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SUPPLEMENTARY INFORMATION

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A nutrient-induced affinity switch controls mTORC1 activation by its Rag GTPase–Ragulator lysosomal scaffold

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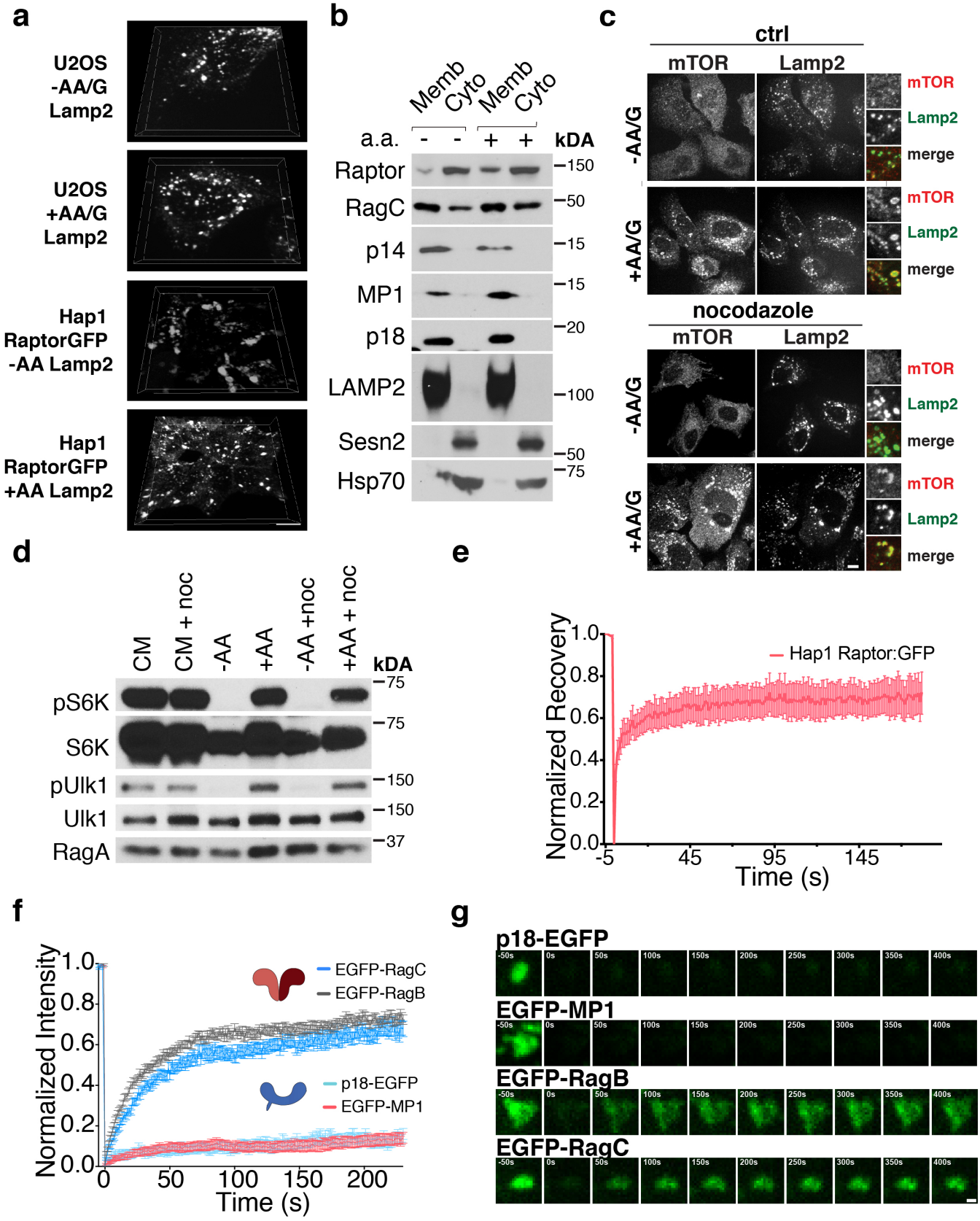
Figure S1: Lawrence, RE et al



Supplementary Figure 1

Control experiments for FRAP analysis of mTORC1 pathway components

(a) Representative 3D images of endogenous LAMP2 staining from U2OS cells that were starved for amino acids and glucose (-AA/G) or starved and restimulated (+AA/G) (*top),* and HAP-1 Raptor:GFP cells that were starved for amino acids (-AA) or starved and restimulated (+AA) *(bottom)*, followed by 3-D volumetric analysis of z-stacks. Scale bar 10 μm. Experiment repeated 2 times.

(b) Subcellular fractionation. HEK-293T cells were starved of amino acids for 1 hour, or starved for 50 minutes then restimulated with amino acids for 10 minutes, followed by fractionation and collection of light membrane and cytoplasm. Fractions were immunoblotted for the indicated proteins. Experiment repeated 2 times. Unprocessed scans are shown in Supplementary Fig. 8

(c) mTOR localization in U2OS cells is not affected by incubation in nocodazole. U2OS cells were starved for amino acids and glucose, or starved and restimulated, and 2.5 μg/mL nocodazole was added for the last 20 minutes where indicated. Cells were fixed and subjected to immunofluorescence for mTOR and LAMP2. Scale bar 10 μm. Experiment repeated 2 times.

(d) mTOR signaling in U2OS cells is not affected by nocodazole treatment. U2OS cells were starved for amino acids and glucose, or starved and restimulated, and 2.5 μg/mL nocodazole was added for the last 20 minutes where indicated. Cells were lysed, followed by immunoblotting for the indicated proteins and phosphor-proteins. Experiment repeated 2 times. Unprocessed scans are shown in Supplementary Fig. 8

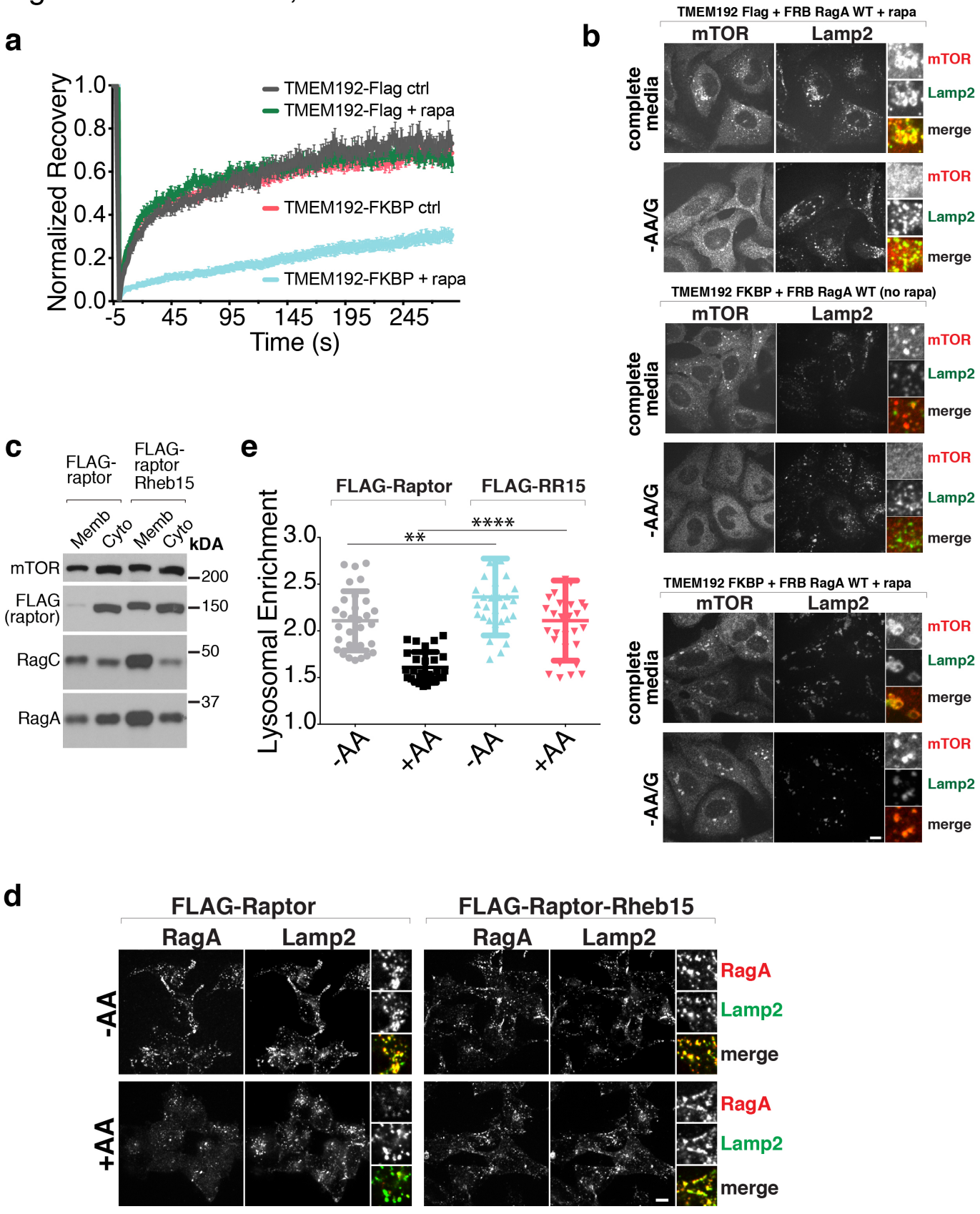
(e) Fluorescence recovery over time curves from FRAP experiments in Raptor:GFP edited HAP-1 cells. Curve is the average ± S.E.M. of N=15 lysosomes.

