Supplemental Figures

Supplemental Figure 1: 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 HEK-293T cells. HEK-293T cell lysate was analyzed by immunoblotting for RagA and RagB with an antibody from CST that recognizes the same epitope in both proteins.

Supplemental Figure 2: HBXIP and C7orf59 are required for the localization of RagC but not p18 to the lysosomal surface, related to Figure 2

A) Images of HEK-293T cells, treated with a non-targeting siRNA or siRNAs targeting HBXIP or C7orf59, co-immunostained for RagC (red) and LAMP2 (green) and processed for imaging.

B) Images of HEK-293T cells, treated with a non-targeting siRNA or siRNAs targeting HBXIP or C7orf59, co-immunostained 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.

Supplemental Figure 3: Amino acids regulate the Ragulator-mTORC1 interaction, related to Figure 3

A) Amino acid stimulation increases the amount of endogenous mTORC1 that co-immunoprecipitates with recombinant p14 and p18. HEK-293T cells stably expressing FLAG-p14 or FLAG-p18 were starved for amino acids for 2 hours 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 co-immunoprecipitates with recombinant C7orf59 and HBXIP. HEK-293T 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. HEK-293T cells, transfected with the indicated cDNAs in expression vectors, were starved for amino acids for 2 hours 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.

Supplemental Figure 4: 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 pre-bound 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 $RagB^{T54N}$ or $RagC^{S75N}$. Proteins were loaded with [^{3}H]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 $RagB^{D163N}$ ($RagB^{X}$) or $RagC^{D181N}$ ($RagC^{X}$). Proteins were loaded with [^{3}H]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 HEK-293T cells transfected with the indicated cDNAs in expression vectors and cell lysates and immunoprecipitates were analyzed by immunoblotting for levels of indicated proteins.

Supplemental Figure 5: The nucleotide state of RagC does not alter Ragulator activity towards 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-$RagC^{D181N}$ was loaded with [^{3}H]GDP and incubated with Ragulator or a control. Dissociation was monitored by a filter-binding assay and is reported as pmols of [^{3}H]GDP per mg of RagB-$RagC^{D181N}$. Each value represents the normalized mean ±SD for n=4.

B) Ragulator increases GTPγS binding to RagA. RagA-RagC^{D181N} loaded with GDP was incubated with Ragulator or a control and [^{35}S]GTPγS. [^{35}S]GTPγS binding was determined as in (A) and is reported as pmols of [^{35}S]GTPγS per mg of RagA-RagC^{D181N}. 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-RagC^{D181N} was loaded with either [^{3}H]GDP or [^{35}S]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-RagC^{D181N} was loaded with either XDP or XTPγS and [^{3}H]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-RagC^{D181N} was loaded with either XDP or XTPγS and [^{35}S]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 HEK-293T 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-RagC^{D181N} was loaded with either [^{3}H]GDP or [^{35}S]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.

Supplemental Figure 6: v-ATPase inhibition decreases the regulated interaction between Rags and Ragulator

A) Inactivation of the v-ATPase inhibits the amino acid dependent regulated interaction between Ragulator and Rags. HEK-293T cells stably expressing FLAG-p18 were starved for amino acids for 2 hours 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.

References

Cole, C., Barber, J.D., and Barton, G.J. (2008). The Jpred 3 secondary structure prediction server. Nucleic Acids Res *36*, W197-201.

