

Figure S1. **SR microscopy analysis of LC3 and Stx17.** HeLa cells were transfected with GFP-Stx17 and induced for autophagy with pp242 for 2 h in the presence of bafilomycin A1 to allow for accumulation of intermediates, and then endogenous LC3 (rabbit anti-LC3) and GFP-Stx17 were sequentially imaged at 647 nm as detailed in the SR microscopy and analysis section of Materials and methods. Shown is a whole-cell SR image. Pseudocolors: green, GFP-Stx17; red, endogenous LC3. The following classification of profiles (marked I–VII) does not imply any order in time or precursor–product relationship: (I) Stx17 and LC3 closely proximal to each other within the arms of phagophores. The profile marked with an asterisk (I*) is shown in Fig. 1 B. (II) Stx17 and LC3 with some proximity in profiles that appear closed. (III) Stx17 and LC3B separated in profiles that appear closed. (IV) Prominent Stx17 rim surrounding internal LC3. (V) Internal LC3 with a less prominent or no rim of Stx17. (VI) Distinct LC3 profiles emanating from Stx17⁺ profiles. (VII) Stx17 profiles of undetermined identity.

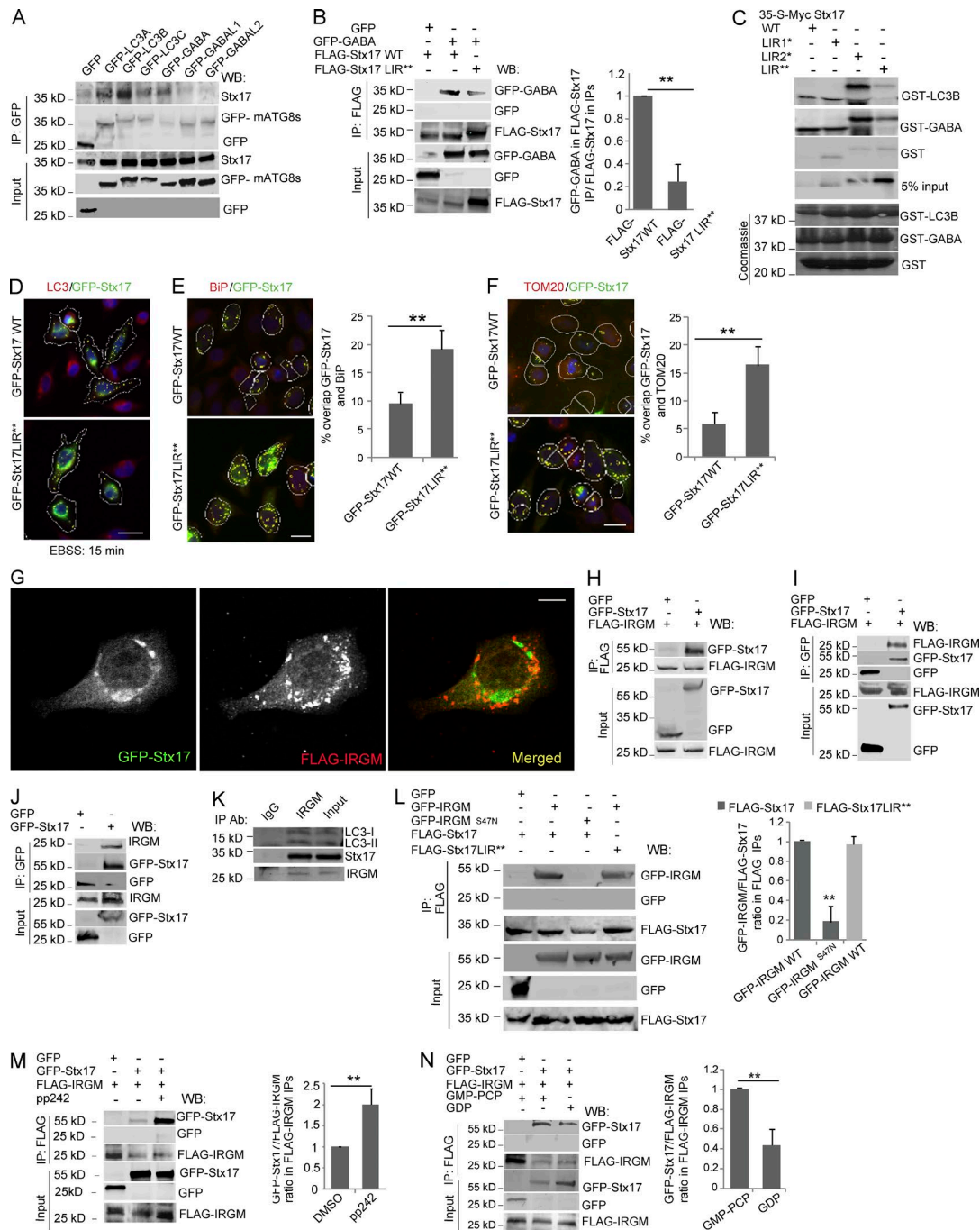


Figure S2. LIR mutations in Stx17 reduce its interactions with mAtg8s, and IRGM interacts with Stx17. (A) CoIP analyses of GFP-mAtg8s and endogenous Stx17 in 293T cells. (B) CoIP analyses using GFP-GABARAP and WT or LIR mutant FLAG-Stx17 in 293T cells. Data indicate relative intensity (normalized to IP) input means \pm SEM. (C) GST pull-down of radiolabeled WT or LIR1/2** mutant [35S]-Myc-Stx17 and GST-GABARAP or GST-LC3B. (D) HC images showing colocalization of LC3 with GFP-Stx17 WT or GFP-Stx17 LIR** in cells incubated with EBSS for 15 min (related to Fig. 2 E). Note that the images shown are cropped from a larger field, so