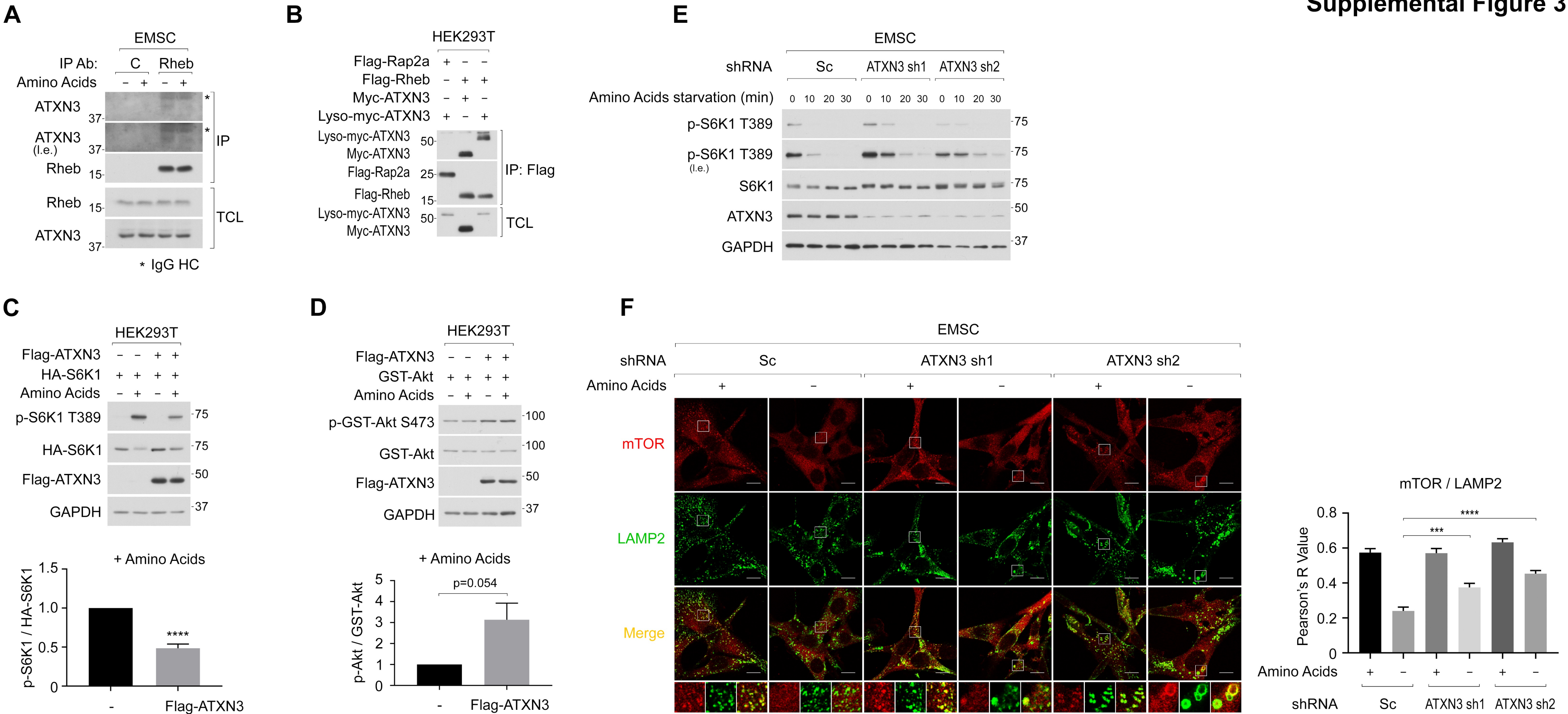
Supplemental Figure 2. ATXN3 interacts with Rheb and reduces levels of Ub-Rheb, related to Figure 2.

S2A. Ub-Rheb co-IPed with ATXN3 is deubiquitinated in the lysate without N-Ethylmaleimide (NEM). Myc-ATXN3 or Myc-Rap2a as control was co-expressed with Flag-Rheb in HEK293T cells. Cells were lysed in the presence or absence of NEM [100 mM], a deubiquitinase inhibitor, and subjected to Myc IP, and levels of co-IPed ubiquitin-free Rheb and Ub-Rheb were monitored.