Supplemental Figure 1

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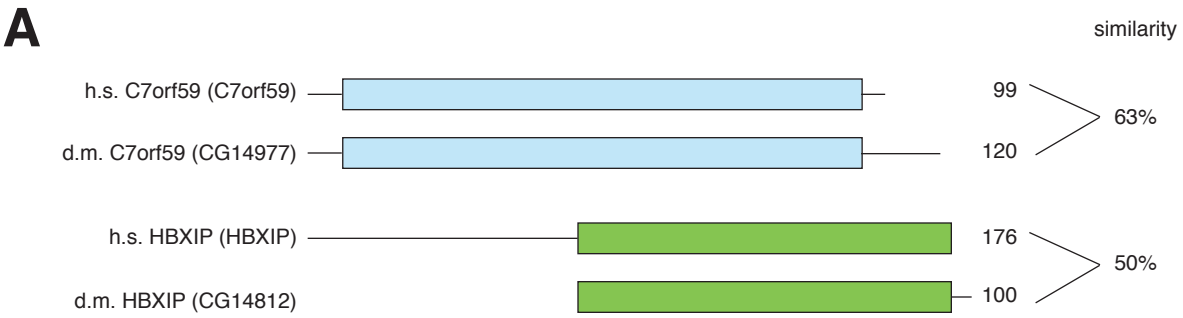
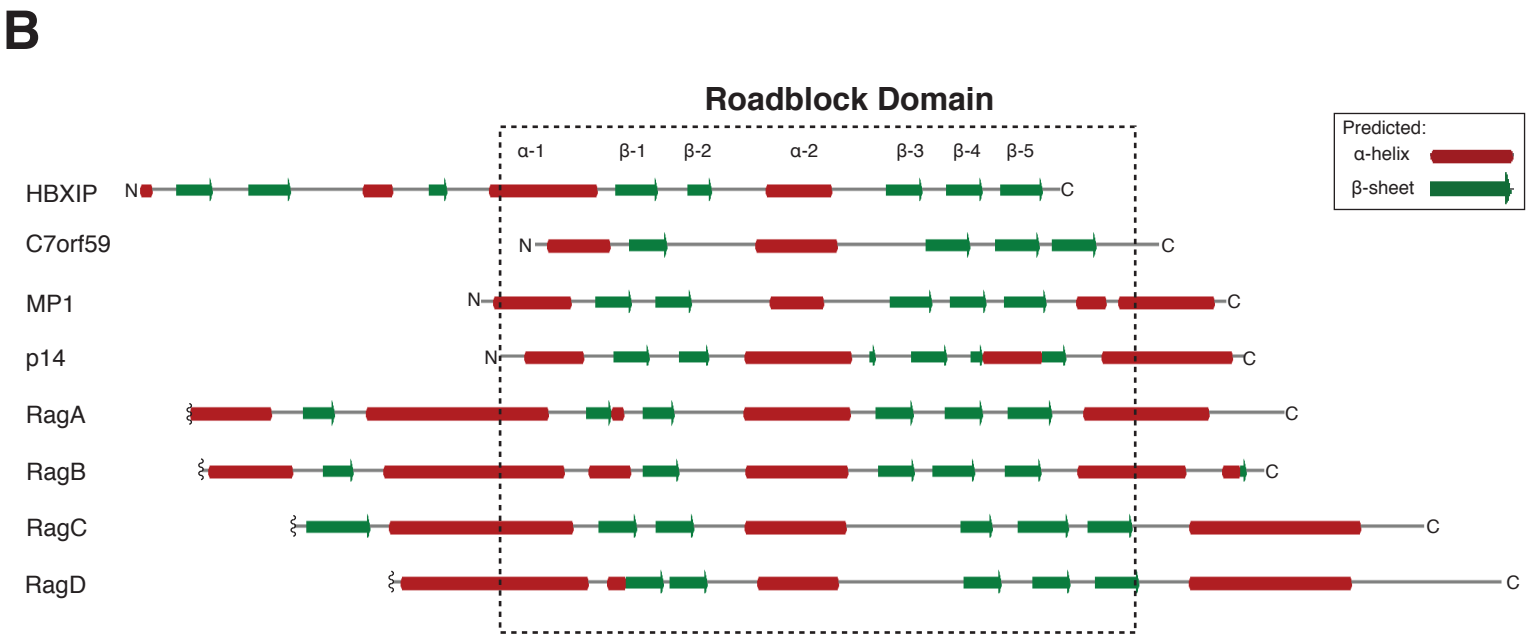
