Supplemental material

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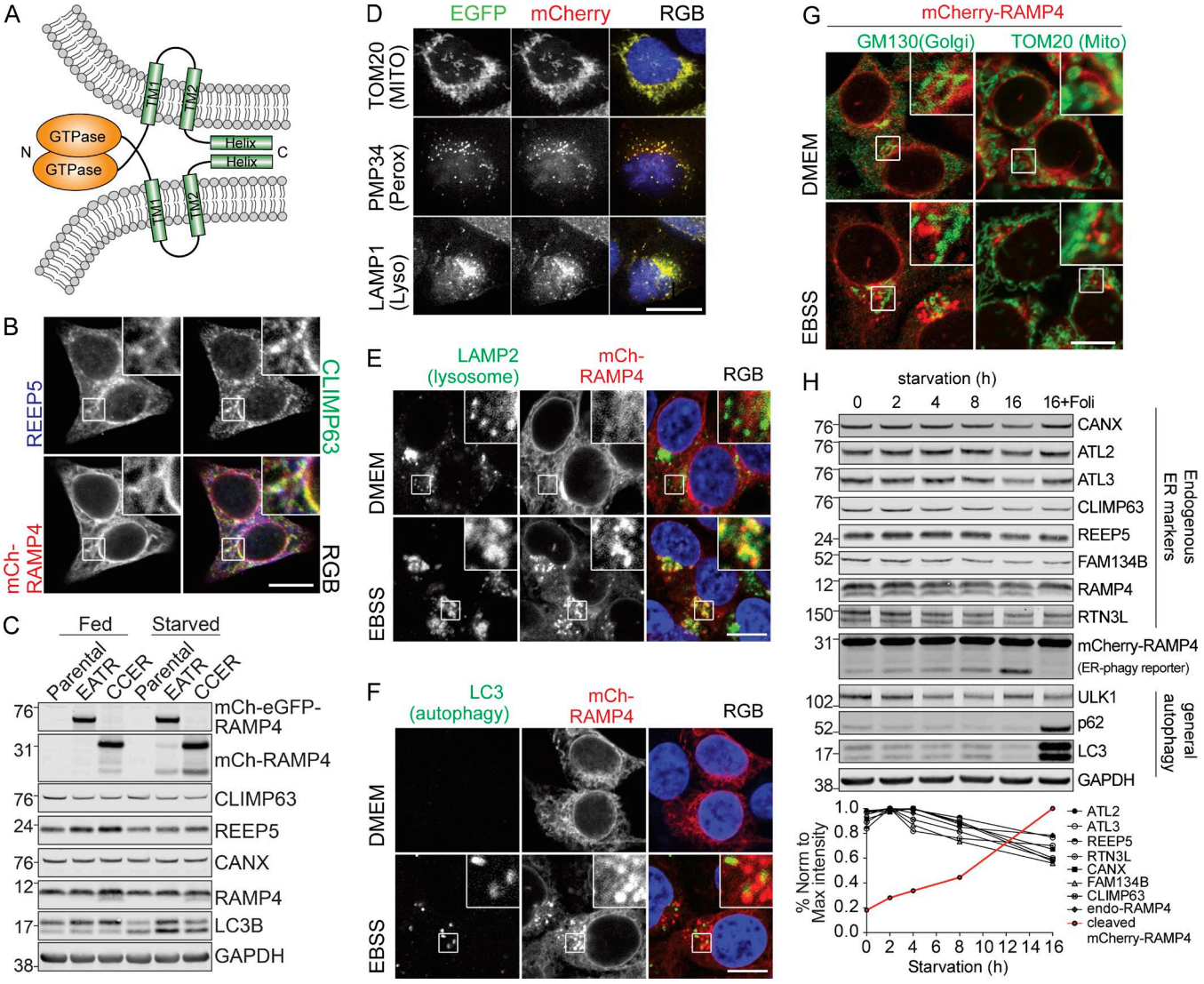

Figure S1. **Development and characterization of EATR and CCER assays for ER-phagy. (A)** Schematic illustrating the topology and dimerization of ATLs during ER membrane fusion. Both the N-terminal GTPase domain and the C-terminal helix face the cytosol, with two TM helixes spanning the ER membrane. ATLs dimerize and undergo GTP hydrolysis to facilitate the fusion of two neighboring ER membranes. **(B)** HCT116 cells stably expressing mCherry-RAMP4 were fixed and immunostained for ER tubule marker (REEP5) and ER sheet marker (CLIMP63). Scale bar represents 10 µm. Inset represents threefold enlargement of boxed area. **(C)** Parental, EATR, and CCER HCT116 cells were starved for 16 h before harvest for Western blot to compare the level of ER protein degradation by ER-phagy. **(D)** U2OS cells expressing each tandem fluorescent reporter with doxycycline regulation. Images were taken in the fed state. Scale bar represents 20 µm. All reporters with the exception of EATR are fluorescently tagged at the C terminus to ensure that the fluorescent tag faces the cytosol. **(E)** HCT116 cells stably expressing mCherry-RAMP4 were starved for 16 h and then fixed to immunoblot for LAMP2 to visualize ER and lysosome