no primary objects were at the edges of the actual fields captured in full images to be rejected. (E and F) HC of GFP-Stx17 WT or GFP Stx17LIR** (double LIR mutant) localization with ER/BiP (E) or mitochondria (TOM20; F). Data indicate percent area overlap (means \pm SEM; **, $P < 0.01$ [$n = 3$] ANOVA; HC, >500 primary objects counted per well; minimum number of wells was four; three independent experiments). Masks: white, primary objects (cells); yellow puncta, overlap. Bars, 10 μ m. (G) Confocal microscopy analysis of GFP-Stx17 and FLAG-IRGM in HeLa cells without any treatment. Bar, 5 μ m. (H) CoIP analysis of interactions between endogenous Stx17 and endogenous IRGM in 293T cells. (I) CoIP analysis of interactions between endogenous Stx17 and endogenous IRGM in 293T cells. (J) CoIP analysis of interactions between GFP-Stx17 and endogenous IRGM in 293T cells. (K) CoIP analysis of interactions between endogenous Stx17 and LC3B with IRGM in THP-1 cells. (L) CoIP analysis in extracts from 293T cells expressing FLAG-Stx17 and WT GFP-IRGM (center left lane) or mutant GFP-IRGM^{S47N} (center right lane) and FLAG-Stx17LIR** and WT GFP-IRGM (far right lane) in 293T cells. Data indicate means and SEM of ratios between FLAG-Stx17 and GFP-IRGM in FLAG IPs. (M) CoIP analysis of interactions between FLAG-IRGM and GFP-Stx17 upon autophagy induction with pp242 in 293T cells. (N) CoIP analysis of interactions between GFP-Stx17 and FLAG-IRGM in the presence of GDP or GMPPCP. 0.1 mM GDP or GMPPCP were added to lysates from 293T cells before coIP. Data indicate means \pm SEM of intensities normalized to IP input. **, $P < 0.01$ ($n = 3$) t test. In IP blots for B and L-N, GFP fusions and GFP-only sections were cropped to avoid IgG bands. WB, Western blot.