(f) Fluorescence recovery over time curves from FRAP experiments in **(g)**. Each curve is the average ± S.E.M. of [EGFP-MP1 N=23, p18-EGFP +AA N=28, EGFP-RagB N=24, EGFP-RagC N=21] lysosomes.

(g) Time-lapse montage of single lysosome fluorescence recovery after photobleaching (FRAP) in U2OS cells expressing the indicated GFP-tagged Ragulator and Rag GTPase constructs (along with non-fluorescent Rag heterodimer partner). Scale bar 1 μm.

See Supplementary Table 1 for statistical source data.

Figure S2: Lawrence, RE et al



Supplementary Figure 2

Supplementary Figure 2: The Rag-mTORC1 complex is transient and can be stabilized by anchoring either component to the lysosomal membrane.

(a) Fluorescence recovery over time curves from FRAP experiments in U2OS cells stably expressing the indicated TMEM192 complex along with FRB-myc RagC and GFP-RagB. Cells were treated with the indicated concentration of rapalogue (rapa) for 30 minutes prior to imaging. Each curve is the average ± S.E.M. of [TMEM192-FLAG ctrl N=27, TMEM192-FLAG +rapa N=23, TMEM192-FKBP ctrl N=31, TMEM192-FKBP +rapa N=23] lysosomes.

(b) Immunofluorescence of mTOR and Lamp2 in U2OS cells stably expressing the indicated constructs. Cells were either kept in complete media or treated for two hours in amino acid- and glucose-depleted media. Where indicated, cells were treated with 50 nM rapalogue (rapa) prior to fixation. Scale bar 10 μm. Experiment repeated 3 times.

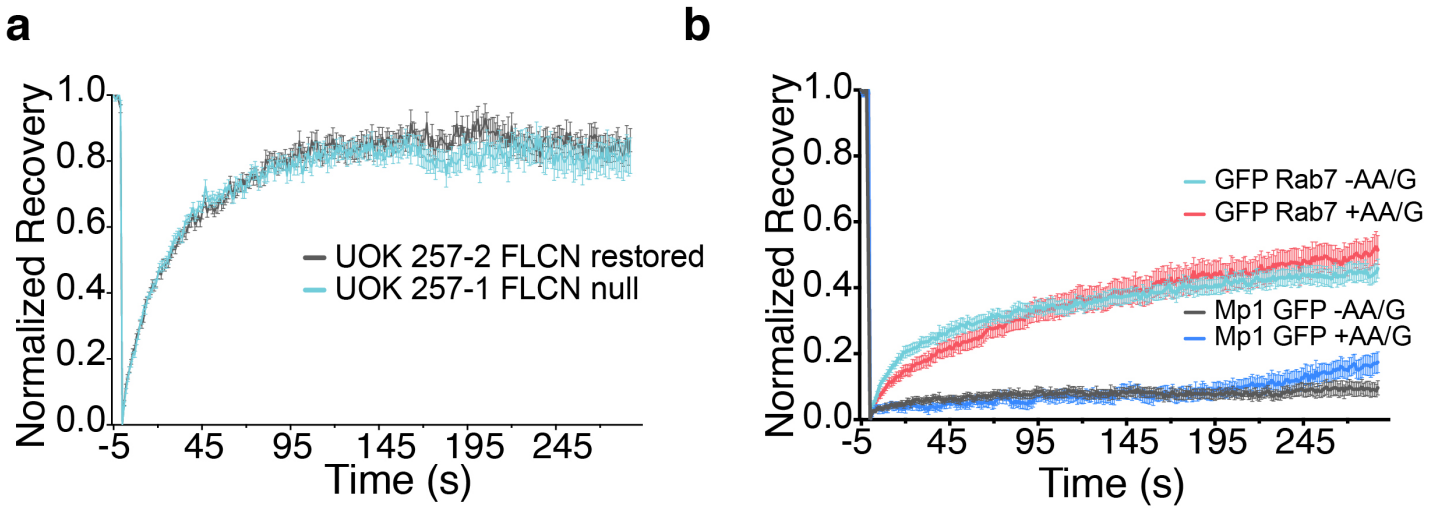
(c) HEK-293T cells stably expressing FLAG-Raptor or FLAG-Raptor-Rheb15 were starved of amino acids for 1 hour, or starved for 50 minutes then restimulated with amino acids for 10 minutes, followed by fractionation and collection of light membrane and cytoplasm fractions. Fractions were immunoblotted for the indicated proteins. Experiment repeated 3 times. Unprocessed scans are shown in Supplementary Fig. 8

(d) HEK- 293T cells expressing FLAG-Raptor or FLAG-Raptor-Rheb15 were starved of amino acids for 1 hour, or starved for 50 minutes then restimulated with amino acids for 10 minutes, followed by immunostaining for the indicated proteins. Scale bar 10 μm.

(e) Quantitation of RagA Lysosomal Enrichment Score for IF images in **(d)** (mean ± S.D., [grey N=30 black N=28, blue N=30, red N=29] cells/condition respectively, $p = 0.0094$; ****$p < 0.0001$ two-sided unpaired t-tests).

See Supplementary Table 1 for statistical source data.

