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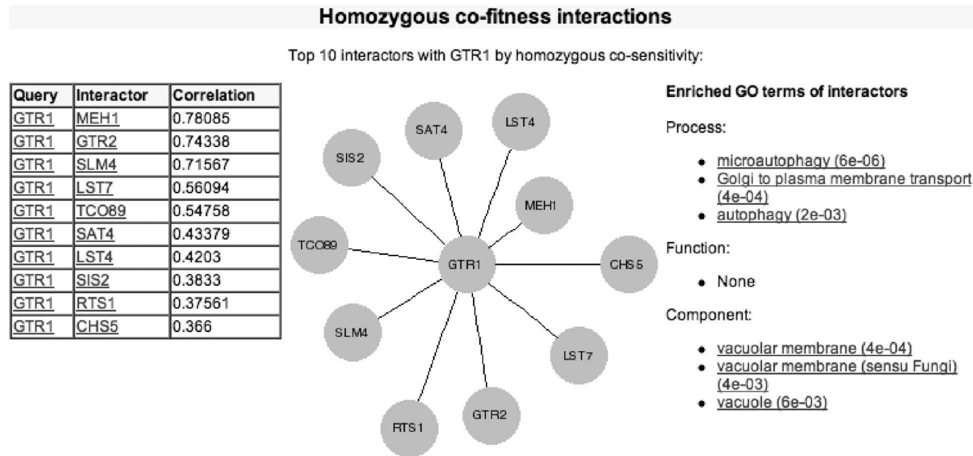

Query	Interactor	Correlation
GTR1	MEH1	0.78085
GTR1	GTR2	0.74338
GTR1	SLM4	0.71567
GTR1	LST7	0.56094
GTR1	TCO89	0.54758
GTR1	SAT4	0.43379
GTR1	LST4	0.4203
GTR1	SIS2	0.3833
GTR1	RTS1	0.37561
GTR1	CHS5	0.366

Figure S1, related to Figure 2. Yeast orthologs of FLCN and Rag GTPases have similar growth sensitivities

(A) Top 10 interactors with GTR1 (Yeast RagA/B ortholog) include GTR2 (Yeast RagC/D ortholog) and LST7 (Yeast FLCN ortholog) in terms of growth sensitivities to various environmental and chemical insults. Output from querying "GTR1" at: http://chemogenomics.stanford.edu/supplements/cofitness/index.html (Hillenmeyer et al., 2008)

Movie S1, related to Figure 3. FLCN localizes to the lysosomal surface

Spinning disk confocal video of HEK293T cells co-expressing FLCN-GFP, HA-FNIP2, and mRFP-LAMP1. FLCN and LAMP1 is pseudo-colored green and red, respectively.

A

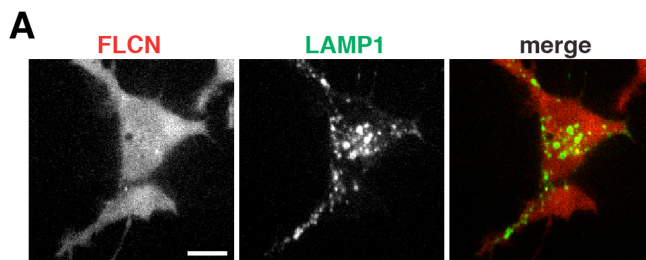

Figure S2, related to Figure 3. FLCN requires FNIP2 to localize to the lysosomal surface

(A) Spinning disk confocal image of a HEK-293T cell co-expressing FLCN-GFP and mRFP-LAMP1 (pseudo-colored red and green in merge, respectively).