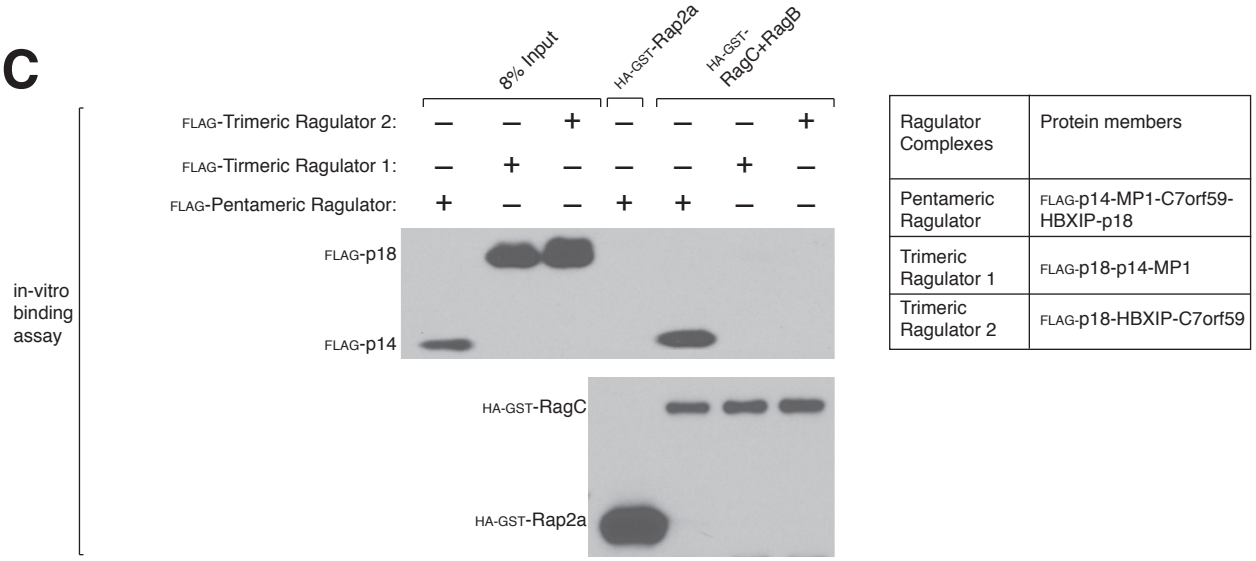
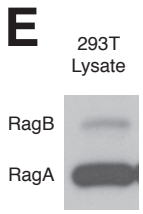
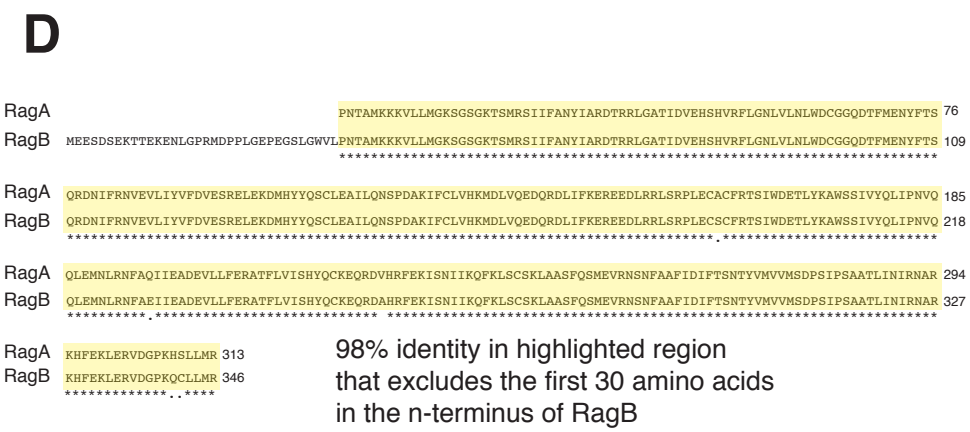






