Supplementary Information Figure Legends

Figure S1. Specificity of Rab21 and Sbf/MTMR13 roles in autophagosome-lysosome fusion. (A-F) Multiple RNAi constructs targeting Drosophila Rab21 or Sbf block starvation-induced LysoTracker puncta (autolysosomes) in fed or starved third instar larval fat body. **(A)** Control (LacZ), fed and **(B)** starved. RNAi depletion of **(C)** Rab21 (hairpin 2, VDRC 109991), **(D)** Rab21 (hairpin 3, TRiP 29403) or **(E)** Sbf (hairpin 1, VDRC 22317) blocks formation of starvation-induced autolysosomes. **(F)** Number of LysoTracker objects normalized to fat body area; SEM. **(G-J)** Drosophila fat body stained for endogenous Atg8 and Ref(2)P. Atg8 and Ref(2)P colocalization is increased in **(H)** *Rab21* or **(I)** *Sbf*-depleted fat body. **(J)** Average percentage of colocalized objects between endogenous Atg8 and Ref(2)P labeling; SEM. **(K)** Ratio of cytoplasmic area occupied by autophagosomes and **(L)** average autophagosome area (size) in transmission electron micrographs of starved larval fat body; SEM.

Figure S2. *RAB21* and *MTMR13* are not required for early steps in autophagy, while *VARP* and *RAB28* mildly affect autophagosome formation. (A-G) RAB21 or MTMR13 knockdown in HeLa cells does not affect early steps in autophagy. **(A)** RAB21 siRNAs and **(B)** MTMR13 siRNAs are specific. Immunoblots show **(A)** RAB21- or **(B)** MTMR13-specific depletion with two independent siRNAs each in HeLa cells. **(C-G)** RAB21 or MTMR13 depletion does not affect the number of Atg5 puncta in starved HeLa cells. HeLa cells stably expressing GFP:Atg5 and grown in EBSS (starved) were treated with **(C)** Scramble, **(D)** RAB21 siRNA or **(E)** MTMR13 siRNA. **(F, G)** Average number of GFP:Atg5 objects per cells with **(F)** RAB21 or **(G)** MTMR13 knockdown;

SEM. **(H-Q)** Autophagy is not grossly affected by VARP or RAB28 siRNA depletion in HeLa cells, as monitored by **(H)** VARP immunoblot or **(J)** *RAB28 mRNA* levels by semi-quantitative RT-PCR. Anti-LC3 immunoblot in **(H)** VARP or **(K)** RAB28 siRNAs-depleted HeLa cells grown in full media with or without Bafilomycin A1. **(I, L)** Ratio of LC3-II to tubulin integrated densities from six or four independent experiments, respectively. **(M)** Scramble (control). **(N)** *VARP* siRNA or **(O)** *RAB28* siRNA results in decreased number of GFP:LC3 autophagosomes. **(P, Q)** Average number GFP:LC3 objects per cell from two independent siRNAs against *VARP* or *RAB28*; SEM. **(R)** Average ratio of the number of LysoTracker objects normalized to cell area (SEM) for image data shown in Figure 3D-F. RAB21, MTMR13 and VAMP8 knockdown each mildly increased the number of LysoTracker staining compartments.

Figure S3. Rab21 and Sbf are required for endolysosomal homeostasis in fly, and RAB21 and VAMP8 localize predominantly on endosomes in human cells. (A-C) Autophagy assessed in Drosophila starved fat body. LysoTracker labels autolysosomes. **(A)** Control (LacZ), starved. Depletion of **(B)** Vamp7 by RNAi blocks autolysosomes in starved conditions. **(C)** Number of LysoTracker puncta normalized to fat body area; SEM. **(D)** Average size in pixels of GFP:Vamp7 puncta (SEM) for image data shown in Figure 4A-4C. **(E-H)** GFP:Rab7 in starved fat body. **(E)** Control. GFP:Rab7 accumulates upon **(F)** Rab21 RNAi or **(G)** Sbf RNAi. **(H)** Number of GFP:Rab7 puncta normalized to fat body area; SEM. GFP:Lamp1 in fat body, **(I-L)** starved or **(M-P)** fed. **(I)** Control. GFP:Lamp1 accumulates in punctae upon **(J)** Rab21 RNAi, **(K)** Sbf RNAi, **(M)** Vamp7 RNAi or **(N)** Rab7 RNAi, and **(O)** abnormally at the plasma membrane with Rab5 RNAi.