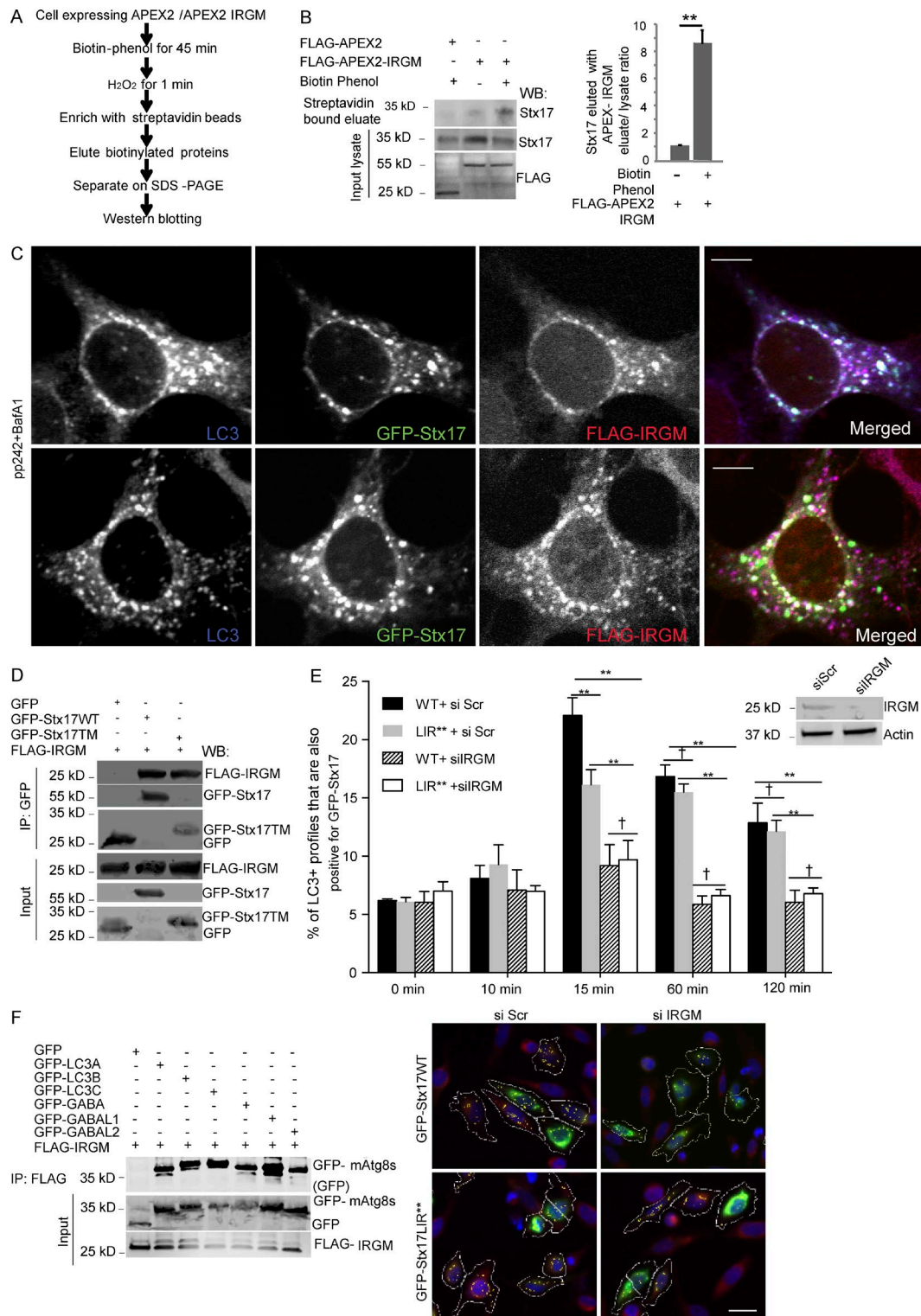


Figure S3. IRGM is in close proximity with Stx17 in cells and interacts with mAtg8s and helps recruiting Stx17 to autophagosomes. (A) Schematic steps in APEX2 proximity biotinylation assay. (B) Proximity biotinylation products using endogenous Stx17 and tagged (FLAG-APEX2-IRGM) IRGM transfected into 293T cells. Cells were incubated with biotin phenol and H₂O₂ to initiate biotinylation reaction, and then biotinylation products were collected from cell lysates on streptavidin beads and analyzed by immunoblotting. Right: Quantification of biotin-eluted Stx17/input ratios. Data indicate means \pm SEM of intensities normalized to input. **, $P < 0.01$ ($n = 3$) t test. WB, Western blot. (C) Confocal microscopy of HeLa cells transfected with FLAG-IRGM and GFP-Stx17 and treated with pp242 and bafilomycin A1 to analyze colocalization between FLAG-IRGM, GFP-Stx17, and LC3. Bar, 5 μ m. (D) ColP analysis of association between FLAG-IRGM and GFP-Stx17 WT or GFP-Stx17TM. (E) HC microscopy analysis of IRGM knockdown effects alone or in combination with GFP-Stx17 LIR** on LC3, and GFP-Stx17 colocalization in HeLa cells incubated in EBSS for indicated time