Ragulator Complexes	Protein members
Pentameric Ragulator	FLAG-p14-MP1-C7orf59-HBXIP-p18
Trimeric Ragulator 1	FLAG-p18-p14-MP1
Trimeric Ragulator 2	FLAG-p18-HBXIP-C7orf59





Supplemental Figure 2

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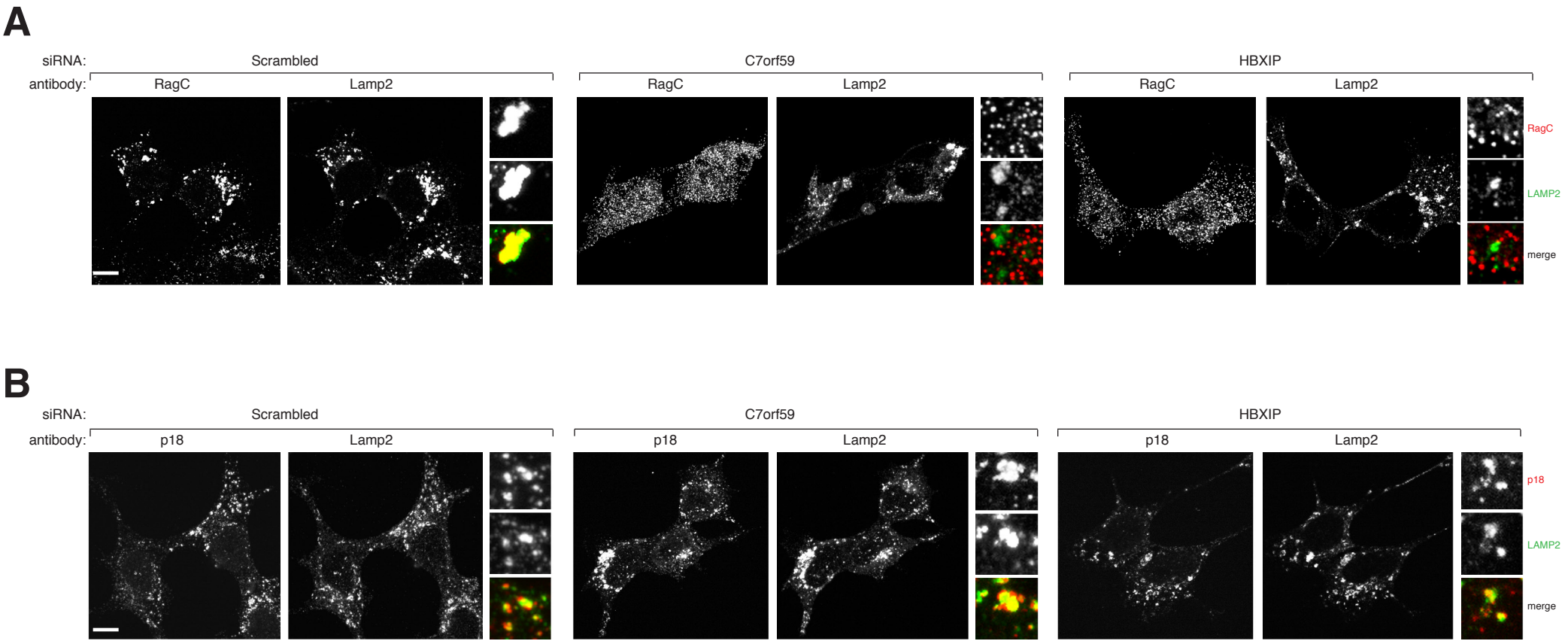