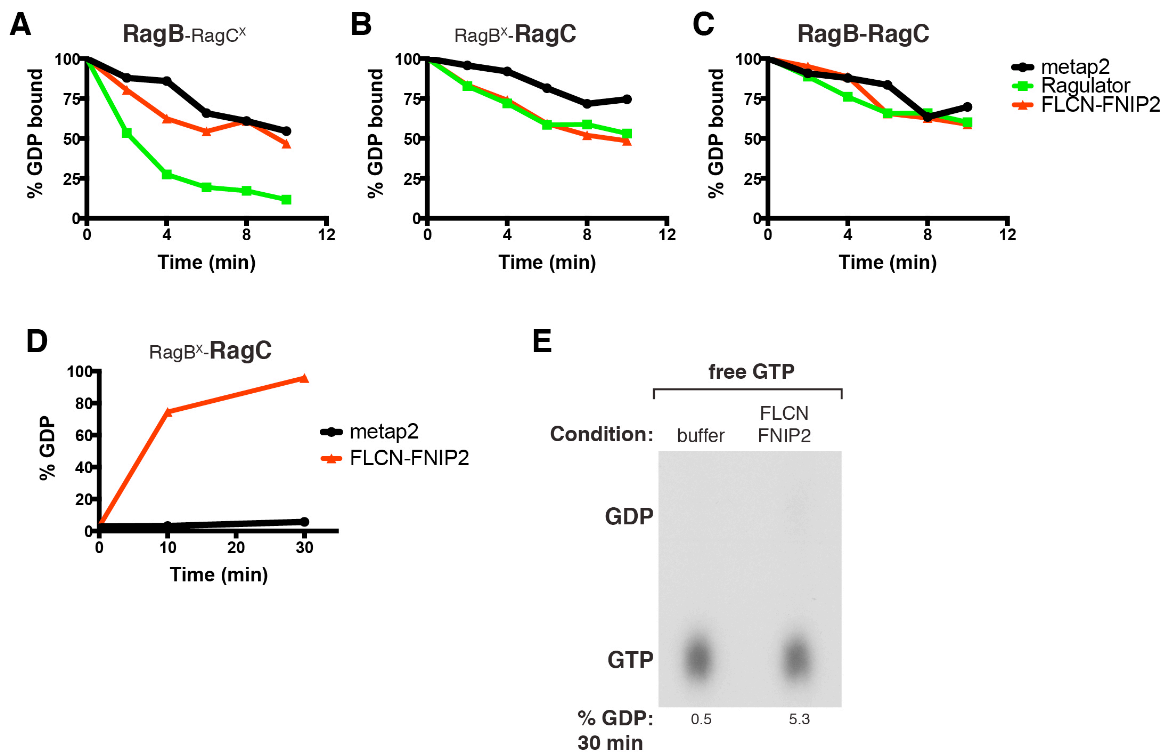

Figure S3, related to Figure 4. FLCN-FNIP2 does not have GEF activity, FLCN-FNIP2 stimulates RagC GTPase activity in a time-dependent manner, and purified FLCN-FNIP2 does not contain significant contaminating phosphatases

(A) FLCN-FNIP2 does not stimulate GDP dissociation from RagB. Dissociation assay in which RagB-RagC^X was loaded with [^{3}H]GDP, and incubated with FLCN-FNIP2, Ragulator positive control, or metap2 control protein. Dissociation was monitored by a filter-binding assay. Each value represents the average of a replicate.

(B) FLCN-FNIP2 does not stimulate GDP dissociation from RagC. Dissociation assay with $RagB^X$-RagC was performed as described in (A).

(C) FLCN-FNIP2 does not stimulate GDP dissociation from wild-type RagB-RagC. Dissociation assay with RagB-RagC was performed as described in (A).

(D) FLCN-FNIP2 stimulates GTP hydrolysis by RagC in a time-dependent manner. 5 pmol of $RagB^X$-RagC was loaded with [α-^{32}P]GTP and incubated with indicated proteins (20 pmol) for indicated times. GTP hydrolysis was determined by thin-layer chromatography. Each value represents the average of two experiments.

(E) Purified FLCN-FNIP2 does not contain significant amounts of contaminating phosphatases. The purified FLCN-FNIP2 used in Figure 4 was incubated with free [α-^{32}P]GTP. This amount of hydrolysis is representative of separate FLCN-FNIP2 purifications. Value represents the quantification in the incubation shown.

Supplemental Experimental Procedures

Materials

Reagents were obtained from the following sources: HRP-labeled anti-mouse and anti-rabbit secondary antibodies and LAMP2 antibody from Santa Cruz Biotechnology; antibodies to phospho-T389 S6K1, S6K1, phospho-S473-Akt, Akt, phospho-T398 dS6K, RagA, RagC, p18, mTOR, FLCN, and the FLAG epitope from Cell Signaling Technology; antibodies to the HA epitope from Bethyl laboratories; antibodies to raptor from Millipore. RPMI, FLAG M2 affinity gel, ATP, GDP, and amino acids were from Sigma Aldrich; DMEM from SAFC Biosciences; XtremeGene9 and Complete Protease Cocktail from Roche; Alexa 488 and 568-conjugated secondary antibodies, Schneider’s media, Express Five-SFM, and Inactivated Fetal Calf Serum (IFS) from Invitrogen; amino acid-free RPMI, and amino acid-free Schneider’s media from US

Biological; Cellulose PEI TLC plates from Sorbent Technologies; [α-^{32}P]GTP from Perkin Elmer; GTP, XTP and XDP from Jena Biosciences; nitrocellulose membrane filters from Advantec; DSP and Glutathione beads from Pierce. Torin1 from Dr. Nathanael Gray (DFCI). The dS6K antibody was a generous gift from Mary Stewart (North Dakota State University).