Figure S3: Lawrence, RE et al



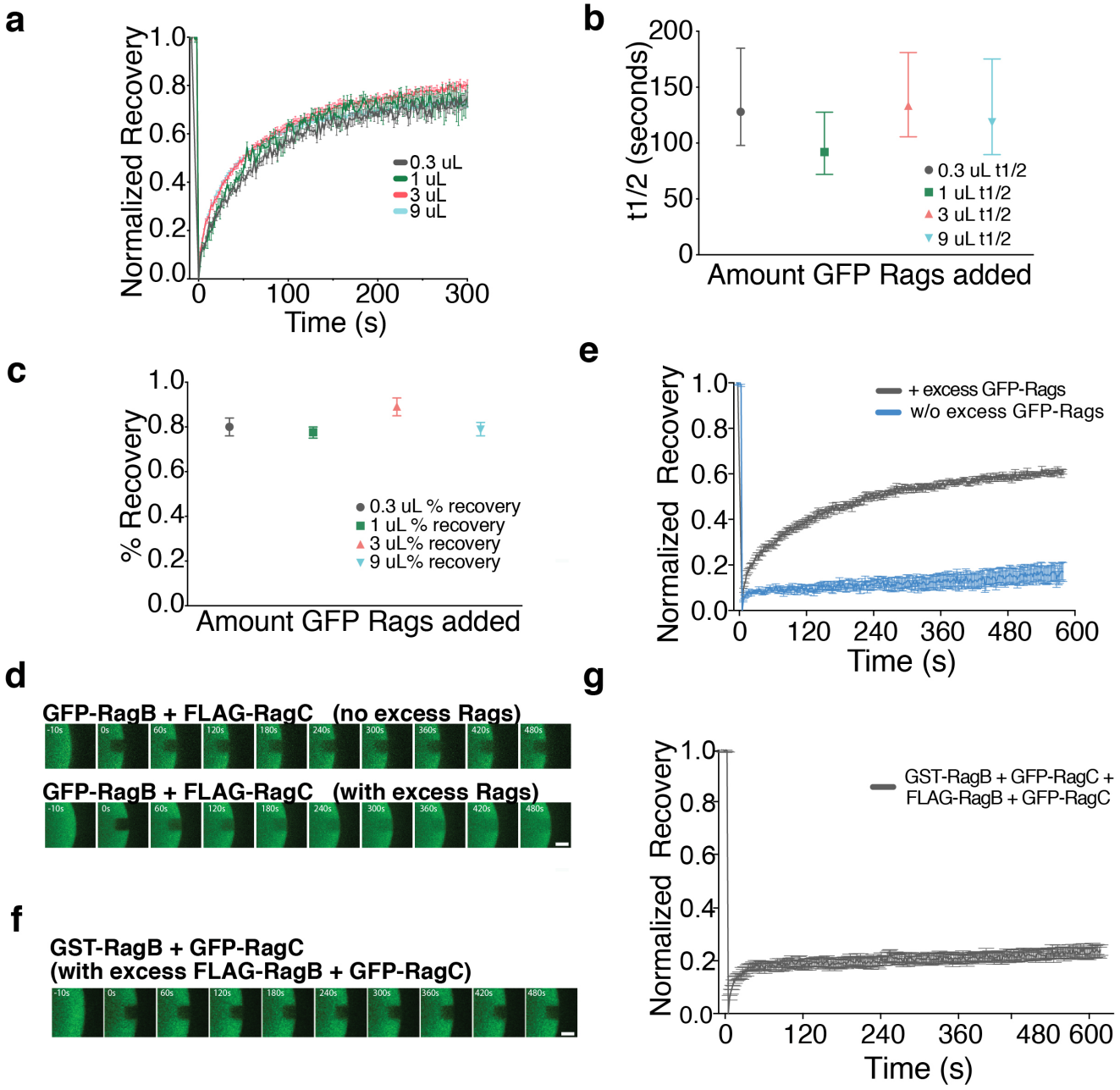
Supplementary Figure 3

Rag GTPase cycling is independent of the RagC/D GAP, FLCN

(a) Fluorescence recovery over time curves from FRAP experiments in UOK257-1 FLCN-null or UOK257-2 FLCN rescue cells. Each curve is the average ± S.E.M. of [UOK257-1 N=25, UOK257-2 N=27] lysosomes.

(b) MP1 and Rab7 FRAP is independent of nutrient conditions. Fluorescence recovery over time curves from FRAP experiments in U2OS cells expressing GFP-tagged MP1 or Rab7. Cells were either starved or restimulated for amino acids and glucose. Each curve is the average ± S.E.M. of [Mp1 –AA/G N=27, Mp1 +AA/G N=27, Rab7 –AA/G N=30, Rab7 +AA/G N= 22] lysosomes.

Figure S4: Lawrence, RE et al



Supplementary Figure 4

Control experiments for *in vitro* FRAP of Rag GTPases

(a) *In vitro* FRAP experiment in which Ragulator-coated beads were incubated with increasing amounts of GFP-RagB + Flag RagC. Each curve is the average ± S.E.M. of [0.3 uL N=11, 1 uL N=8, 3 uL N=11, 9 uL N=12] bead regions. Experiment repeated 2 times.

(b) Halftime ($t_{1/2}$) calculations on single exponential fits of FRAP recovery curves in **(a)**. Shown are best fit

values with 95% confidence intervals.

(c) Recovery fraction calculations on single exponential fits of FRAP recovery curves in **(a)** the presence of increasing concentrations of soluble GFP-tagged Rag GTPases. Shown are best fit values with 95% confidence intervals.

(d) Montages showing fluorescence recovery over time for *in vitro* FRAP experiment of GFP-labeled Rag GTPases bound to GST-tagged Ragulator on beads, either in the presence or absence of excess GFP-labeled Rag GTPase heterodimers. Scale bar 10 μm.

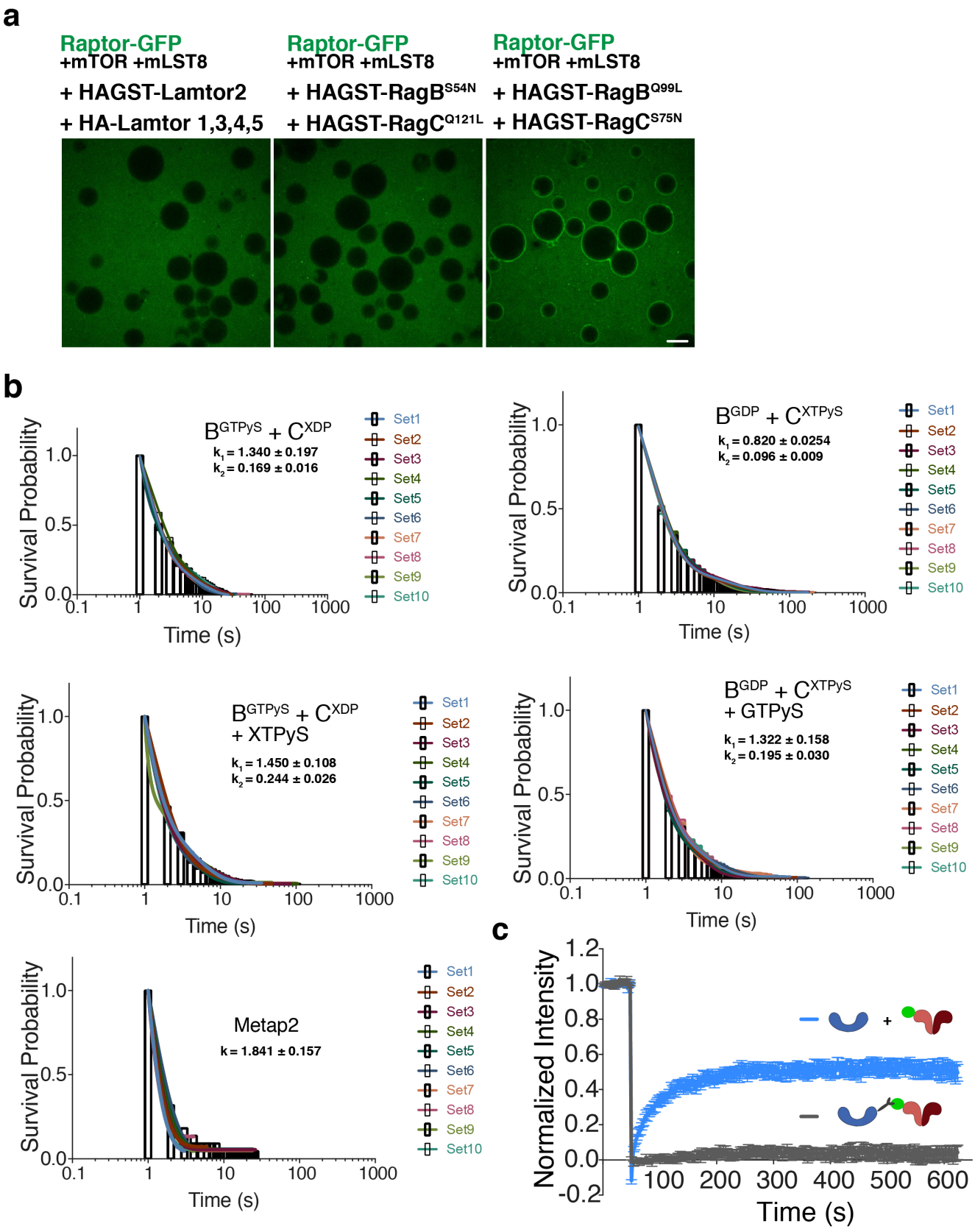
(e) Fluorescence recovery over time curves for **(d)**. Fluorescence recovery occurs only when excess GFP-Rag heterodimers are present, indicating absence of lateral diffusion of the bead-bound Ragulator-Rag complexes into the bleached area. Each curve is the average ± S.E.M. of [+excess GFP-Rags N=12, w/o excess GFP Rags N=9] bead regions.