Supplemental Figure 3

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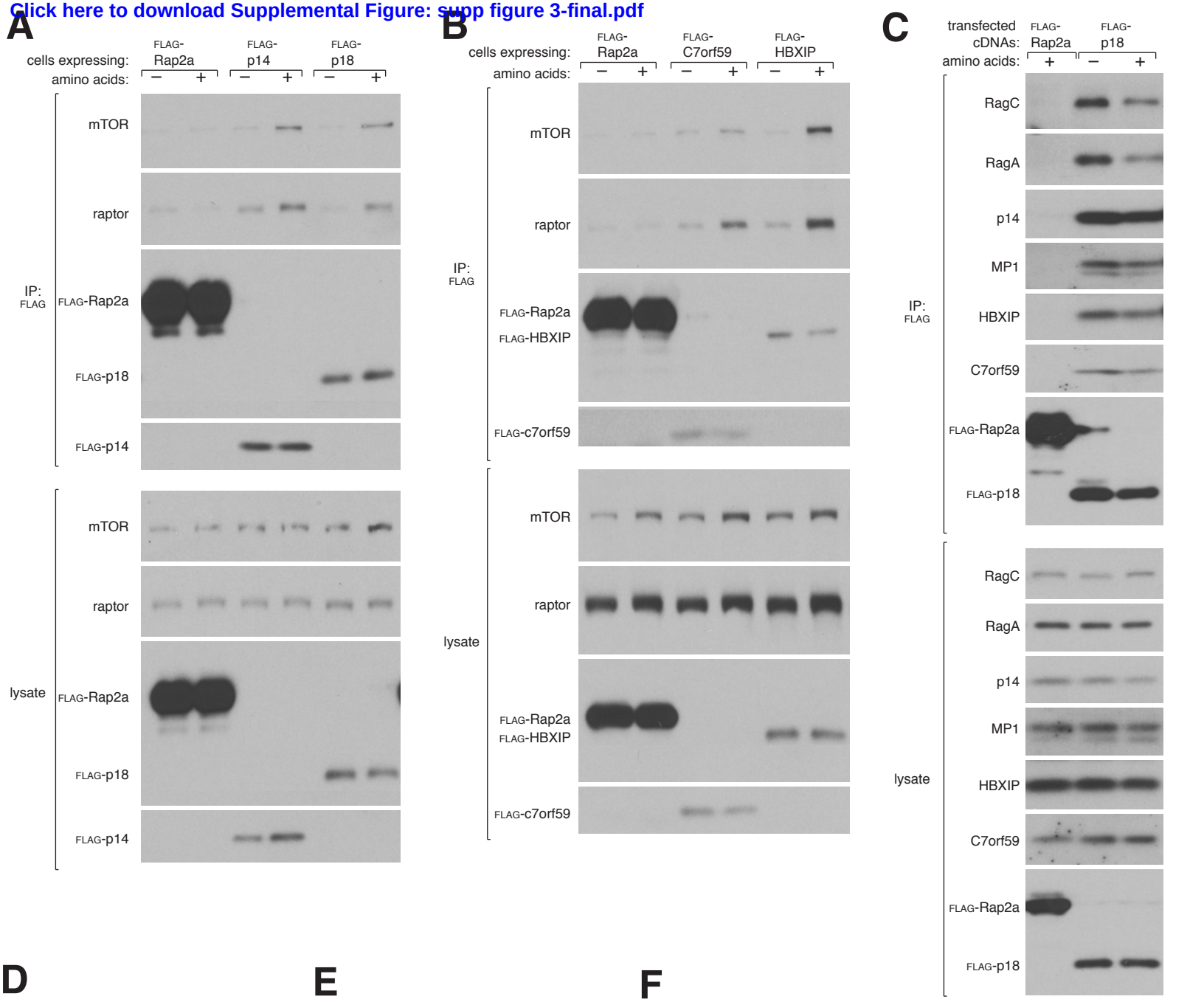