points. Bottom: Images shown are from 15 min after starvation. Bar, 10 μ m. Masks: white, primary objects (cells); yellow puncta, overlap. Data indicate means \pm SEM. †, $P \geq 0.05$; **, $P < 0.01$ ($n = 3$) ANOVA (>500 primary objects counted per well; minimum number of wells was six; three independent experiments). (F) ColP analysis of interactions between GFP-mAtg8s and FLAG-IRGM in 293T cells.

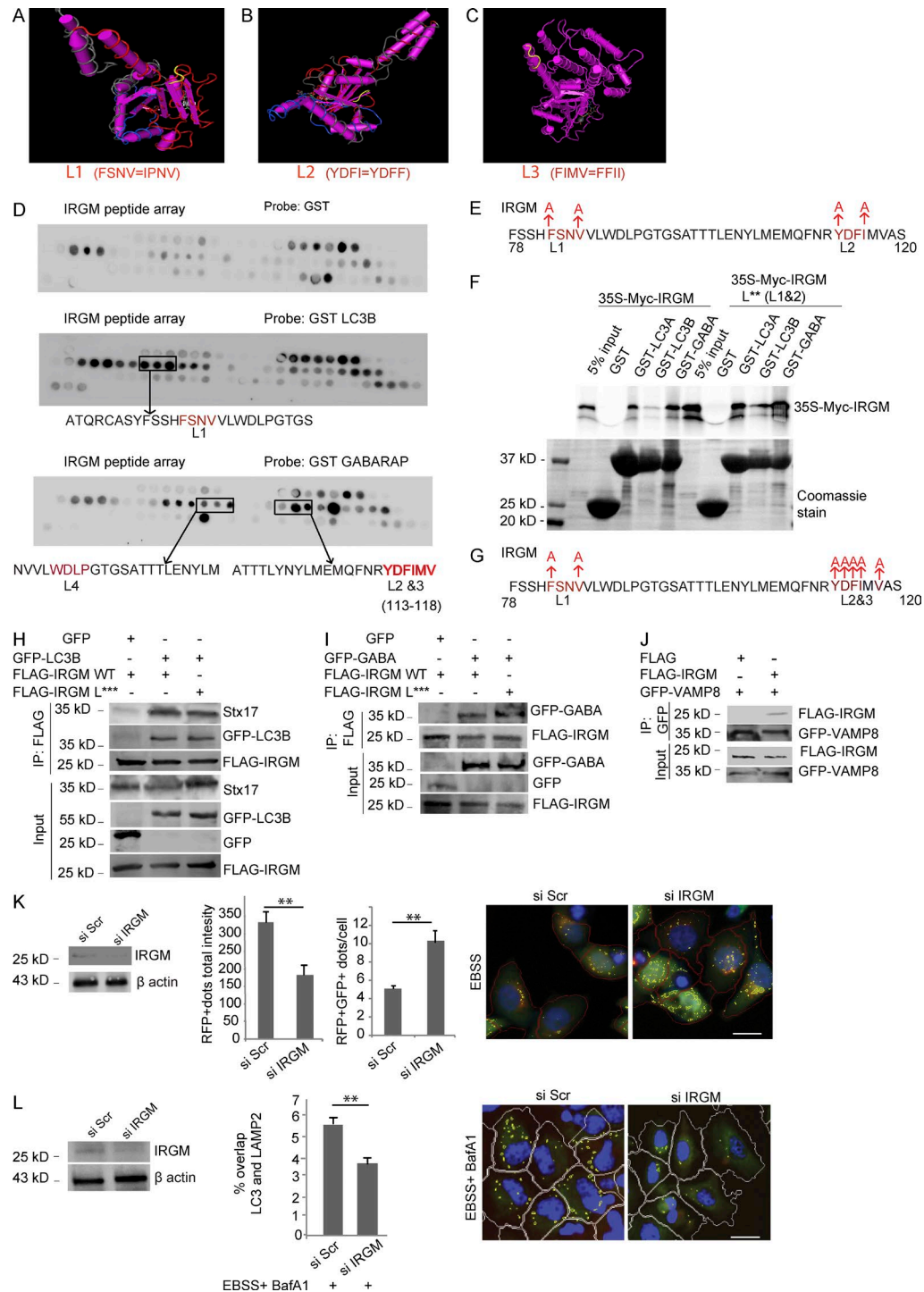


Figure S4. IRGM interacts with mAtg8s. (A–C) Potential sites (designated as L1, L2, and L3) in IRGM identified by published algorithms for LIR motifs were modeled on available crystal structure of the IRGM homologue 1TQ6 (Irga6). Yellow, surface loops where potential motifs aligned with 1TQ6. FSNV in IRGM corresponds with IPNV in 1TQ6 (A), YDFI in IRGM corresponds with YDFF in 1TQ6 (B), and FIMV in IRGM corresponds with FFII in 1TQ6 (C). (D) Peptide array dot blot analysis to analyze linear epitopes in IRGM binding to LC3B and GABARAP. (E) Mutational analysis of L1 and L2 sites in IRGM. (F) GST pulldown assay to test the effect of double L1 and L2 mutations (L**) in IRGM on binding to LC3B or GABARAP. (G) Triple-L site mutation (L***) in IRGM. (H and I) Effects