S2B. Wild-type ATXN3 but not ATXN3 C14A mutant deubiquitinates Ub-Rheb in vitro. Myc-ATXN3 or Myc-ATXN3 C14A mutant was co-expressed with Flag-Rheb in HEK293T cells. Cell lysates were subjected to Myc IP for the indicated time, and co-IPed ubiquitin-free Rheb and Ub-Rheb were monitored.

S2C. Rheb-4Rs mutant fails to interact with ATXN3. Flag-Rheb or Flag-Rheb-4Rs mutant was co-expressed with Myc-ATXN3 in HEK293T cells. Cell lysates were subjected to Myc IP for the indicated times (20 and 60 min), and levels of co-IPed ubiquitin-free Rheb and Ub-Rheb were monitored. The arrows indicate the Ub-Rheb co-IPed with Myc-ATXN3. The ubiquitination of the four lysine residues was required for Rheb to interact with ATXN3.

S2D. The ablation of ATXN3 increases levels of Ub-Rheb. Endogenous ATXN3 was ablated by the CRISPR-Cas9 system in HEK293T cells stably expressing Flag-Rheb. The cells were starved for amino acids for 50 min and stimulated with a 1x amino acid mixture for 15 min. Flag-Rhebs were IPed by Flag antibody-conjugated beads and eluted with Flag peptides. Levels of Ub-Rheb were monitored with the Rheb antibody. The Ub-Rheb was accumulated, but the level of Ub-Rheb was not further increased in response to amino acids.


Supplemental Figure 3
A
EMSC
IP Ab: C Rheb
Amino Acids
ATXN3
ATXN3 (l.e.)
Rheb
IP
TCL
* IgG HC
B
HEK293T
Flag-Rap2a
Flag-Rheb
Myc-ATXN3
Lyso-myc-ATXN3
IP: Flag
TCL
C
HEK293T
Flag-ATXN3
HA-S6K1
Amino Acids
p-S6K1 T389
HA-S6K1
Flag-ATXN3
GAPDH
+ Amino Acids
p-S6K1 / HA-S6K1
****
Flag-ATXN3
D
HEK293T
Flag-ATXN3
GST-Akt
Amino Acids
p-GST-Akt S473
GST-Akt
Flag-ATXN3
GAPDH
+ Amino Acids
p=0.054
p-Akt / GST-Akt
Flag-ATXN3
E
EMSC
shRNA
Sc
ATXN3 sh1
ATXN3 sh2
Amino Acids starvation (min)
p-S6K1 T389
p-S6K1 T389 (l.e.)
S6K1
ATXN3
GAPDH
F
EMSC
shRNA
Sc
ATXN3 sh1
ATXN3 sh2
Amino Acids
mTOR
LAMP2
Merge
mTOR / LAMP2
Pearson's R Value
Amino Acids
shRNA
Sc
ATXN3 sh1
ATXN3 sh2

Supplemental Figure 3. ATXN3 inhibits amino acid-induced mTORC1 activation, related to Figure 3.

S3A. Amino acid replenishment decreases the interaction between endogenous Rheb and ATXN3. EMSC cells were starved for amino acids for 50 min in the amino acid-free DMEM containing 2% dialyzed-FBS then stimulated with a 1x amino acid mixture for 15 min. Endogenous Rheb was IPed, and levels of co-IPed ATXN3 were monitored. * indicates IgG heavy chain.

S3B. Lyso-ATXN3 preferentially interacts with Rheb. Cells co-expressed with Flag-Rheb and Myc-ATXN3 or Lyso-Myc-ATXN3 were subjected to Flag IP. Levels of co-IPed ATXN3 were monitored. Note that the expression of Lyso-Myc-ATXN3 in the lysate was significantly lower than Myc-ATXN3.