colocalization. Scale bar represents 10 µm. Inset represents 3× enlargement of boxed area. **(F)** HCT116 cells stably expressing mCherry-RAMP4 were starved for 16 h and then fixed to immunoblot for LC3. Scale bar represents 10 µm. Inset represents 3× enlargement of boxed area. **(G)** HCT116 cells stably expressing mCherry-RAMP4 were starved for 16 h and then fixed to immunoblot for either GM130 (Golgi marker) or TOM20 (mitochondria marker). Scale bar represents 10 µm. Inset represents 3× enlargement of boxed area. **(H)** CCER HCT116 cells were starved for the indicated duration in EBSS (± folimycin) to measure the loss of ER-resident proteins and proteins involved in general autophagy by Western blotting. **(I)** Densitometry measurement of Western blots in H. Band intensities were normalized to the highest signal.

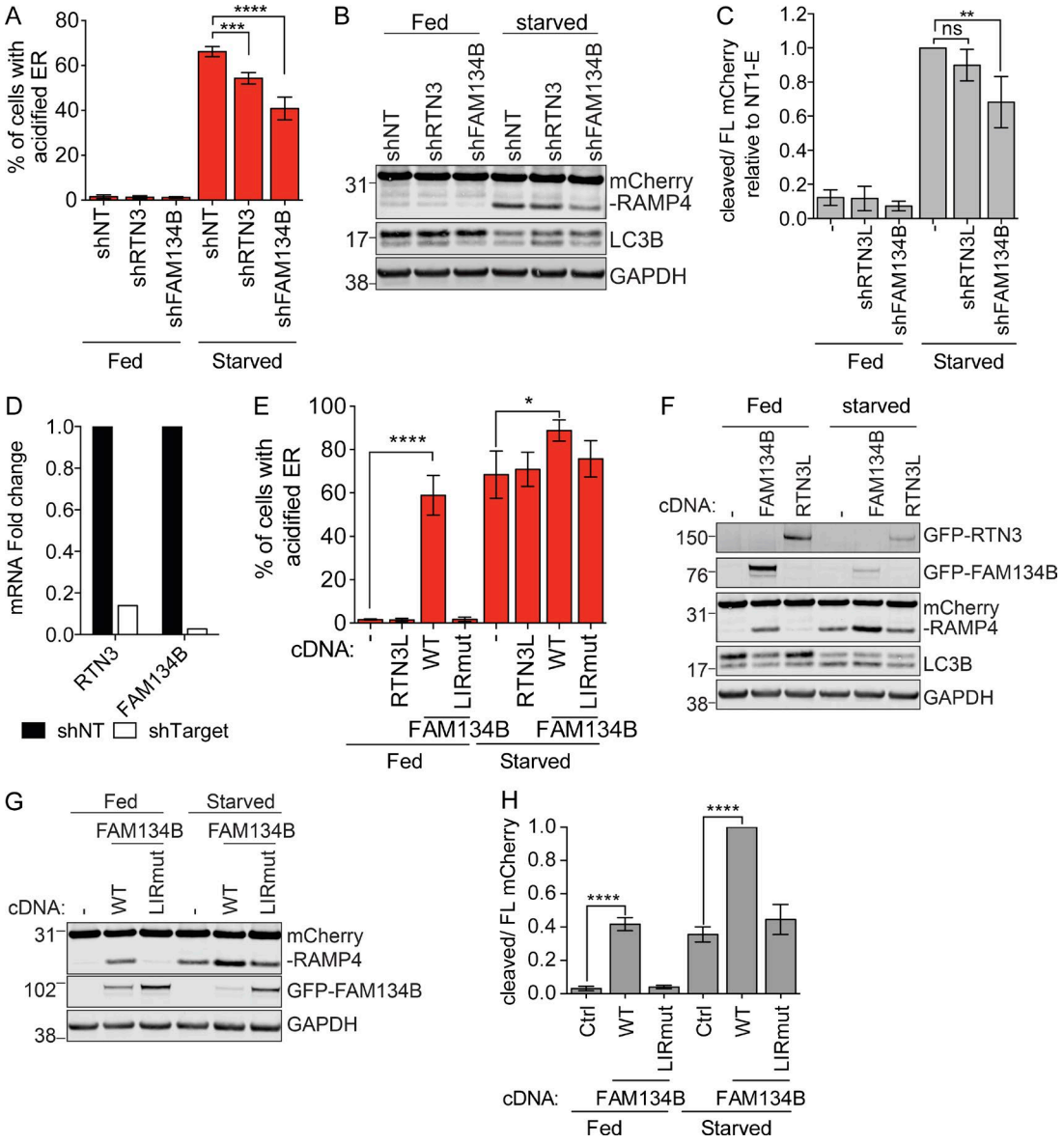

Figure S2. **EATR and CCER assays report on FAM134B-mediated ER-phagy. (A)** EATR HCT116 cells stably transduced with shRTN3 or shFAM134B were starved for 16 h for flow cytometry measurement. Data presented as mean ± SD of three biological replicates. P value indicates one-way ANOVA with Dunnett's multiple comparisons test (***, P < 0.0005; ****, P < 0.0001). **(B)** CCER HCT116 cells were transduced and starved as in A and then subjected to Western blot