(f) Montages showing fluorescence recovery over time for *in vitro* FRAP experiment of bead-bound GST-RagB + GFP-RagC in the presence of excess FLAG-RagB + GFP-RagC. Scale bar 10 μm.

(g) Fluorescence recovery over time curves for **(f).** No fluorescence recovery occurs, indicating that Rag heterodimers are stable and that no GFP-RagC exchanges between bead-bound GST-RagB and soluble FLAG-RagB. Curve is the average ± S.E.M. of N=12 bead regions.

See Supplementary Table 1 for statistical source data.

Figure S5: Lawrence RE et al



Supplementary Figure 5

Single molecule studies of mTORC1 scaffolding interactions

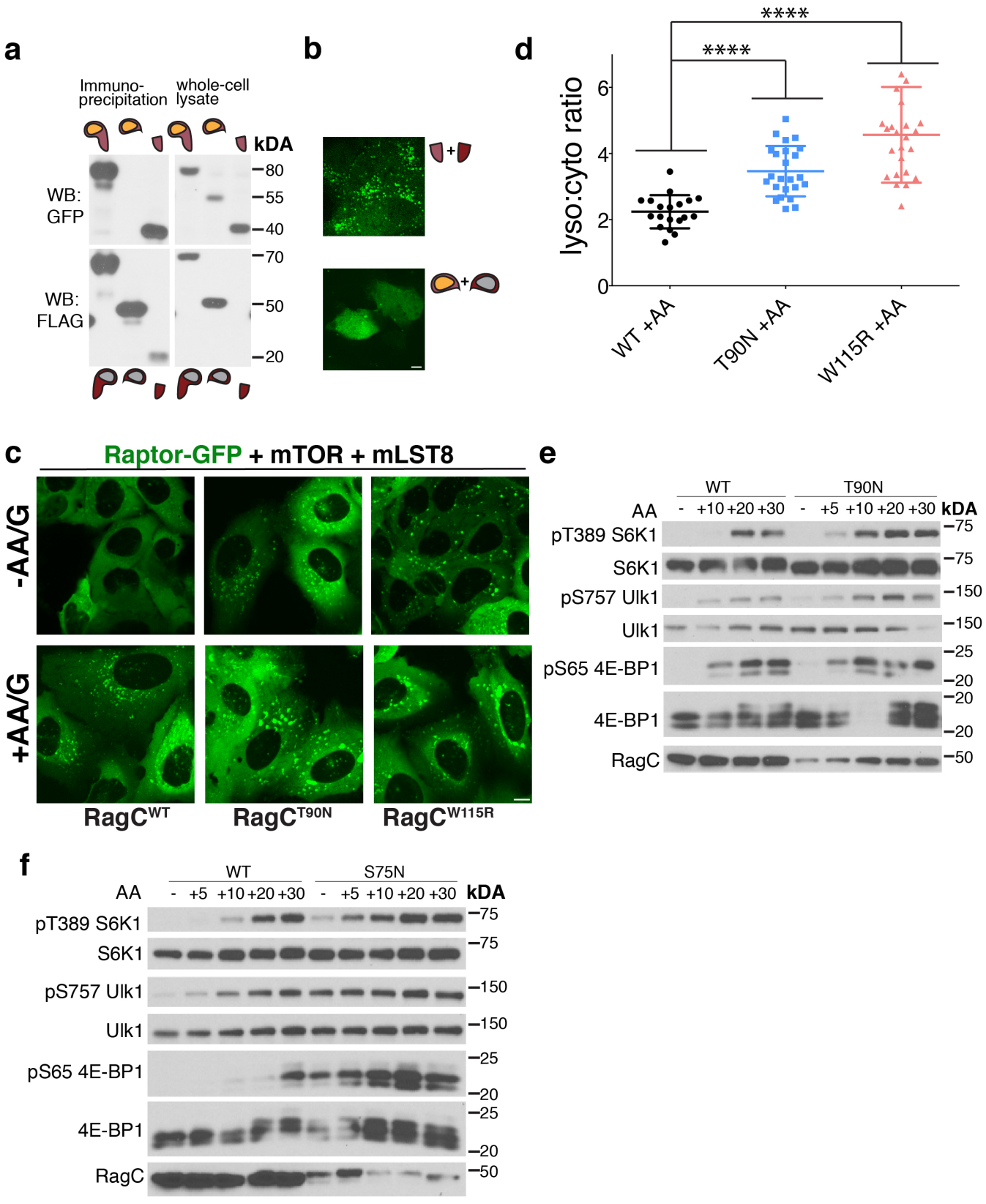
(a) Confocal images of glutathione beads coated with GST-Ragulator (left) or GST-inactive-loaded Rags (middle) or GST-active-loaded Rags (right) and incubated with an excess of GFP-Raptor (co-expressed along with mTOR and mLST8). Notice binding of GFP-Raptor to the surface of beads bearing active Rags, but not inactive Rags or Ragulator. Scale bar 100 μm. Experiment repeated 3 times.

(b) Two-component exponential fits of survival probability curves determined from single molecule imaging of the indicated nucleotide loading combinations of GFP-tagged Rag GTPase heterodimers binding to GST-Ragulator on GST affinity beads. Graphs were randomly generated from ten percent of all single-molecule detections per condition. Datasets were each modeled by a two-component exponential fit and slow and fast time constants are reported as average +/- SEM.

(c) Fluorescence recovery over time curves from FRAP experiments in U2OS cells expressing p18-VhH (p18 fused with a GFP nanobody) or p18 alone along with GFP-tagged Rag GTPases. Each curve is the average ± S.E.M. [blue N=15, grey, N=17] lysosomes. Experiment repeated 2 times.

See Supplementary Table 1 for statistical source data.

Figure S6: Lawrence, RE et al



Supplementary Figure 6

Investigation of Rag truncation interactions and the effects of RagC cancer mutant expression on mTORC1 accumulation on lysosomes

(a) HEK-293T cells were transiently transfected with FLAG-tagged full-length or truncated RagB, along with GFP-tagged, full-length or truncated RagC. Cells were subjected to lysis and FLAG immunoprecipitation, followed by western blotting for FLAG and GFP. Experiment performed 1 time, Unprocessed scans are shown in Supplementary Fig. 8

(b) Subcellular localization of the indicated truncated GFP-RagB + FLAG-RagC heterodimers in U2OS cells. Scale bar 10 μm. Experiment repeated 2 times.

