EXTENDED EXPERIMENTAL PROCEDURES

Materials
Reagents were obtained from the following sources: antibodies to ATP6V1B2 and LAMP2 from Abcam; antibodies to phospho-T389 S6K1, S6K1, RagA, RagC, p14, p18, MP1, C7orf59, HBXIP mTOR, phospho-T398 dS6K, and the FLAG epitope from Cell Signaling Technology; HRP-labeled anti-mouse, and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; antibody to the HA tag from Bethyl laboratories; RPMI, FLAG M2 affinity gel, GTP, S, GDP, Chaps, Triton, and amino acids from Sigma Aldrich; [3H]GDP and [35S]GTP;S from Perkin Elmer; protein G-sepharose and immobilized glutathione beads from Pierce; FuGENE 6 and Complete Protease Cocktail from Roche; Alexa 488 and 568-conjugated secondary antibodies, Schneider’s media, Express Five Drosophila-SFM, and Inactivated Fetal Calf Serum (IFS) from Invitrogen; amino acid-free RPMI, and amino acid free Schneider’s media from US Biological; siRNAs targeting indicated genes and siRNA transfection reagent from Dharmacon; human cDNA encoding HBXIP from Open Biosystems; Concanamycin A from A.G. Scientific; nitrocellulose membrane filters from Advantec; calf-alkaline phosphatase from NEB. The dS6K antibody was a generous gift from Mary Stewart (North Dakota State University). Salicylhalamide A (SalA) was a generous gift from Jeff DeBrabander (UT Southwestern).

RNAi in Drosophila S2 cells
dsRNAs against Drosophila HBXIP and C7orf59 genes were designed as described in (Sancak et al., 2008). Primer sequences used to amplify DNA templates for dsRNA synthesis for dHBXIP and, dC7orf59 including underlined 5’ and 3’ T7 promoter sequences, are as follows. For dHBXIP (CG14812), forward primer: GAATTAATACGACTCACTATAGGGAGA; reverse primer: GAATTAATACGACTCACTATAGGGAGA. For dC7orf59 (CG14977), forward primer: GAATTAATACGACTCACTATAGGGAGA; reverse primer: GAATTAATACGACTCACTATAGGGAGA.

dsRNAs targeting GFP and dRagC were used as positive and negative 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 (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 hr. To stimulate with amino acids, the amino acid-free medium was replaced with complete Schneider’s medium for 30 min. Cells were then washed with ice cold PBS, lysed, and subjected to immunoblotting for phospho-T398 dS6K and total dS6K.

Immunofluorescence Assays
Immunofluorescence assays were performed as described in (Sancak et al., 2010). Briefly, 200,000 HEK293T cells were plated on fibronectin-coated glass cover slips 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. After rinsing twice with PBS, the slides were incubated with primary antibody in 5% normal donkey serum, and starved for amino acids by replacing the media with amino acid-free Schneider’s medium for 1.5 hr. To stimulate with amino acids, the amino acid-free medium was replaced with complete Schneider’s medium for 30 min. Cells were then washed with ice cold PBS, lysed, and subjected to immunoblotting for phospho-T398 dS6K and total dS6K.

Cell Size Determinations
For measurements of cell size, HEK293T cells treated with siRNAs as described above were harvested by trypsinization 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.

Protein Purification of Recombinant Rag Heterodimers and Ragulator
To produce protein complexes used for GEF or in vitro binding assays, 4,000,000 HEK293T cells were plated in 15 cm culture dishes. Forty-eight hours later, cells were transfected separately with the following constructs (all cDNAs were expressed from pRK5 expression plasmid). For pentameric Ragulator: 4 μg Flag-p14, 8 μg HA-MP1, 8 μg HA-p18G2A (a lipidation defective mutant), 8 μg HA-HBXIP, and 8 μg HA-C7orf59. For trimeric Ragulator complexes: 8 μg Flag-p14, 16 μg HA-MP1 and 16 μg HA-p18G2A; or 8 μg Flag-HBXIP, 16 μg HA-C7orf59 and 16 μg HA-p18G2A. For dimeric complexes: 8 μg Flag-p14 and 16 μg HA-MP1; 8 μg Flag-HBXIP and 16 μg HA-C7orf59; 8 μg Flag-RagCD181N and 16 μg HA-RagB; 8 μg of HA-GST-p14 and 16 μg of MP1; 8 μg Flag-RagBD163N and 16 μg HA-RagC; 8 μg Flag-RagCΔ181N and 16 μg HA-RagB; or 8 μg Flag-RagB and 16 μg HA-RagC. For individual
proteins: 10 μg Flag-p18G2A; 10 μg Flag-Metap2; 15 μg Flag-VPS39; 10 μg HA-GST-HBXIP, 10 μg HA-GST-C7orf59; or 10 μg HAGST-Rap2a.