of triple L*** mutation (L1^{83FSNV/ASNA86}, L2^{113YDFI/ADFA116}, and L3^{115FIMV/AAMA118}) on interactions between FLAG-IRGM and GFP-LC3B on Stx17 (H) or GFP-GABARAP (I); colP analysis of in 293T cells. (J) ColP analysis of FLAG-IRGM and GFP-VAMP8. (K) IRGM was knocked down in HeLa cells stably expressing mRFP-GFP-LC3, and autophagy was induced by incubating cells in EBSS for 2 h. Left: IRGM knockdown was tested by Western blotting; left graph: HC quantification of red (mRFP) puncta total intensity; right graph: HC quantification of yellow puncta (GFP⁺ mRFP⁺). Data indicate means \pm SEM. Representative images from HC microscopy analysis. Masks: red, primary objects (cells); yellow puncta, overlap. (L) HeLa cells were knocked down for IRGM using siRNA and incubated with EBSS in presence of bafilomycin A1; HC microscopy was used to analyze the effect of IRGM knockdown on colocalization between LC3 and LAMP2. Data indicate percent area overlap (means \pm SEM). **, $P < 0.01$ ($n = 3$) t test (K) or ANOVA (L; HC, >500 primary objects counted per well; minimum number of wells was four; three independent experiments). Masks: white, primary objects (cells); yellow puncta, overlap. Bars, 10 μ m.