(L, P) Number of GFP:Lamp1 puncta normalized to fat body area; SEM. **(Q-W)** GFP:RAB21 colocalization with early endosomes is not affected by starvation. Immunofluorescence analysis of fed or starved HeLa cells expressing GFP:RAB21 and labeled with anti- **(Q)** RAB5, **(R)** RAB7, **(S)** RAB11, **(T)** LAMP1, **(U)** Syntaxin6 (STX6) or **(V)** clathrin heavy chain (CHC). **(W)** Per cell average Pearson Correlation of GFP:RAB21 with different compartments; SEM. **(X-Dd)** GFP:VAMP8 localizes mainly on early and late endosomes, with endolysosomal localization responsive to starvation. Immunofluorescence analysis of fed or starved HeLa cells expressing GFP:VAMP8 and labeled with anti- **(X)** RAB5, **(Y)** RAB7, **(Z)** RAB11, **(Aa)** LAMP1 **(Bb)** STX6 or **(Cc)** CHC. **(Dd)** Per cell average Pearson Correlation of GFP:VAMP8 with different compartments; SEM.

Figure S4. RAB21 and VAMP8 do not co-localize at Golgi or autophagosomes, and internalized VAMP8 traffics to early endosomes in RAB21 or MTMR13 depleted cells. (A-C) RAB21 and VAMP8 show weak or no colocalization with Golgi markers. Colocalization analysis of FLAG:RAB21-WT (green) and VAMP8-3xHA (red) with **(A)** GFP:GOLPH3 or **(B)** GFP:SialT (blue) **(A''' and B''')** Merge images of individual panels. **(C)** Per cell average Pearson Correlation of FLAG:RAB21 or VAMP8-3xHA with GFP:GOLPH3 or GFP:SialT; SEM. **(D-I)** RAB21 and VAMP8 show no to weak colocalization with autophagic compartments. Colocalization analysis in fed or starved HeLa cells. mCherry:RAB21 and **(D)** GFP:LC3 or **(E)** GFP:Atg5. **(F)** Per cell average Pearson Correlation of mCherry:RAB21 with GFP:LC3 or GFP:Atg5; SEM. mCherry:VAMP8 and **(G)** GFP:LC3 or **(H)** GFP:Atg5. **(I)** Per cell average Pearson

Correlation of mCherry:VAMP8 with GFP:LC3 or GFP:Atg5; SEM. **(J-L)** Validation of VAMP8 antibody uptake assay. **(J)** VAMP8-3xHA is specifically labeled at the plasma membrane of non-permeabilized cells. **(K)** Cell surface-labeled VAMP8-3xHA is internalized following a 30 minute chase at 37°C. **(L)** Steady state localization of VAMP8-3xHA in permeabilized cells. **(M-P)** VAMP8 delivery to early endosomes is unaffected in *RAB21*- or *MTMR13*-depleted cells. Antibody uptake assay of VAMP8:3xHA (internalized anti-HA, red) with EEA1 (green). **(M)** Scramble siRNA, **(N)** *RAB21* siRNA and **(O)** *MTMR13* siRNA treated cells. **(P)** Per cell average object colocalization of internalized VAMP8:3xHA with EEA1 over time; SEM. **(Q-S)** VAMP8 early endosomal co-localization increases in *RAB21*- or *MTMR13*-depleted cells treated with chloroquine. Antibody uptake assay of VAMP8:3xHA (internalized anti-HA, red) with EEA1 (green) in chloroquine treated cells. **(Q)** Scramble siRNA, **(R)** *RAB21* siRNA and **(S)** *MTMR13* siRNA treated cells. Quantification shown in Figure 4Q.

Figure S5. RAB21 interaction with VAMP8 requires full starvation and MTMR13. **(A)** RAB21 interaction with VAMP8 requires full starvation. FLAG IP FLAG:RAB21 and immunoblot of coexpressed GFP:VAMP8 from differentially starved HeLa cells as shown. Lysates were immunoblotted for phospho-Akt-S473 and phospho- S6K-T389 to assess starvation efficacy. **(B)** RAB21 interacts with VAMP8 independent of VAMP8 trans-SNARE complex involved in autophagosome-lysosome fusion. FLAG IP of FLAG:RAB21 and immunoblot of co-expressed GFP:VAMP8 or GFP:SYNTAXIN17 in fed or starved conditions. **(C-F)** Wild type and constitutively active RAB21 preferentially colocalize with VAMP8. Colocalization analysis of starved HeLa cells expressing

GFP:VAMP8 and **(C)** mCherry:RAB21-WT, **(D)** mCherry:RAB21-CA or **(E)** mCherry:RAB21-DN. **(F)** Per cell average Pearson Correlation of GFP:VAMP8 with the different m:Cherry:RAB21 forms. **(G-I)** RAB21 and VAMP8 colocalization requires MTMR13. GFP:RAB21-WT and mCherry:VAMP8 colocalization in **(G-G')** Scramble siRNA or **(H-H')** MTMR13 siRNA (probe 1). **(I)** Per cell average Pearson Correlation of GFP:RAB21-WT with mCherry:VAMP8; SEM.