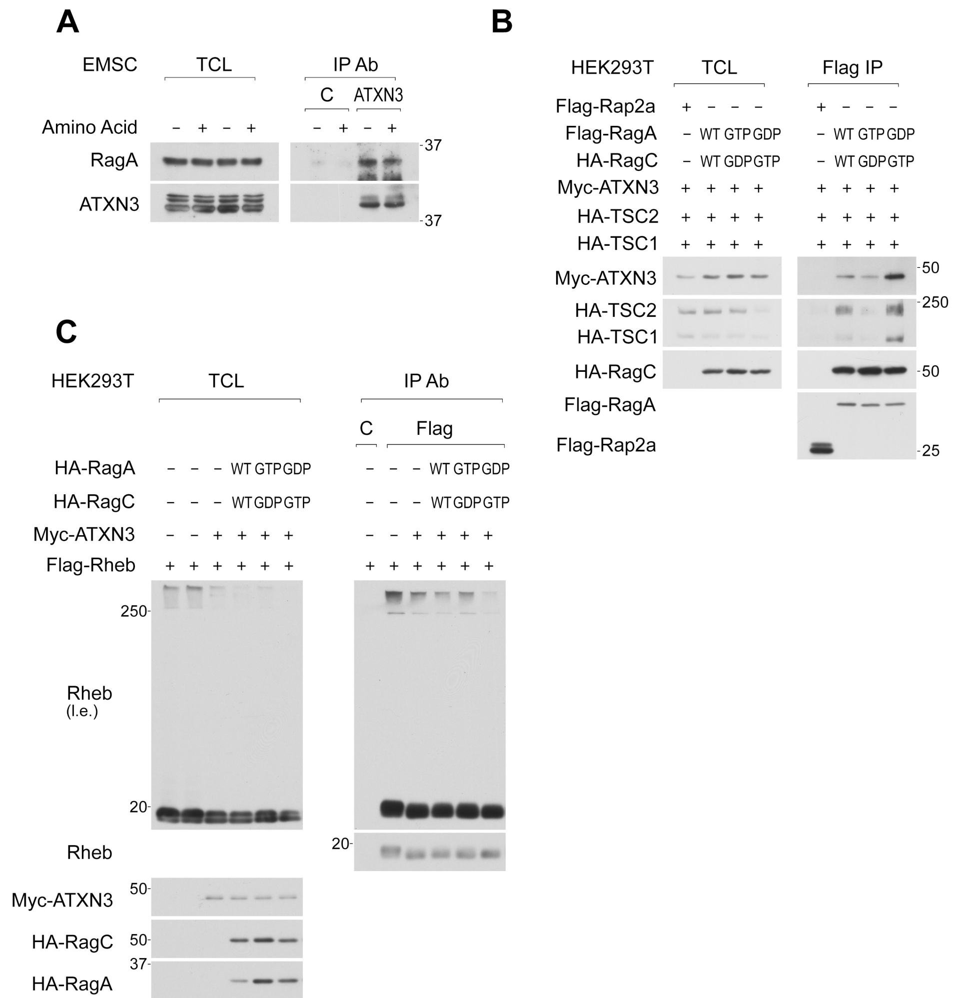
S3C. Overexpression of ATXN3 partially decreases cellular mTORC1 activity in the presence of amino acids. HEK293T cells were co-transfected with Flag-ATXN3 and HA-S6K1 as a reporter for cellular mTORC1 activity. Cells were treated as Fig. S3A. Levels of phosphorylated S6K1 were monitored and quantified. Data were expressed as the ratio of pS6K1/total S6K1 under amino acid-replete conditions. ****$p<0.0001$, mean±SEM, n=4.

S3D. Overexpression of ATXN3 has little effect on Akt phosphorylation. HEK293T cells were co-transfected with Flag-ATXN3 and GST-Akt as a reporter for cellular PI3K and mTORC2 activity. Cells were treated as Fig. S3A. Levels of phosphorylated Akt (Ser473), a site phosphorylated by mTORC2, were monitored and quantified. Data were expressed as the ratio of pAkt/total Akt under amino acid replete conditions. $p=0.054$, mean±SEM, n=3.

S3E. The ablation of ATXN3 renders mTORC1 activity partially resistant to amino acid starvation. EMSC cells treated with control (Sc: scramble) or two distinct shRNAs targeting ATXN3 were starved for amino acids for the indicated times in the amino acid-free DMEM/F12 containing 2% dialyzed-FBS. Levels of mTORC1 activity were monitored.