for the indicated proteins. **(C)** Densitometry measurement from B of the ratio of cleaved mCherry band versus full-length mCherry-RAMP4 normalized to the starved shNT sample. Data presented as mean ± SD of three biological replicates. P value indicates one-way ANOVA with Dunnett's multiple comparisons test (**, P < 0.005). **(D)** qRT-PCR measurement of HCT116 cells transduced with shRTN3 or shFAM134B to validate their knockdown efficiencies. **(E)** EATR HCT116 cells stably expressing either HA-tagged RTL3L or FAM134B (WT vs. LIRmut) were starved for 16 h for flow cytometry measurement. P value indicates one-way ANOVA with Tukey's multiple comparisons test (*, P < 0.05; ****, P < 0.0001). **(F)** CCER HCT116 cells stably expressing either GFP-tagged FAM134B or RTN3L were starved for 16 h and then subjected to Western blot for the indicated proteins. **(G)** CCER HCT116 cells stably expressing either WT or LIRmut GFP-FAM134B were starved for 16 h and then subjected to Western blot for the indicated proteins. **(H)** Densitometry measurement from G of the ratio of cleaved mCherry band versus full-length mCherry-RAMP4 normalized to the starved FAM134B-WT sample. Data presented as mean ± SD of three biological replicates. P value indicates one-way ANOVA with Dunnett's multiple comparisons test (****, P < 0.0001).