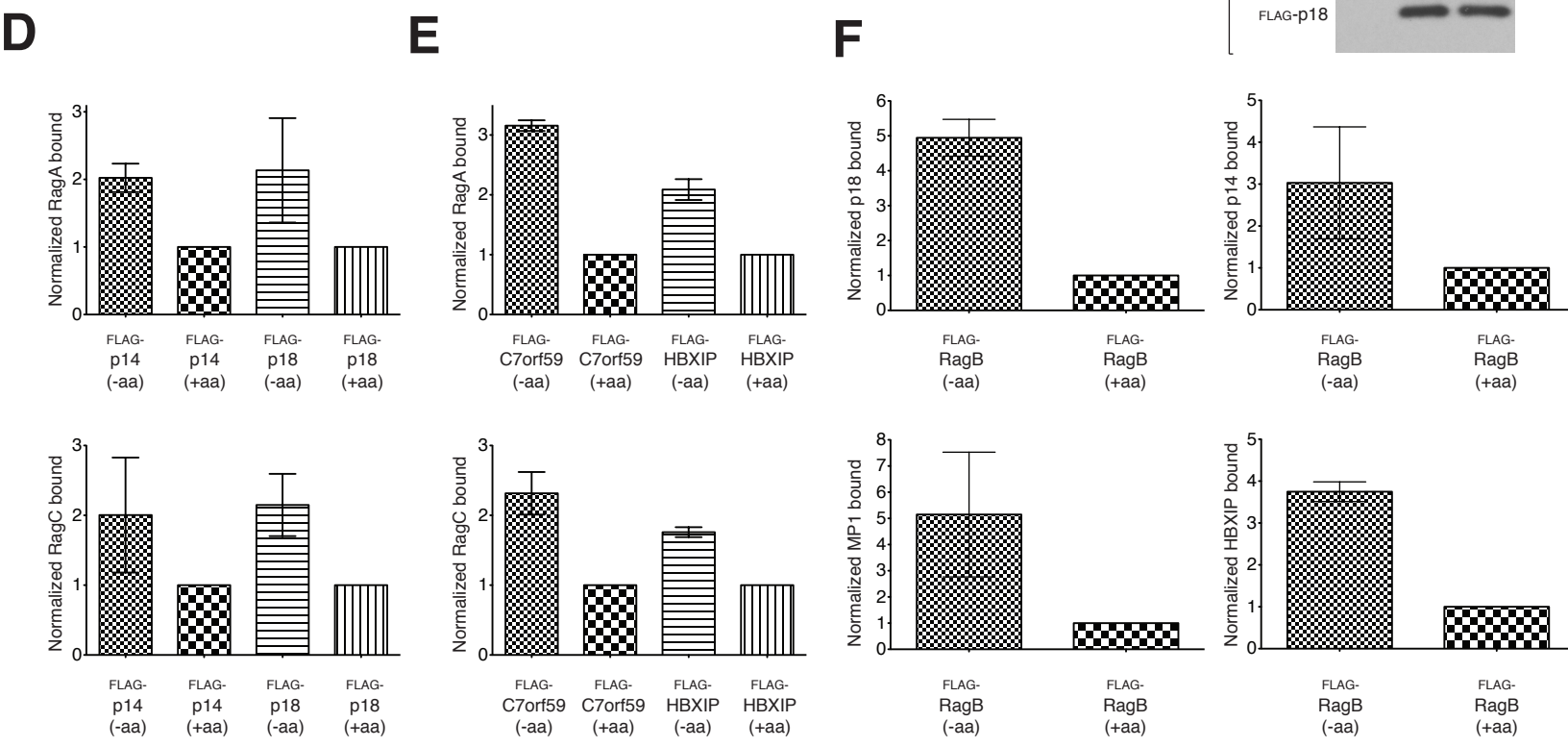

Supplemental Figure 4

