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Supplemental Information

Enteroviruses Remodel Autophagic Trafficking through Regulation of Host SNARE Proteins to Promote Virus Replication and Cell Exit

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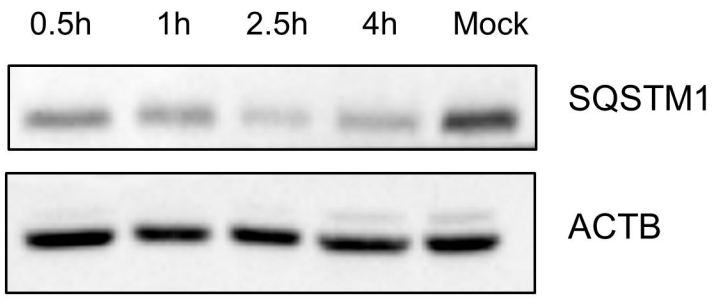

Figure S1: Validation of starvation medium in H1HeLa cells, related to Figure 1F. H1HeLa cells were placed in starvation media for times indicated. Cells were collected and immunoblotted for SQSTM1 and ACTB.

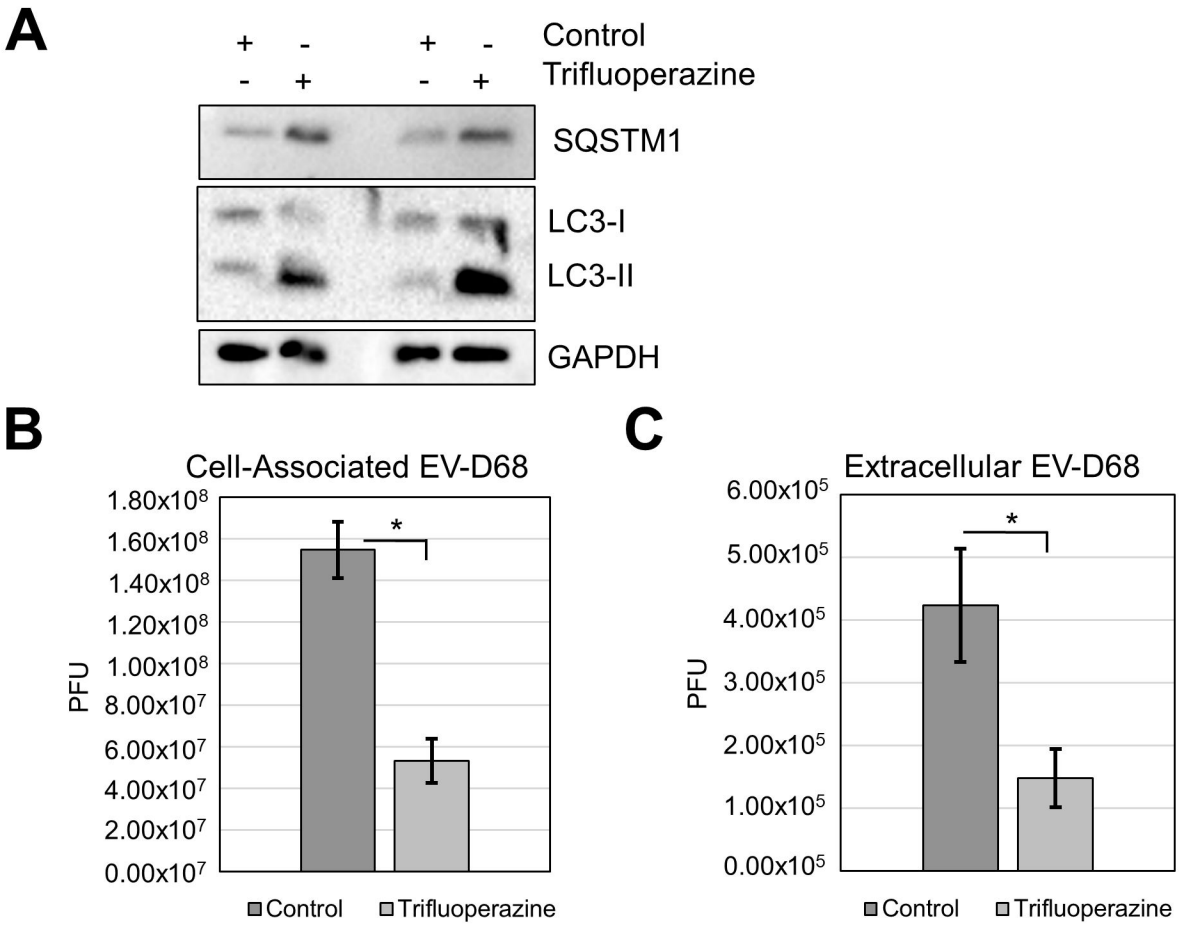

Figure S2: Trifluoperazine treatments inhibit EV-D68 replication, related to Figure 1. (A) H1HeLa cells were treated for 18 hr with 8.3μM Trifluoperazine. Treated cells were collected and subjected to western blot analysis, probed for SQSTM1 and LC3. Two replicates shown. **(B, C)** Treated cells were infected at an MOI of 0.1 with EV-D68 for 5 hr in the presence of trifluoperazine. Cells were collected and both cell-associated and extracellular viral titers were determined by plaque assay. Cell-associated (B, $p=0.016$) and extracellular (C, $p=0.033$) titers are represented as the mean ± SEM. Statistical tests were done using an unpaired Student's t test with statistical significance set at $*p \leq 0.05$.