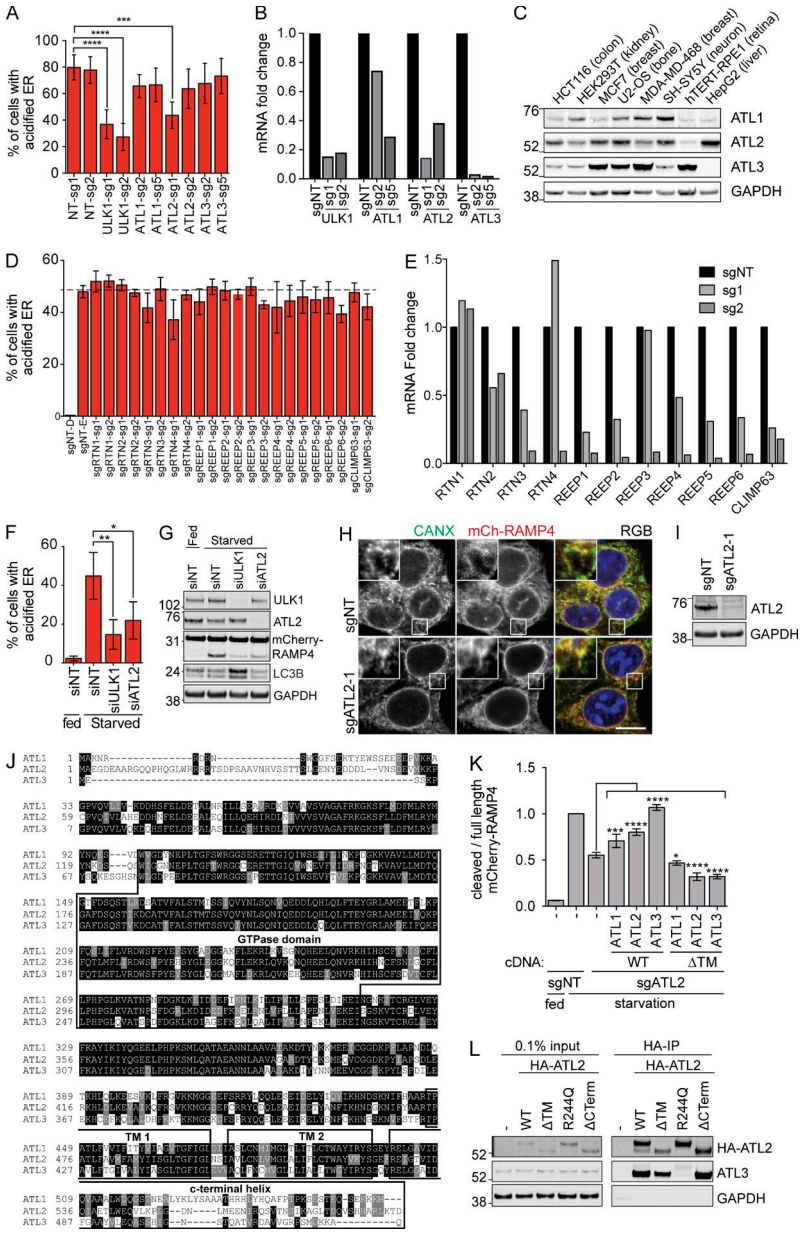

Figure S3. **ATL2 is a specific ER remodeler that is involved in ER-phagy progression. (A)** EATR CRISPRi HCT116 cells stably expressing sgRNAs targeting the indicated genes were starved for 16 h for flow cytometry measurement. Data presented as mean ± SD of three biological replicates. P value indicates one-way ANOVA with Dunnett's multiple comparisons test (***, P < 0.001; ****, P < 0.0001). **(B)** CRISPRi HCT116 cells from A were harvested to assess the knockdown efficiencies by qRT-PCR. The Ct values were normalized to β-actin and NT sgRNA (sgNT). Two previously validated ULK1 sgRNAs were used as positive controls. **(C)** Cell lysates from different cell lines in the fed condition were loaded in equal amounts to measure the protein expression profiles of ATL1, ATL2, and ATL3. **(D)** EATR CRISPRi HCT116 cells stably expressing sgRNAs targeting the different ER remodeling proteins (two guides per target) were starved for 16 h for flow cytometry measurement. Data presented as mean ± SD of three biological replicates. Analysis with one-way ANOVA with Dunnett's multiple comparisons test revealed no significant difference in ER-phagy upon knockdown of any of the genes. **(E)** EATR CRISPRi HCT116 cells from D were harvested for qRT-PCR to assess the knockdown efficiency of each guide. The Ct values were normalized to β-actin and sgNT. **(F)** EATR CRISPRi HCT116 cells were transiently transfected with siRNA targeting either ULK1 or ATL2 for 48 h before starvation for 16 h. Data presented as mean ± SD of three biological replicates. P value indicates one-way ANOVA with