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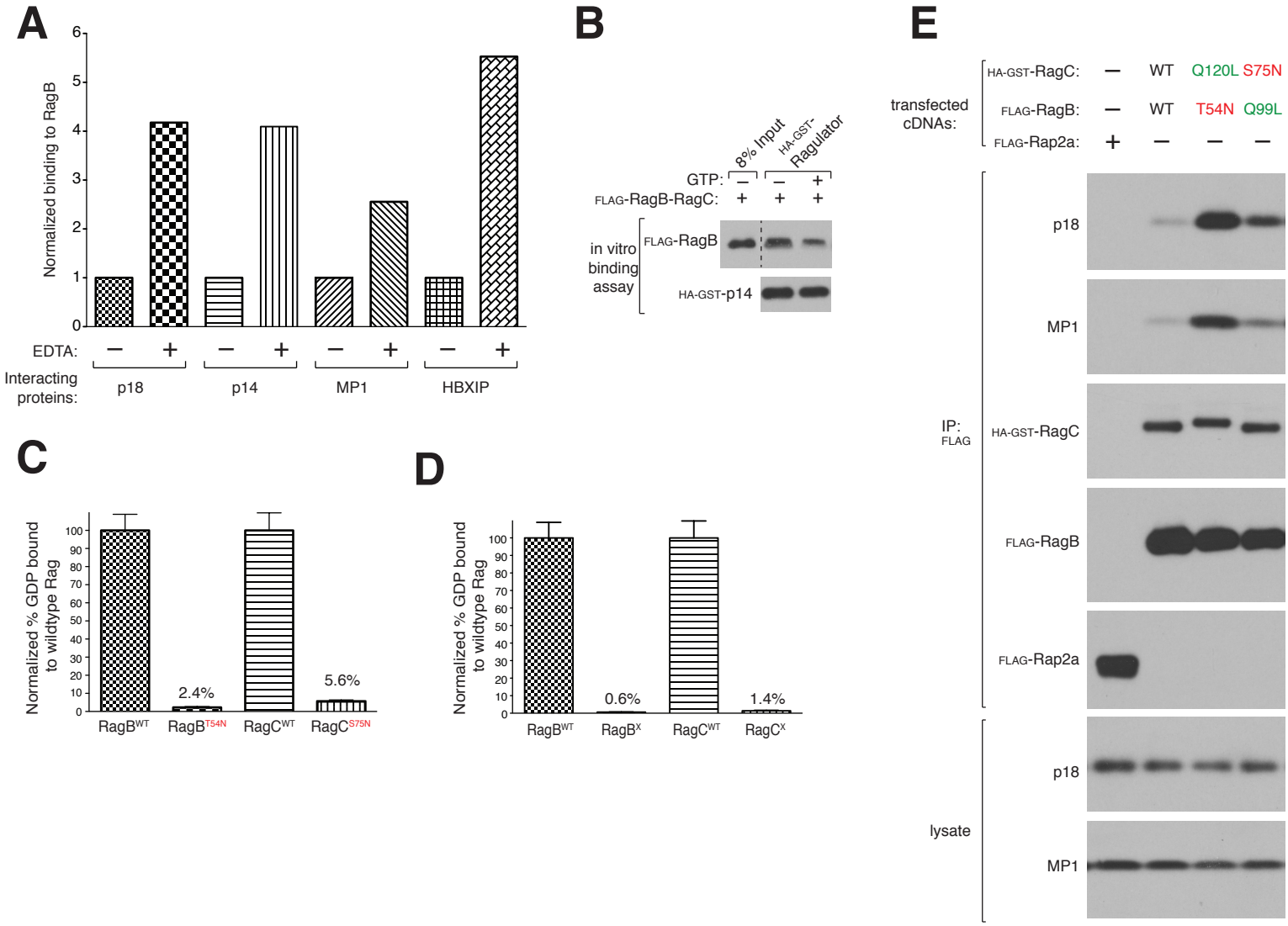