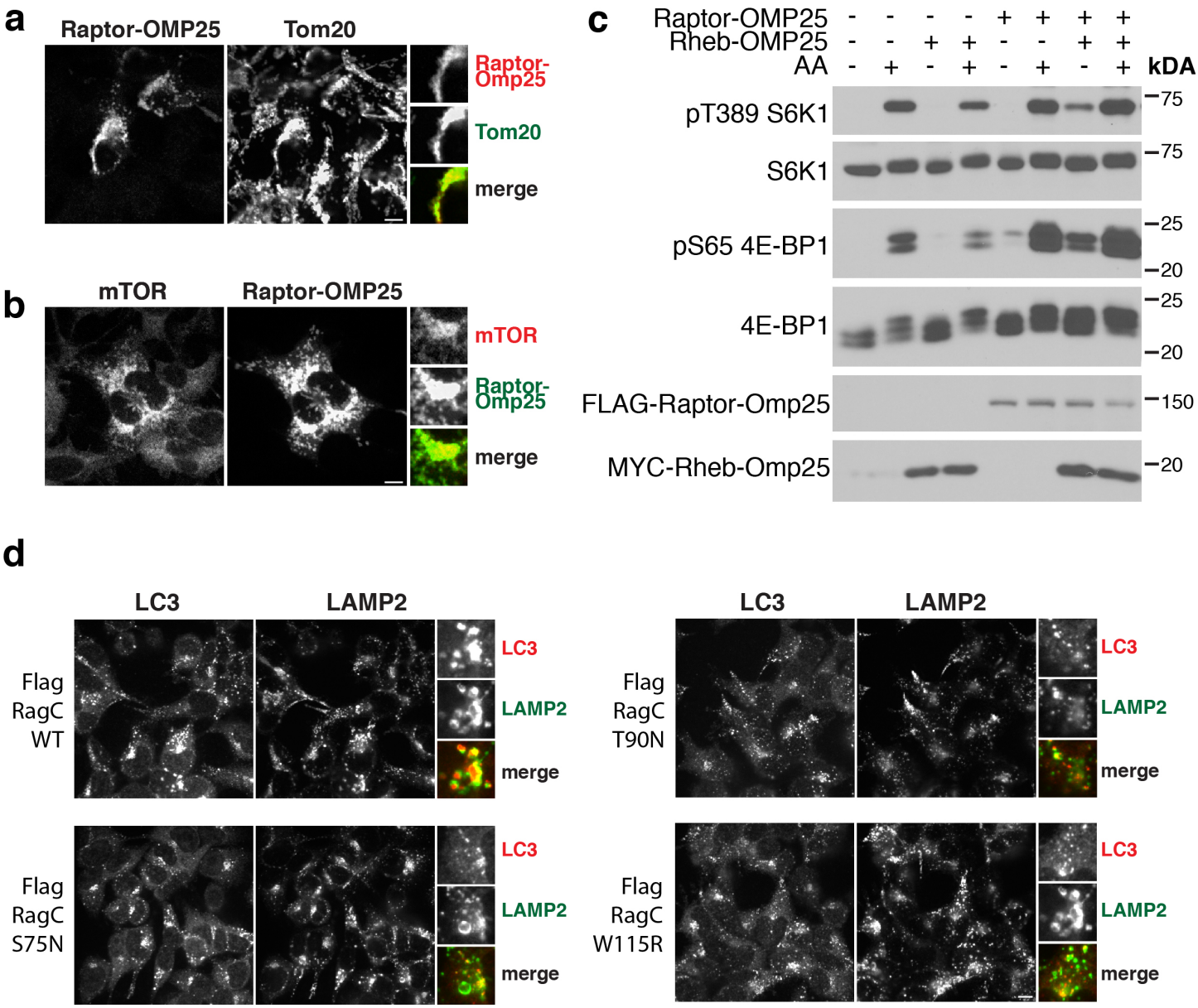
(c) Images of Raptor:GFP localization in U2OS cells stably coexpressing RaptorGFP and Flag-tagged RagC containing the indicated mutations. Cells were maintained in the indicated nutrient conditions for 2 hours. Scale bar 10 μm. Experiment repeated 2 times.

(d) Quantitation of the ratio between lysosomal and cytosolic fluorescence intensities (lyso:cyto ratios) from the images in **(c)** (mean ± S.D., N =19, 24, 24 cells/condition respectively, left to right, **** $p<.0001$, two-sided unpaired t-tests).

(e,f) 293T cells stably expressing the indicated FLAG-RagC constructs were starved for amino acids for 90 min, or starved and restimulated for the indicated times (5 min, 10 min, 20 min, or 30 min), followed by cell lysis and immunoblotting for the indicated proteins and phosphor-proteins. Experiment repeated 3 times. Unprocessed scans are shown in Supplementary Fig. 8

See Supplementary Table 1 for statistical source data.

Figure S7: Lawrence, RE et al



Supplementary Figure 7

mTORC1 efficiently phosphorylates substrates when localized to mitochondrial membranes that contain Rheb

(a) Immunofluorescence images of HEK-293T cells expressing FLAG-Raptor-Omp25 stained for FLAG and for the TOM20 mitochondrial marker. Scale bar 10 μm. Experiment repeated 2 times.

(b) Immunofluorescence images of HEK-293T cells expressing Flag-Raptor-Omp25 (Raptor-OMP25) stained for endogenous mTOR and for FLAG (Raptor-OMP25). Experiment repeated 2 times.

(c) U2OS cells transiently overexpressing FLAG-Raptor-Omp25 (Raptor-OMP25) and/or MYC-Rheb-Omp25

(Rheb-OMP25) were starved for amino acids for 50 min, or starved and restimulated for 10 min, followed by cell lysis and immunoblotting for the indicated proteins and phosphor-proteins. Experiment repeated 4 times. Unprocessed scans are shown in Supplementary Fig. 8

(d) HEK-293T cells stably expressing Flag-RagC containing the indicated mutations were treated with BafA for 2h, fixed and immunostained for LC3 and LAMP2. Images correspond to quantitative Lysosomal Enrichment Scores reported in Figure **7c**. Scale bar 10 μm. Experiment repeated 1 time.

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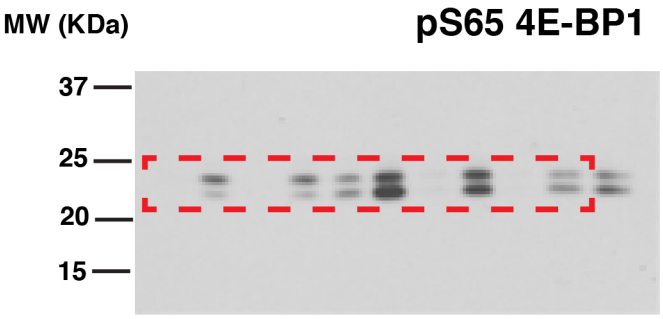

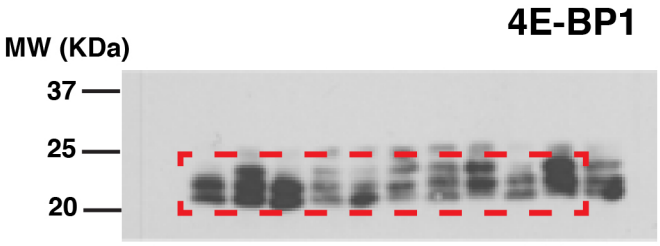

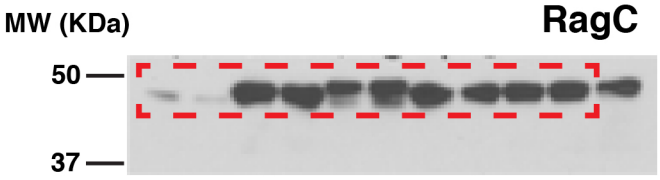

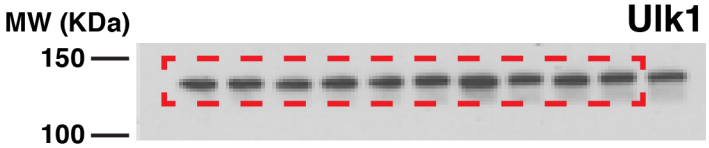

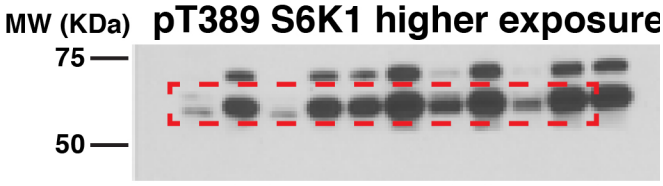

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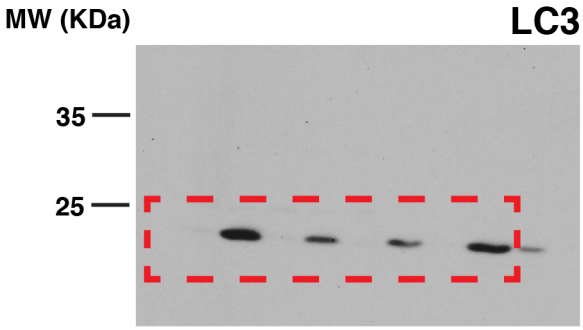