S3F. The ablation of ATXN3 renders mTOR retention on the lysosome in response to amino acid starvation. Similar experiments were performed as Fig. S3D. The indicated EMSC cells were fixed in the presence (+AA) or absence (-AA for 20 min) of amino acids in the medium. Pearson's correlation coefficient, R values were determined for quantifying the colocalization of mTOR and LAMP2. ***$p<0.001$; ****$p<0.0001$, n (Sc –AA, Sc +AA, ATXN3 sh1 –AA, ATXN3 sh1 +AA, ATXN3 sh2 –AA, ATXN3 sh2 +AA) =(13, 9, 12, 16, 18, 17).

Supplemental Figure 4



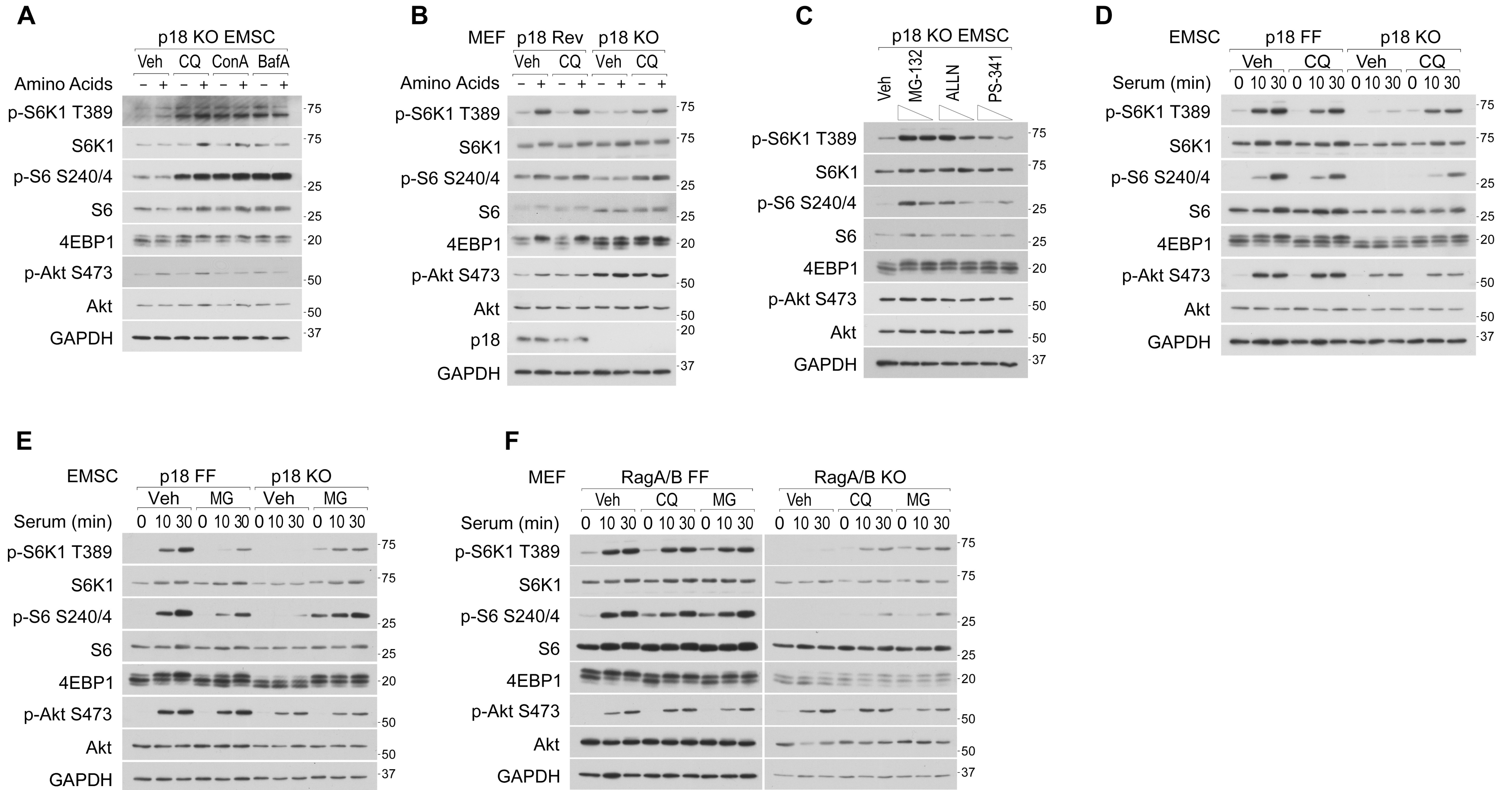
Supplemental Figure 4. The interaction between ATXN3 and Rheb is negatively regulated by amino acids through Rag small GTPase activity, related to Figure 4.