Supplemental Figure 5

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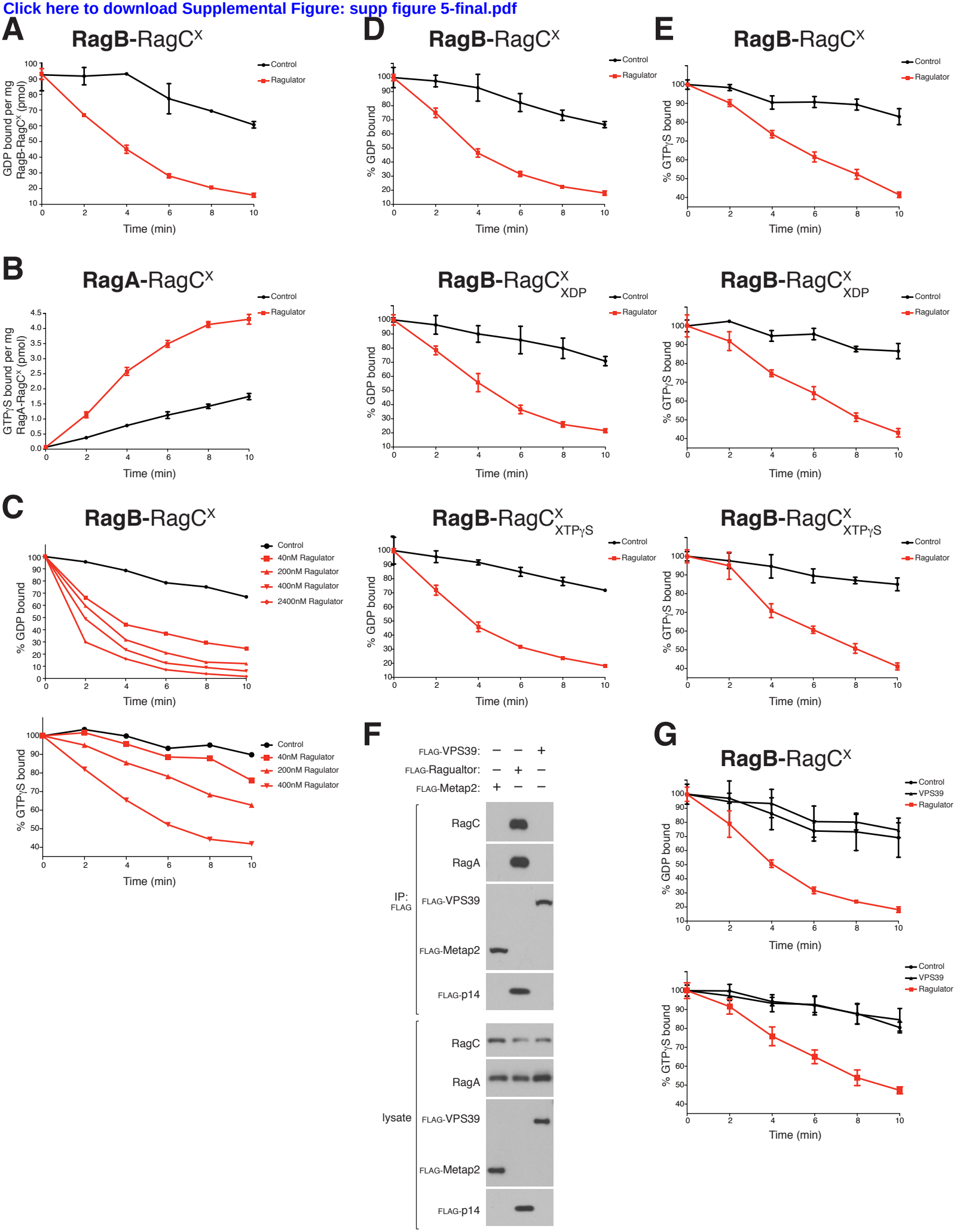

Supplemental Figure 6

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