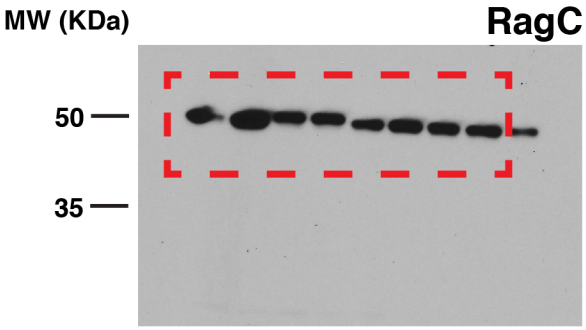

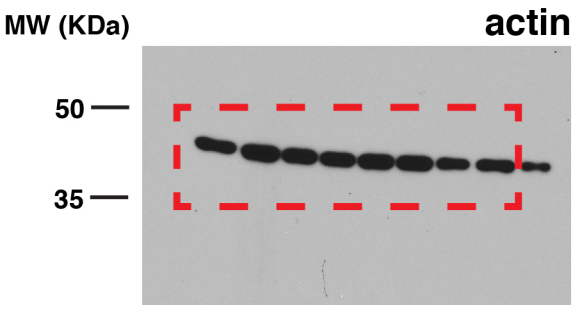

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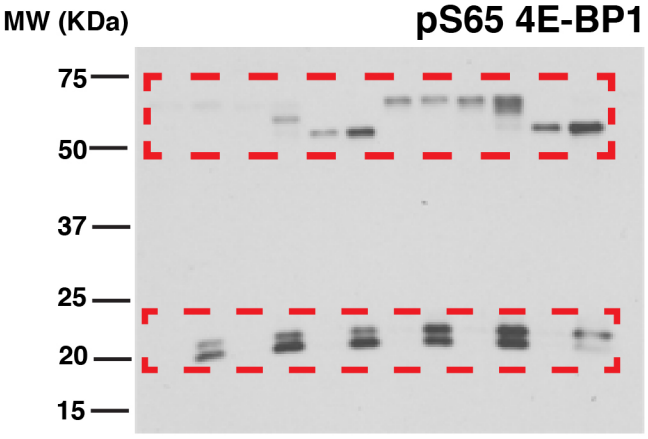

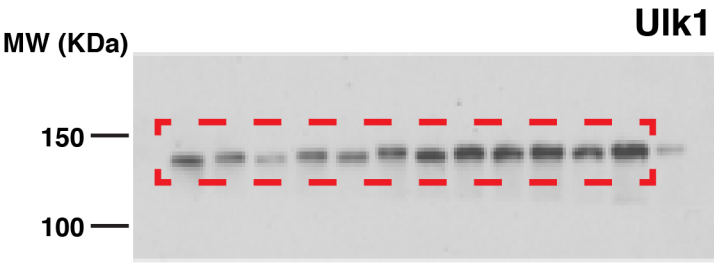

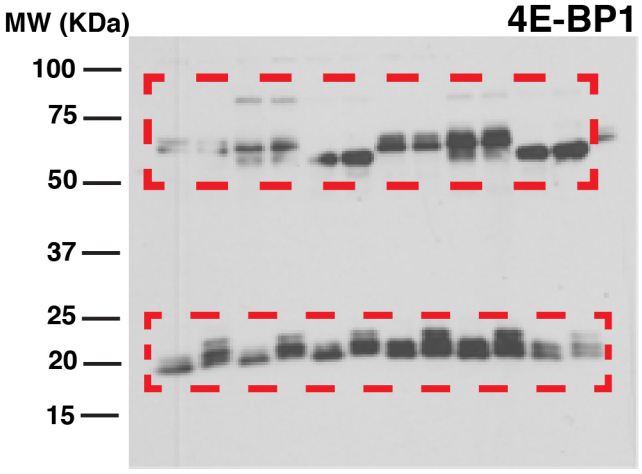

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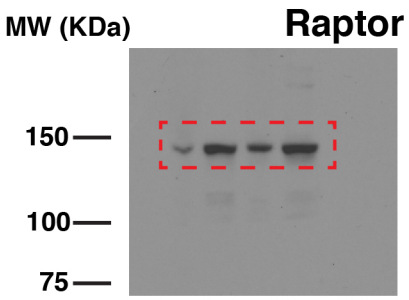

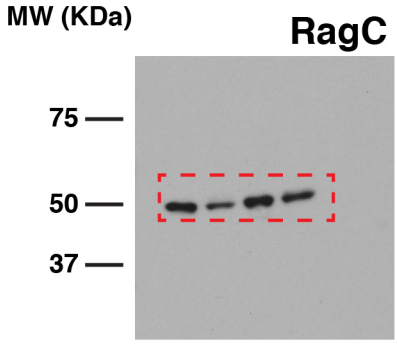

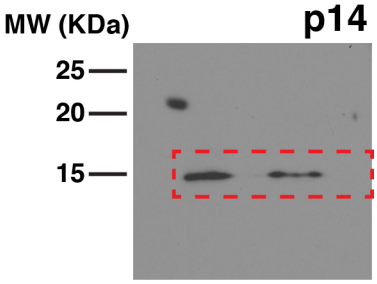

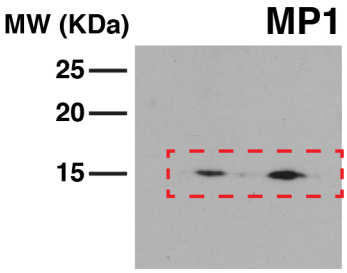

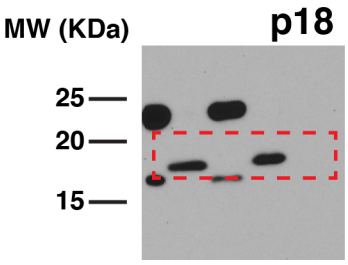

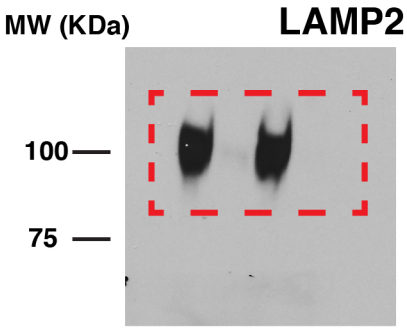

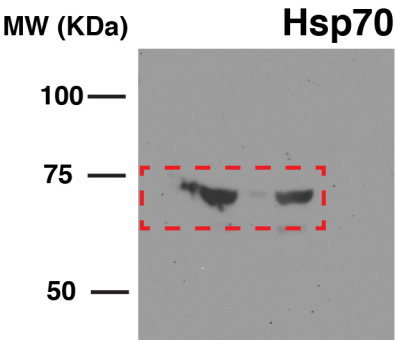

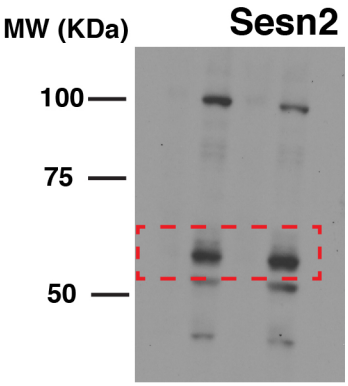

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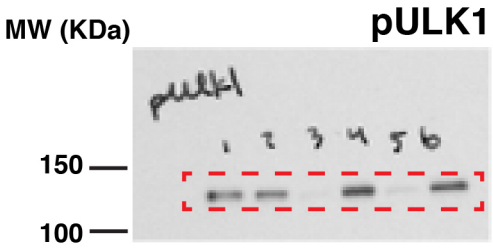