Thirty-six hours after transfection cell lysates were prepared as described above and either 200 μl of a 50% slurry of glutathione affinity beads or 200 μl of a 50% slurry of FLAG-M2 affinity gel were added to lysates from cells expressing HA-GST-tagged or FLAG-tagged proteins, respectively. Recombinant proteins were immunoprecipitated for 3 hr 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. Samples containing FLAG-tagged proteins were eluted from the FLAG-M2 affinity gel with a competing FLAG peptide as described above.

**Monomeric Rag GDP Loading**

40 pmols of FLAG-RagB, FLAG-RagB^{S54N}, FLAG-RagB^{D163N}, FLAG-RagC, FLAG-RagC^{T75N} or FLAG-RagC^{D181N} were loaded with 2 μM of [3H]GDP as described for Rag heterodimers, but MgCl₂ stabilization lasted for 5 min at 25°C. The amount of [3H]GDP bound to monomeric Rags was determined with a filter binding assay and was normalized to [3H]GDP binding by wild-type RagB or RagC.

**SUPPLEMENTAL REFERENCES**


Figure S1. Secondary Structure Predictions of Rag and Ragulator Proteins Indicate the Presence of the Roadblock Domain and Rags Preferentially Interact with a Pentameric Ragulator Complex, Related to Figure 1

(A) Schematic amino acid alignment of human HBXIP and C7orf59 with their corresponding Drosophila orthologs.

(B) The presence of roadblock domains in Ragulator and Rag proteins. Secondary structure predictions of the indicated proteins using Jpred 3 secondary structure prediction server (Cole et al., 2008). The dashed box outlines the canonical roadblock domain predicted in each protein.

(C) Rags preferentially interact with a pentameric Ragulator complex. In vitro binding assay in which recombinant HA-GST-tagged-RagB-RagC, were incubated with the indicated purified FLAG-tagged Ragulator complexes. HA-GST precipitates were analyzed by immunoblotting for levels of the indicated proteins.

(D) RagA and RagB are highly similar. Amino acid sequence alignment of RagA and RagB indicates that the two proteins are 98% identical. RagB contains an N-terminal extension that increases its molecular weight compared to RagA.

(E) RagA is much more abundant than RagB in HEK293T cells. HEK293T cell lysate was analyzed by immunoblotting for RagA and RagB with an antibody from CST that recognizes the same epitope in both proteins.
Figure S2. HBXIP and C7orf59 Are Required for the Localization of RagC but Not p18 to the Lysosomal Surface, Related to Figure 2
(A) Images of HEK293T cells, treated with a nontargeting siRNA or siRNAs targeting HBXIP or C7orf59, coimmunostained for RagC (red) and LAMP2 (green) and processed for imaging.
(B) Images of HEK293T cells, treated with a nontargeting siRNA or siRNAs targeting HBXIP or C7orf59, coimmunostained for p18 (red) and LAMP2 (green). Cells were treated and processed as in (A). In all images, insets show selected fields that were magnified five times and their overlays. Scale bars represent 10 μm. See also Figure S2.
Figure S3. Amino Acids Regulate the Ragulator-mTORC1 Interaction, Related to Figure 3

(A) Amino acid stimulation increases the amount of endogenous mTORC1 that coimmunoprecipitates with recombinant p14 and p18. HEK293T cells stably expressing FLAG-p14 or FLAG-p18 were starved for amino acids for 2 hr or starved and stimulated with amino acids for 15 min. After in-cell crosslinking, anti-FLAG immunoprecipitates were prepared from cell lysates and analyzed by immunoblotting for levels of indicated proteins.

(B) Amino acids regulate the amount of endogenous mTORC1 that coimmunoprecipitates with recombinant C7orf59 and HBXIP. HEK293T cells stably expressing FLAG-C7orf59 or FLAG-HBXIP were treated as in (A) and anti-FLAG immunoprecipitates were analyzed for the levels of the indicated proteins.

(C) Inter-Ragulator interactions are moderately regulated by amino acids. HEK293T cells, transfected with the indicated cDNAs in expression vectors, were starved for amino acids for 2 hr or starved and stimulated for 15 min and anti-FLAG immunoprecipitates were analyzed for the levels of the indicated proteins.

(D) Quantification of endogenous RagA and RagC binding to recombinant p14 and p18 upon amino acid starvation and re-stimulation. Each value represents the normalized mean ± SE for n = 2.