Identification of FLCN, FNIP1, and FNIP2 as Rag-interacting proteins

HEK-293T cells stably expressing FLAG-tagged metap2, RagA, RagB, RagC or RagD were subjected to FLAG-immunoprecipitation as described above. Proteins were eluted with the FLAG peptide (sequence DYKDDDDK) from the FLAG-M2 affinity gel, resolved on 4-12% NuPage gels (Invitrogen), and stained with simply blue stain (Invitrogen). Each gel lane was sliced into 10-12 pieces and the proteins in each gel slice digested overnight with trypsin. The resulting digests were analyzed by mass spectrometry as described (Sancak et al., 2008). Peptides corresponding to FLCN and FNIP1 and FNIP2 were detected in the FLAG-RagA, -B, -C, and -D immunoprecipitates, while no such peptides were detected in negative control immunoprecipitates of FLAG-metap2.

RNAi in *Drosophila* S2 cells

dsRNAs against *Drosophila FLCN* were designed as described in (Sancak et al., 2008). Primer sequences used to amplify DNA templates for dsRNA synthesis for dFLCN including underlined 5' and 3' T7 promoter sequences, are as follows:

dFLCN (CG8616)

Forward primer CG8616_1F:

GAA<u>TTAATACGACTCACTATAGGGAGA</u> AGAATAACAATGCGATCTACAGCAG

Reverse primer CG8616_1R:

GAATTAATACGACTCACTATAGGGAGA GAAGGTGTGACTCAGGATGTGA

Forward primer CG8616_2F:

GAATTAATACGACTCACTATAGGGAGA GTACAAAATCATATCCGTGTCCAAT

Reverse primer CG8616_2R:

GAATTAATACGACTCACTATAGGGAGA GAAGGTGTGTGCTCCAGTAGTTAAT

dsRNAs targeting GFP and dRagB (Sancak et al., 2008) were used as negative and positive controls, respectively. On day one, 4,000,000 S2 cells were plated in 6-cm culture dishes in 4 ml of Express Five SFM media. Cells were transfected with 1 μg of dsRNA per million cells using XtremeGene9. Two days later, a second round of dsRNA transfection was performed. On day four, cells were transferred to a fibronectin coated 6-cm culture dish. 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 hours. To stimulate with amino acids, the amino acid-free medium was replaced with complete Schneider's medium for 30 minutes. Cells were then washed with ice cold PBS, lysed in Triton lysis buffer, and subjected to immunoblotting for phospho-T398 dS6K and total dS6K.

In Vitro Binding Assays

For the binding reactions, 20 μl of a 50% slurry containing immobilized HA-GST-tagged proteins were incubated in binding buffer (0.3% CHAPS, 2.5 mM $MgCl_2$, 40 mM HEPES [pH 7.4], 2 mM DTT, and 1 mg/ml BSA) with 2 μg of FLAG-raptor, FLAG-metap2, or FLAG-FNIP2-HA-FLCN in a total volume of 50 μl for 1 hr and 30 min at 4°C. Where indicated, Rags were loaded with the indicated nucleotides as previously described (Bar-Peled et al., 2012). To terminate binding assays, samples were washed twice times with 1 mL of ice-cold binding buffer supplemented with 150 mM NaCl

followed by the addition of 50 μl of sample buffer.

Nucleotide Exchange Assays

GEF assays were performed as described in (Bar-Peled et al., 2012). Briefly, the indicated purified Rag GTPases were loaded with XTPγS and [^{3}H]GTP at room temperature followed by an incubation with $MgCl_2$ to stabilize the nucleotide. To initiate GEF assay, purified FLCN-FNIP2, Ragulator, or metap2 proteins were added to Rags along with GTPγS. Samples were taken every 2 minutes and spotted on nitrocellulose filters, which were washed. Filter-associated radioactivity was measured using a TriCarb scintillation counter (PerkinElmer).

Supplemental References

Bar-Peled, L., Schweitzer, L.D., Zoncu, R., and Sabatini, D.M. (2012). Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. Cell *150*, 1196-1208.

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Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., and Sabatini, D.M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science *320*, 1496-1501.