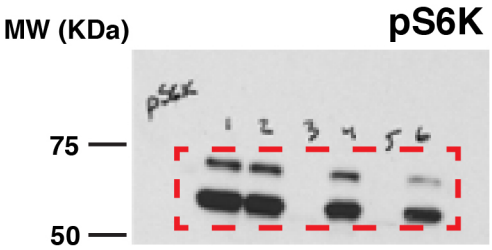

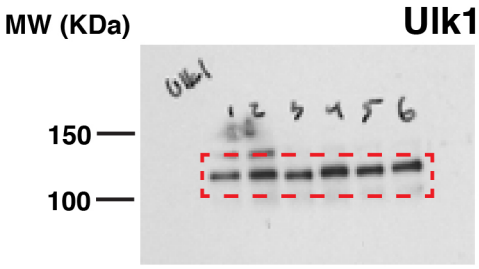

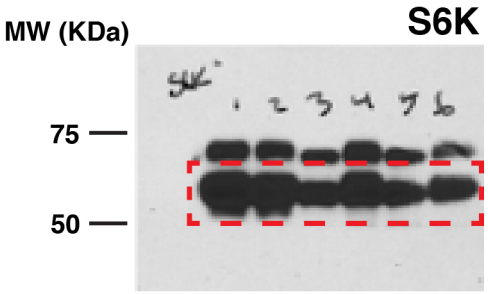

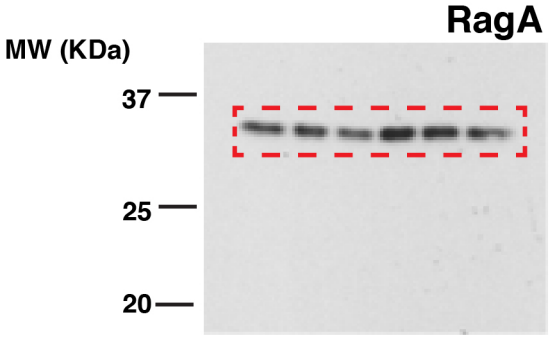

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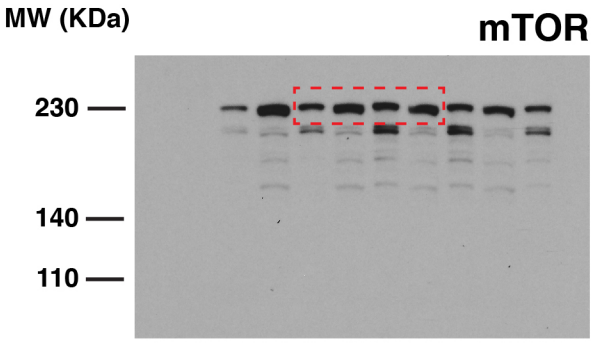

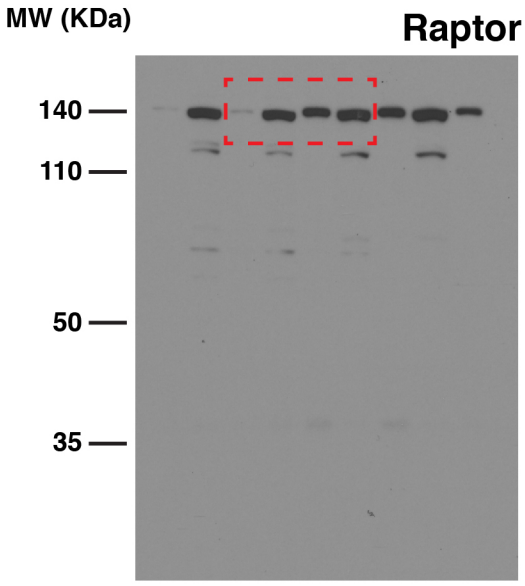

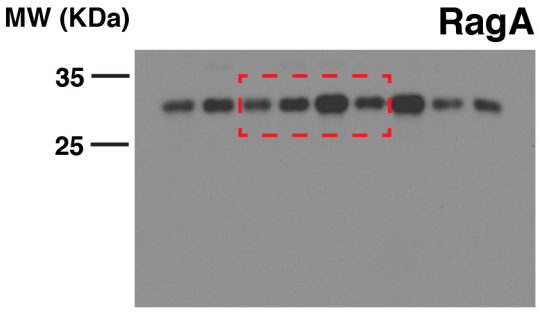

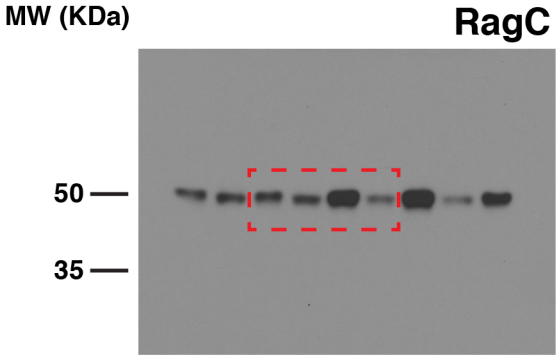

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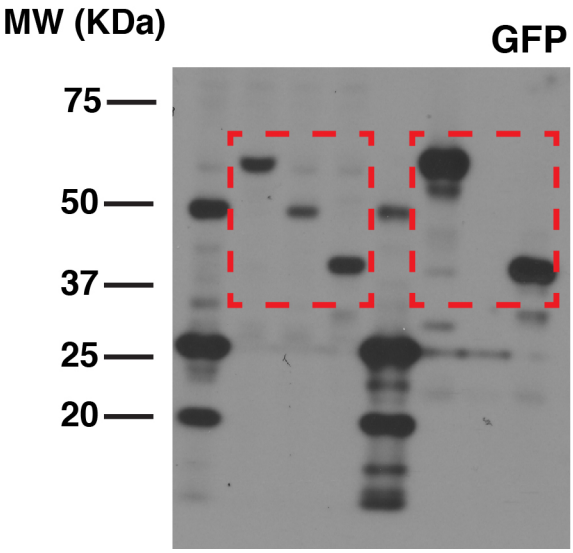

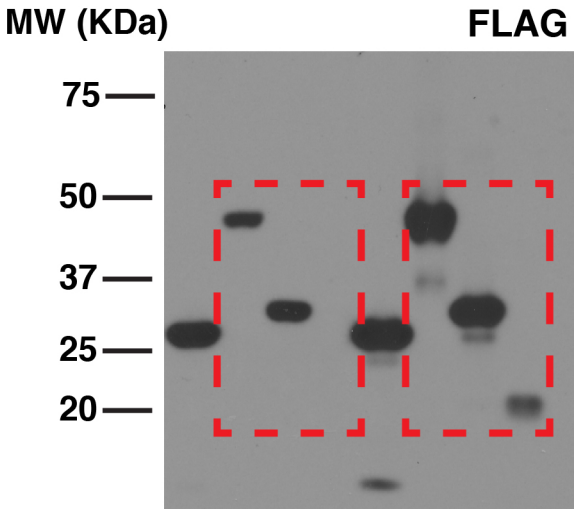

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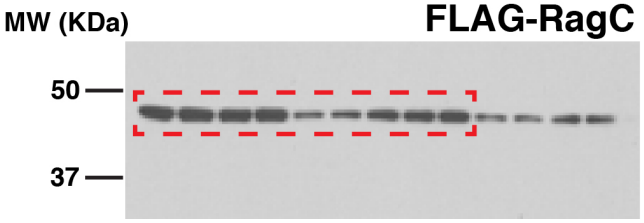
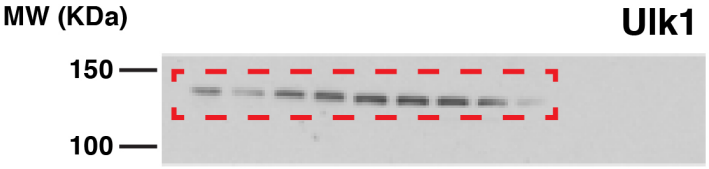
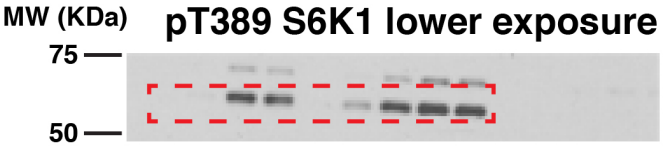
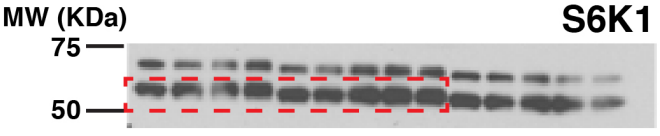
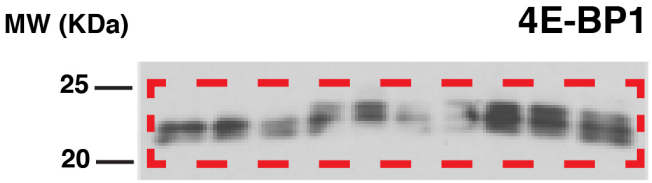