S4A. Amino acid stimulation decreases the interaction between endogenous ATXN3 and RagA. EMSC cells were starved for amino acids in the amino acid-free DMEM/F12 containing 2% dialyzed-FBS for 50 min and stimulated with a 1x amino acid mixture for 15 min. Endogenous ATXN3 was IPed, and levels of co-IPed endogenous RagA were monitored.

S4B. ATXN3 and TSC2 preferentially interact with the inactive Rag heterodimer. HEK293T cells were co-expressed with Myc-ATXN3, HA-TSC1/2, and the indicated Flag-Rag heterodimer. Flag-Rap2a was used as a control for the Rag heterodimers. Cell lysates were subjected to Flag IP, and levels of co-IPed Myc-ATXN3 and HA-TSC1/2 were monitored.

S4C. Active Rag heterodimer partially blocked while inactive Rag heterodimer facilitated ATXN3-induced deubiquitination of Rheb. HEK293T cells were co-expressed with Flag-Rheb, Myc-ATXN3, and the indicated Rag heterodimers (wild-type, active, or inactive). Flag-Rheb was IPed, and levels of Ub-Rheb were monitored.

Supplemental Figure 5



Supplemental Figure 5. The inhibition of lysosome- or proteasome-mediated cellular degradation machinery activates mTORC1 in Rag-Ragulator deficient cells independent of amino acid availability, related to Figure 5.

S5A. Multiple lysosome inhibitors activate mTORC1 activity in p18 KO EMSC cells in a manner independent of amino acid availability. p18 KO EMSC cells were starved for amino acids in the amino acid-free DMEM/F12 containing 2% dialyzed-FBS in the presence or absence of a lysosomal inhibitor, Chloroquine (CQ, 50 μM), Concanamycin A (ConA, 5 μM) or Bafilomycin A1 (BafA, 1 μM) for 50 min, then replenished with 1x amino acid mixture for another 10 min. Levels of mTORC1 activity and the indicated protein expression were monitored.

S5B. Blocking lysosome function by chloroquine also stimulates mTORC1 activity in p18 KO MEF cells independent of amino acid availability. p18 KO MEF cells and p18 KO MEF cells re-expressing p18 (p18 Rev) were treated as Fig. S5A.

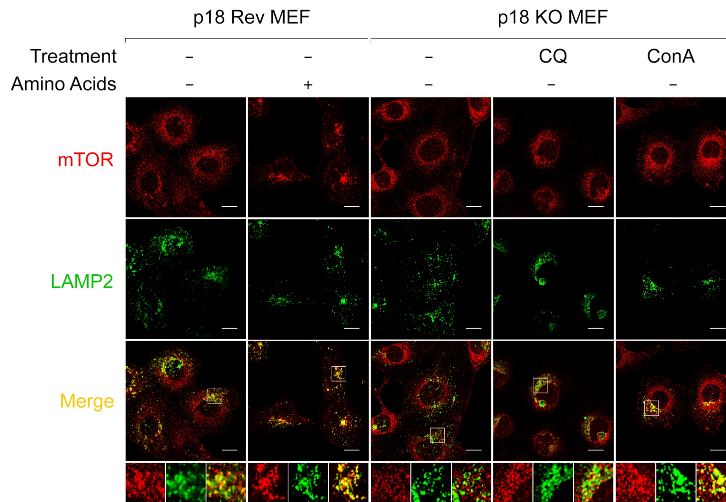
S5C. Multiple proteasome inhibitors enhance mTORC1 activity in amino acid-starved p18 KO EMSC cells. p18 KO EMSC cells were amino acid-starved for 50 min in the presence or absence of the indicated proteasome inhibitors (MG-132; 20 μM, 0.5 μM; ALLN, 25 μM, 1 μM; Bortezomib (PS-341), 1μM, 0.1 μM). Levels of mTORC1 activity and the indicated protein expression were monitored.