Tukey's multiple comparisons test (*, P < 0.05; **, P < 0.001). **(G)** CCER CRISPRi HCT116 cells were transiently transfected with siRNA targeting either ULK1 or ATL2 for 48 h before starvation for 16 h. A representative blot is shown. **(H)** CCER CRISPRi HCT116 cells stably expressing sgRNA targeting ATL2 were fixed and immunostained with CANX to assess the effect of ATL2 knockdown on the localization and insertion of mCherry-RAMP4. Scale bar represents 10 µm. Inset represents threefold enlargement of boxed area. **(I)** CCER CRISPRi HCT116 cells from H were harvested for Western blotting to assess the knockdown efficiency of sgATL2-1. **(J)** Protein sequence alignment between human ATL1, ATL2, and ATL3 performed using Clustal Omega. The boxed areas indicate the respective GTPase, TM, and C-terminal helix domains. **(K)** Densitometry measurement from Fig. 2 M of the ratio of cleaved mCherry band versus full-length mCherry-RAMP4 normalized to the starved NT sample. Data presented as mean ± SD of three biological replicates. P value indicates one-way ANOVA with Dunnett's multiple comparisons test (*, P < 0.05; ***, P < 0.0005; ****, P < 0.0001). **(L)** HCT116 cells stably expressing HA-tagged ATL2 or its mutant variants were immunoprecipitated with anti-HA antibody to measure their interaction with ATL3. Three biological replicates were performed, and a representative Western blot is shown.

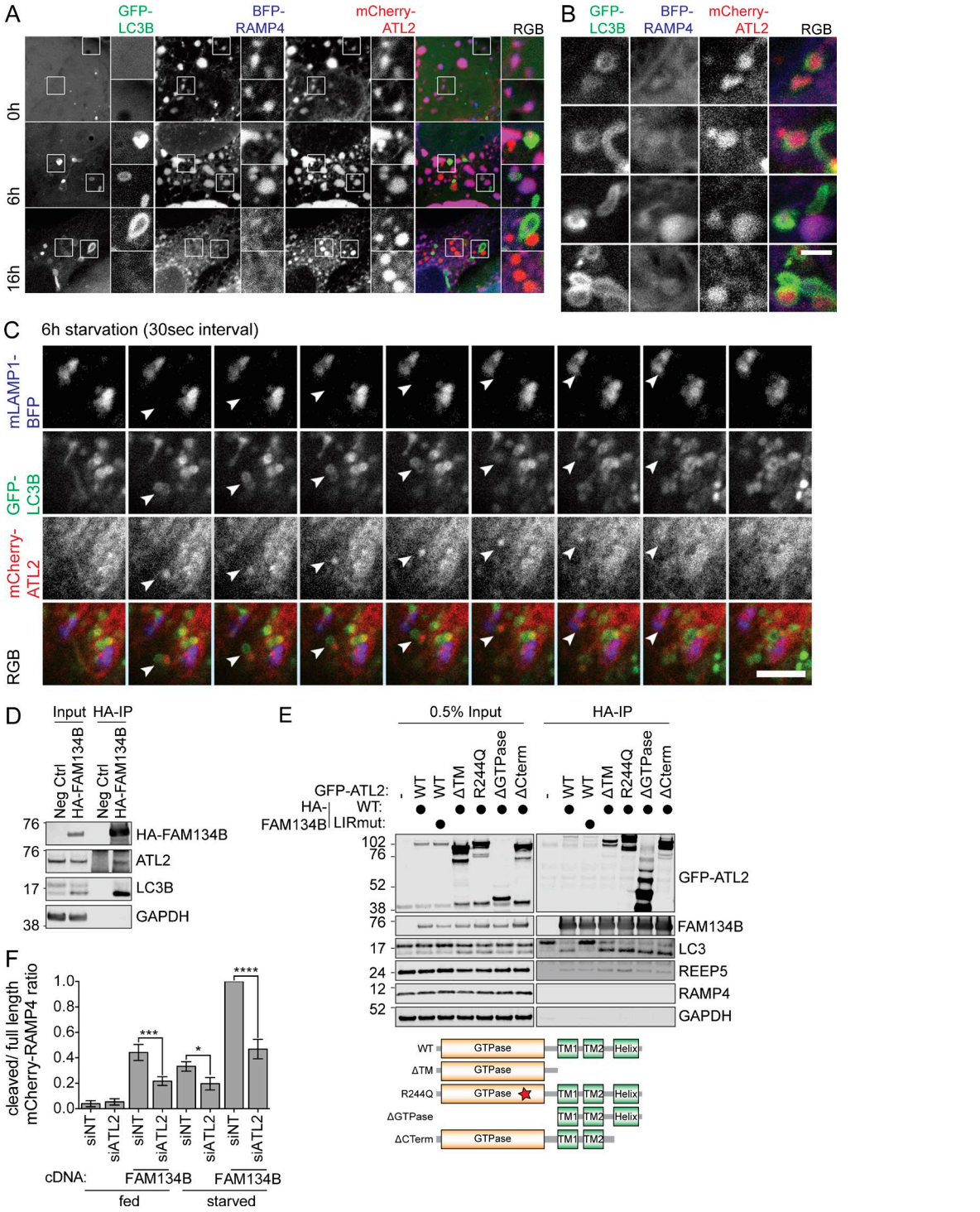