(E) Quantification of endogenous RagA and RagC binding to recombinant C7orf59 and HBXIP upon amino acid starvation and re-stimulation. Each value represents the normalized mean ± SE for n = 2.

(F) Quantification of endogenous Ragulator proteins binding to recombinant RagB upon amino acid starvation and re-stimulation. Each value represents the normalized mean ± SE for n = 2.
Figure S4. GTP Destabilizes the Rag-Ragulator Complex Both In Vitro and In Vivo, Related to Figure 4
(A) Quantification of endogenous Ragulator proteins binding to recombinant RagB in the absence and presence of EDTA.
(B) Excess GTP destabilizes the Rag-Ragulator interaction. In vitro binding assay in which FLAG-RagB-RagC was prebound to HA-GST-Ragulator and then further incubated in the absence or presence of GTPγS. HA-GST precipitates were analyzed by immunoblotting for the levels of the indicated proteins.
(C) Quantification of the binding of GDP to RagBT54N or RagCS75N. Proteins were loaded with [3H]GDP and the amount of GDP bound was determined by filter-binding assays. Each value represents the normalized mean ± SD of four independent samples.
(D) Quantification of binding to GDP to RagBD163N (RagBX) or RagCD181N (RagCX). Proteins were loaded with [3H]GDP and the amount of GDP bound was determined by filter-binding assays. Each value represents the normalized mean ± SD of four independent samples.
(E) The nucleotide binding state of RagB governs the Rag-Ragulator interaction. Anti-FLAG immunoprecipitates were prepared from HEK293T cells transfected with the indicated cDNAs in expression vectors and cell lysates and immunoprecipitates were analyzed by immunoblotting for levels of indicated proteins.
Figure S5. The Nucleotide State of RagC Does Not Alter Ragulator Activity toward RagB and VPS39 Does Not Function as a GEF for RagB, Related to Figure 5

(A) Ragulator stimulates GDP dissociation from RagB. Nucleotide dissociation assay in which RagB-RagCD181N was loaded with [3H]GDP and incubated with Ragulator or a control. Dissociation was monitored by a filter-binding assay and is reported as pmoles of [3H]GDP per mg of RagB-RagCD181N. Each value represents the normalized mean ± SD for n = 4.

(B) Ragulator increases GTPγS binding to RagA. RagA-RagCD181N loaded with GDP was incubated with Ragulator or a control and [35S]GTPγS. [35S]GTPγS binding was determined as in (A) and is reported as pmoles of [35S]GTPγS per mg of RagA-RagCD181N. Each value represents the normalized mean ± SD for n = 4.

(C) Ragulator stimulates GDP and GTPγS dissociation in a dose dependent manner. Dissociation assay in which RagB-RagCD181N was loaded with either [3H]GDP or [35S]GTPγS, and incubated with the indicated amounts of Ragulator and analyzed as in (A). Each value represents the normalized mean of two independent samples.

(D) The nucleotide-binding state of RagC does not alter Ragulator-mediated GDP dissociation from RagB. Dissociation assay in which RagB-RagCD181N was loaded with either XDP or XTPγS and [3H]GDP and incubated with Ragulator or a control and analyzed as in (A). Each value represents the normalized mean ± SD for n = 4.

(E) Ragulator-mediated GTP dissociation from RagB is not affected by RagC nucleotide binding. Dissociation assay in which RagB-RagCD181N was loaded with either XDP or XTPγS and [35S]GTPγS and incubated with Ragulator or a control and analyzed as in (A). Each value represents the normalized mean ± SD for n = 4.

(F) VPS39 does not interact with endogenous Rags. Anti-FLAG immunoprecipitates were prepared from HEK293T cells transfected with the indicated cDNAs in expression vectors. Cell lysates and immunoprecipitates were analyzed by immunoblotting for levels of indicated proteins.

(G) VPS39 does not stimulate GDP or GTP dissociation from RagB. Dissociation assay in which RagB-RagCD181N was loaded with either [3H]GDP or [35S]GTPγS, and incubated with either VPS39, Ragulator or a control and analyzed as in (A). Each value represents the normalized mean ± SD for n = 4.
Figure S6. v-ATPase Inhibition Decreases the Regulated Interaction between Rags and Ragulator, Related to Figure 6

(A) Inactivation of the v-ATPase inhibits the amino acid dependent regulated interaction between Ragulator and Rags. HEK293T cells stably expressing FLAG-p18 were starved for amino acids for 2 hr or starved and stimulated with amino acids for 15 min in the absence or presence of the v-ATPase inhibitor ConA. Cell lysates and anti-FLAG immunoprecipitates were analyzed by immunoblotting for the levels of the indicated proteins.