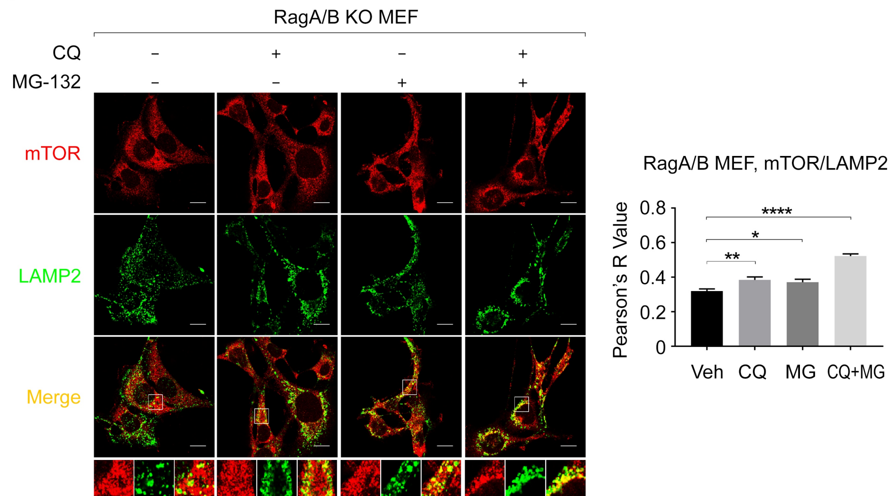
S5D. Blocking lysosome activity restores growth factor-induced mTORC1 activation in p18 KO EMSC cells. Wild-type (p18 FF) or p18 KO EMSC cells were serum-starved in the serum-free DMEM/F12 containing 1x amino acids for 12 hrs. The cells were treated with or without Chloroquine (CQ, 50 μM) for 60 min, then stimulated with serum (2 % final concentration) for the indicated time periods.

S5E. Blocking proteasome activity restores growth factor-induced mTORC1 activation in p18 KO EMSC cells. Similar experiments were performed as Fig. S5D using a proteasome inhibitor (MG-132, 20 μM).

S5F. Inhibiting lysosome or proteasome function restores growth factor-induced mTORC1 activation in RagA/B double KO MEF cells. Similar experiments were performed as in Fig. S5D and S5E in wild-type (Rag A/B FF) or RagA/B double KO (RagA/B KO) MEF cells.