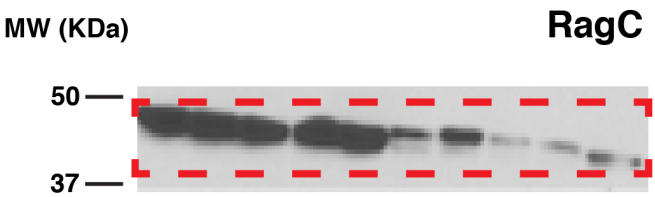


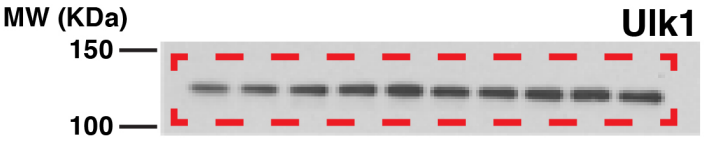


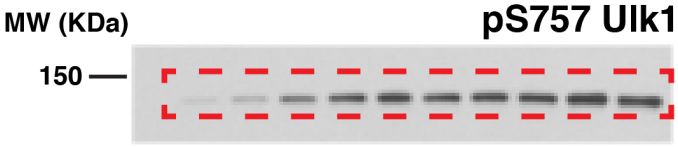


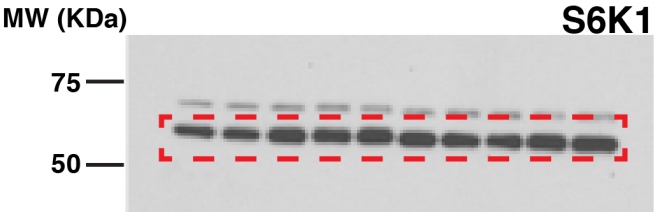
S6F unprocessed film

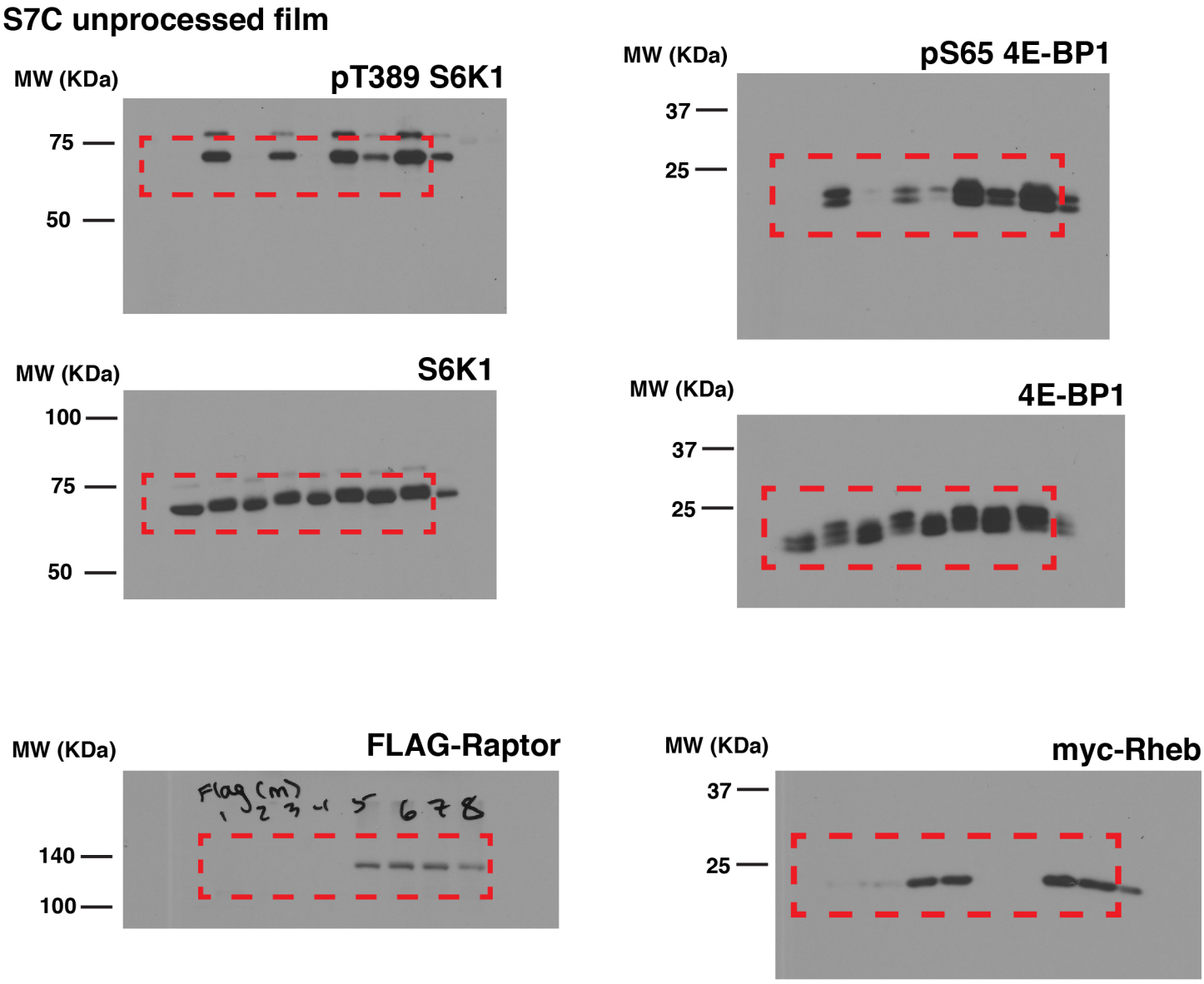












Supplementary Figure 8

Supplementary Figure 8: Unprocessed scans for all films presented in figures

Included here are uncropped blots corresponding to the indicated figures.

Supplementary Table 1 : Statistical Source Data. Source data for Figures 1-7 and Supplementary Figures 2, 4, 5, and 6 are provided.

Supplementary Table 2 : Antibody Information.

Supplementary Table 3 : Reagents and Resources. Reagents used in this study including bacterial and viral strains, chemicals, papetides and recombinant proteins, cell lines, and recombinant DNA.

Supplementary Videos

Supplementary Video 1: Rag GTPases cycle between cytoplasmic and lysosomal pools more rapidly in nutrient restimulated cells than in nutrient starved cells. Shown are side-by-side FRAP time series from U2OS cells expressing GFP-RagB + Flag RagC either starved for amino acids and glucose (left) or starved and restimulated for amino acids and glucose (right). Images were acquired at 1s intervals. These videos correspond to Figure 2c-e.

Supplementary Video 2: *in vitro* single molecule measurement of Rag residence times. Shown are side-by-side single molecule TIRF time series of Halo-tagged Rags on glutathione beads coated with GST-Metap2 (left) or GST-Ragulator (right). Images were acquired at 1s intervals. These videos correspond to Figure 3b.

Supplementary Video 3: Rag heterodimers lacking a G domain have impaired cycling *in vitro*. Shown are side-by-side *in vitro* FRAP time series for GFP-tagged $RagB^{FL}$ + $RagC^{FL}$ (left) or $RagB^{CTD}$ + $RagC^{FL}$ (right) bound to GST-Ragulator on glutathione beads. Images were acquired at 2s intervals. These videos correspond to Figure 5d,e.