Figure S4. **ATL2 acts downstream of FAM134B and is degraded during ER-phagy. (A)** U2OS cells stably expressing BFP-RAMP4, GFP-LC3B, and mCherry-ATL2 were starved for the indicated durations and imaged in live-cell conditions to visualize the engulfment of mCherry-ATL2 and BFP-RAMP4 by GFP-LC3B. Scale bar represents 5 µm. Scale bar for twofold enlarged insets represents 2 µm. **(B)** Additional examples from the same experiment as A of GFP-LC3B-engulfed mCherry-ATL2 (± BFP-RAMP4). Scale bar represents 2 µm. **(C)** U2-OS cells stably expressing mLAMP1-BFP, GFP-LC3B, and mChertry-ATL2 were starved for 6 h. ER-phagy was captured by live-cell imaging at 30-s intervals. Scale bar represents 4 µm. The arrow tracks the movement of GFP-LC3-tagged mCherry-ATL2 toward BFP-LAMP2 tagged lysosome over time. **(D)** HCT116 cells stably expressing HA-tagged FAM134B were immunoprecipitated with anti-HA antibody to detect its interaction with ATL2 and LC3B. **(E)** HEK293T cells were cotransfected with either WT or HA-FAM134B LIRmut and different GFP-ATL2 mutant constructs for 48 h. Immunoprecipitation was performed using anti-HA antibody and then Western blotted for the indicated proteins. The schematic at the bottom illustrates the domains present in each ATL2 mutant construct. **(F)** Densitometry measurement from Fig. 5 E of the ratio of cleaved mCherry band versus full-length mCherry-RAMP4 normalized to the starved siNT with FAM134B overexpression sample. Data presented as mean ± SD of three biological replicates. P value indicates one-way ANOVA with Tukey's multiple comparisons test (*, P < 0.05; ***, P < 0.0005; ****, P < 0.0001).

Provided online are two tables in Excel. Table S1 lists all overexpression constructs used in this study. Table S2 lists all primary antibodies used in this study.