Supplemental Figure 6



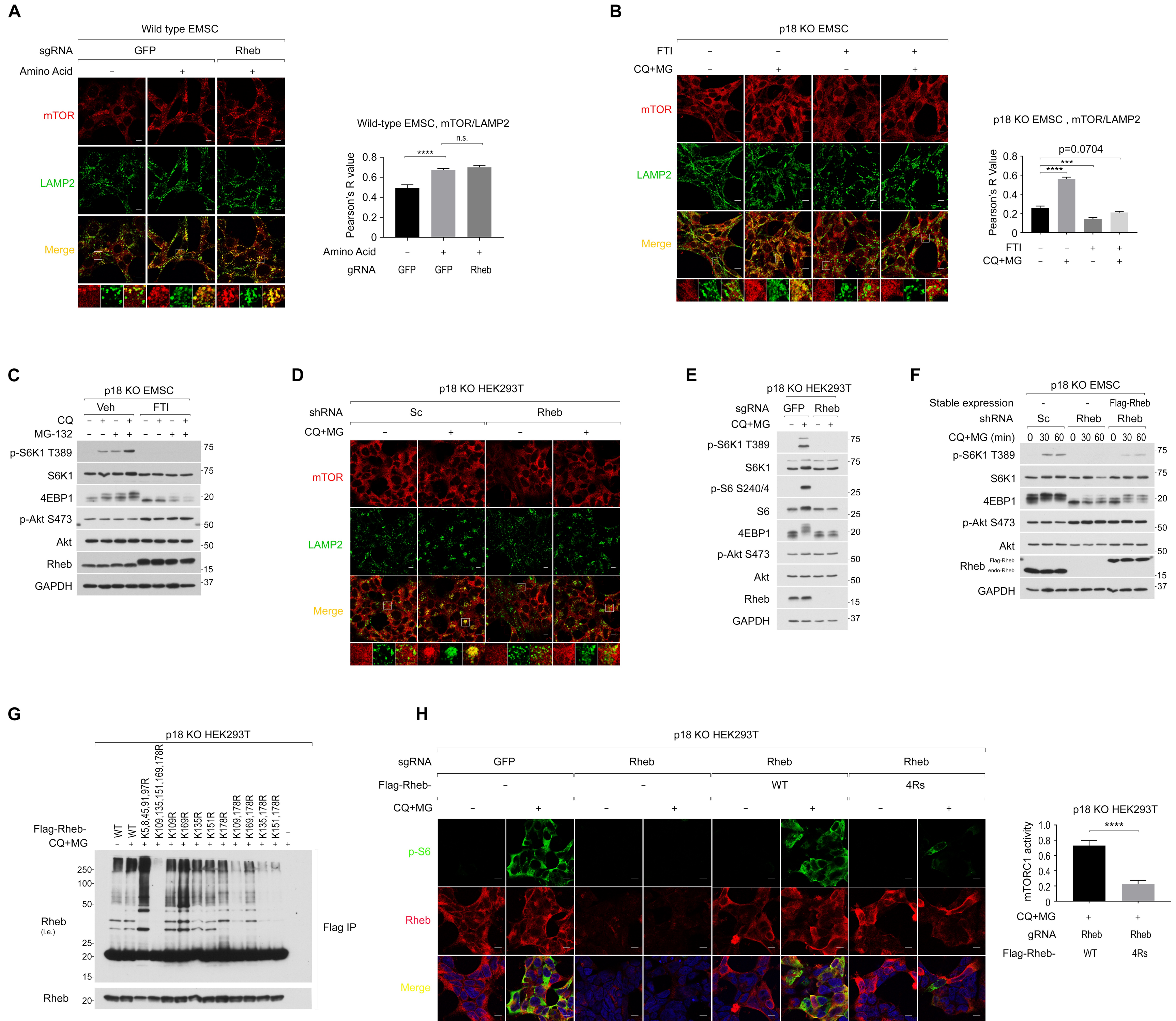


Supplemental Figure 6. The inhibition of lysosome- or proteasome-mediated cellular degradation machinery induces lysosomal mTOR localization in Rag-Ragulator deficient cells independent of amino acid availability, related to Figure 6.

S6A. Blocking lysosome function by Chloroquine (CQ, 50 μM) or Concanamycin A (ConA, 5 μM) induces lysosomal mTOR localization in amino acid-starved MEF cells. p18 KO MEF cells re-expressing p18 (p18 Rev) were starved for amino acids for 50 min and replenished with 1x amino acids for 10 min. p18 KO MEF cells were starved for amino acids for 50 min in the presence or absence of the indicated lysosome inhibitors for 50 min. Lysosomal mTOR localization was monitored by double immunofluorescent staining with mTOR and LAMP2 antibodies.

S6B. Blocking both lysosome and proteasome function additively induces lysosomal mTOR localization in RagA/B deficient cells. RagA/B KO MEF cells were starved for amino acids in the amino acid-free DMEM/F12 containing 2% dialyzed-FBS for 15 min then treated with the indicated inhibitors for 45 min. Pearson's correlation coefficient, R values were determined for quantifying colocalization of mTOR and LAMP2 and graphed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$; ****$p < 0.0001$ compared to vehicle treatment (first column), n (number of cells analyzed: Veh, CQ, MG, CQ+MG)=(17, 24, 25, 36).

Supplemental Figure 7



Supplemental Figure 7. Poly-ubiquitination of Rheb contributes to lysosomal mTOR localization and its activation in Ragulator deficient cells, related to Figure 7.

S7A. Ablation of endogenous Rheb showed little effect on amino acid-induced lysosomal mTORC1 localization in wild-type EMSC cells. Endogenous Rheb was knocked out by the sgRNA in wild-type EMSC cells. Cells were starved for amino acids for 50 min and then stimulated with a 1x amino acid mixture for 10 min. Pearson's correlation coefficient, R values were determined for quantifying colocalization of mTOR and LAMP2. mean ± SEM, ****$p<0.0001$, n.s., not significant, n (sgGFP –AA, sgGFP +AA, sgRheb +AA) = (20, 18, 18).

S7B. The farnesyltransferase inhibitor (FTI), which blocks membrane localization of Rheb, effectively inhibits CQ+MG-induced lysosomal mTOR localization in Ragulator deficient cells. p18 KO EMSC cells were treated with the FTI (20 μM) for 24 hr, and then amino acid-starved with or without CQ or MG-132 treatment for 50 min. Levels of lysosomal localization of mTOR were monitored. Pearson's R values are graphed as the mean ± SEM. ***$p< 0.001$, ****$p< 0.0001$ compared to control treatment (first column), n (number of cells analyzed: Veh, CQ+MG, FTI, CQ+MG+FTI) = (17, 16, 19, 18).

S7C. The FTI effectively inhibits CQ+MG-induced mTORC1 activation in Ragulator deficient cells. p18 KO EMSC cells were treated as Fig. S7B. Levels of mTORC1, mTORC2 activity, and Rheb expression were monitored.

S7D. shRNA-mediated Rheb knockdown inhibits CQ+MG-induced lysosomal mTORC1 localization in p18 KO HEK293T cells. Endogenous Rheb was knocked down by the shRNA in p18 KO HEK293T cells. Cells were starved for amino acids for 60 min and then treated with Chloroquine (CQ, 50 μM) and MG-132 (MG, 20 μM) for another 75 min. Levels of lysosomal mTOR localization were monitored.

S7E. CRISPR-Cas9-mediated Rheb KO effectively inhibits CQ+MG-induced mTORC1 activation in p18 KO HEK293T cells. Endogenous Rheb was knocked out by the sgRNA in p18 KO HEK293T cells. Cells were treated as Fig. S7D, and levels of mTORC1 activity were monitored.

S7F. Rheb is required for mTORC1 activation induced by the CQ+MG treatment in p18 KO EMSC cells. Endogenous Rheb was knocked down (KD) by the Rheb shRNA, and the exogenous Flag-Rheb was re-expressed in p18 KD EMSC cells. The control cells (Sc: scramble shRNA), Rheb KD cells, and Rheb KD cells stably expressing Flag-Rheb were starved for amino acids for 50 min then treated with or without CQ+MG for another 75 min.

S7G. Identification of the lysine residues responsible for poly-ubiquitination of Rheb in p18 KO HEK293T. A panel of lysine to arginine (KR) mutations was made in the Rheb protein, and levels of ubiquitination of the indicated Rheb KR mutants were

monitored in p18 KO HEK293T cells. While levels of poly-ubiquitination in the K109, 135, 151, 169, 178R (5 KRs) Rheb mutant were largely diminished, levels of poly-ubiquitination in the K169R Rheb mutant were not decreased compared to the wild-type Rheb. In contrast, the levels of poly-ubiquitination in the K109R, K135R, K151R, or K178R mutant were partially decreased compared to the wild-type Rheb. Thus, the four lysine residues, which are evolutionarily conserved from *Drosophila* to human, are key lysine ubiquitination sites on Rheb protein.

S7H. Ub-Rheb contributes to CQ+MG-induced mTORC1 activation in p 18 KO HEK293T cells. CQ+MG treatment-induced mTORC1 activation was abolished in p18 KO HEK293T cells lacking endogenous Rheb expression (left panels). Wild-type Rheb (Flag-Rheb-WT) or the ubiquitination-deficient Rheb mutant (Flag-Rheb-4Rs) was re-expressed in the p18/Rheb KO HEK293T cells, and levels of CQ+MG treatment-induced mTORC1 activation were monitored at the single-cell level by staining pS6 and Rheb (middle panels). The ratio of p-S6 positive cell/Rheb positive cell was determined and quantified. Data were expressed as the mean±SEM, ****$p < 0.0001$, n (WT, 4Rs) = (98, 111).