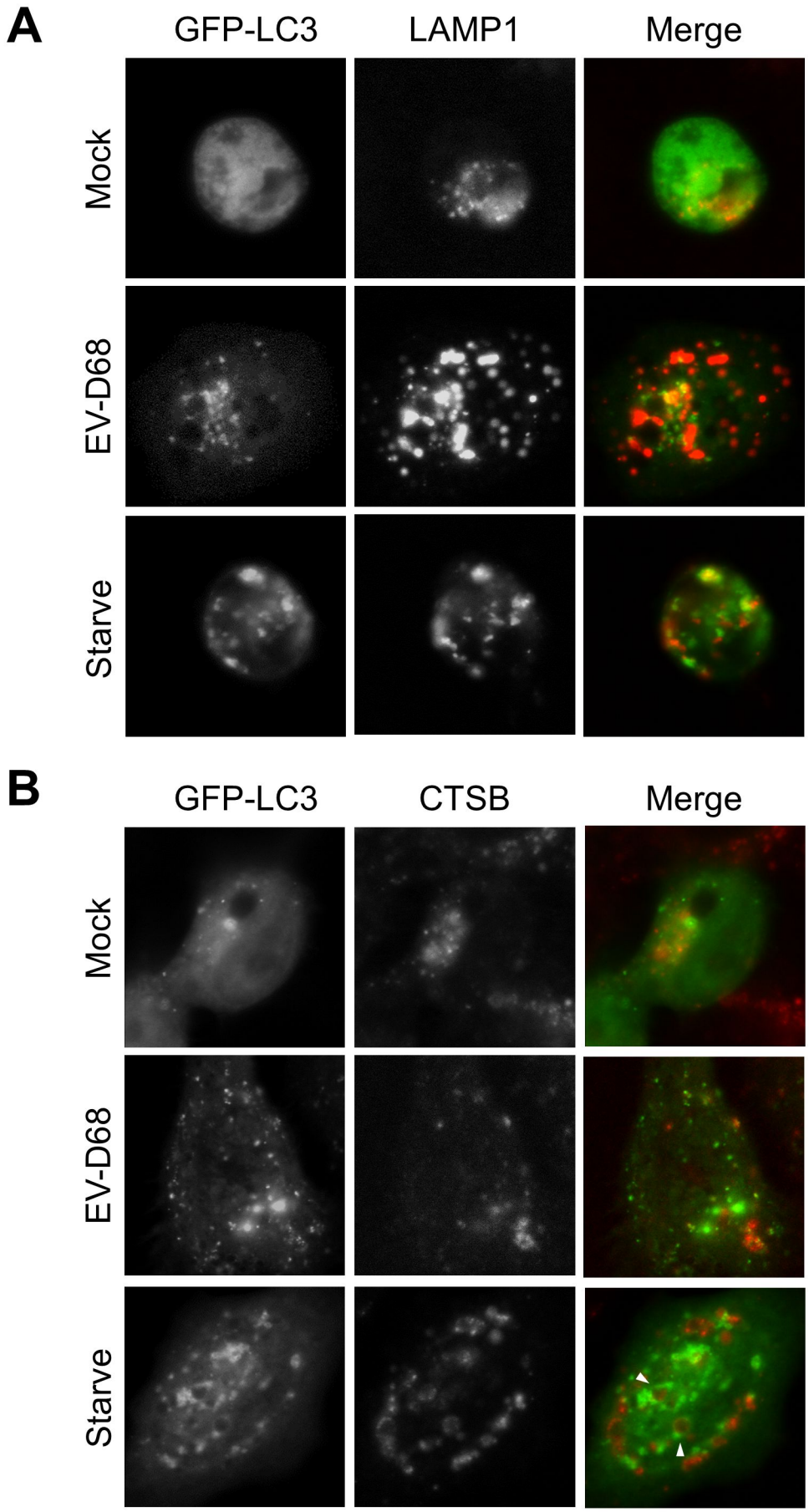

Figure S3: Enterovirus D68-Induced Autophagosomes costain with LAMP1 but not CTSB, related to Figures 2 and 3A. H1HeLa cells were transfected with GFP-LC3 24 hr prior to experiment. Cells were either untreated/mock, infected with EV-D68 for 4 hr or treated with starvation medium for 4 hr. Cells were then fixed in methanol and stained for LAMP1 **(A)** or CTSB **(B)**. Cells were imaged for GFP and AF568 fluorescence. Representative images shown.

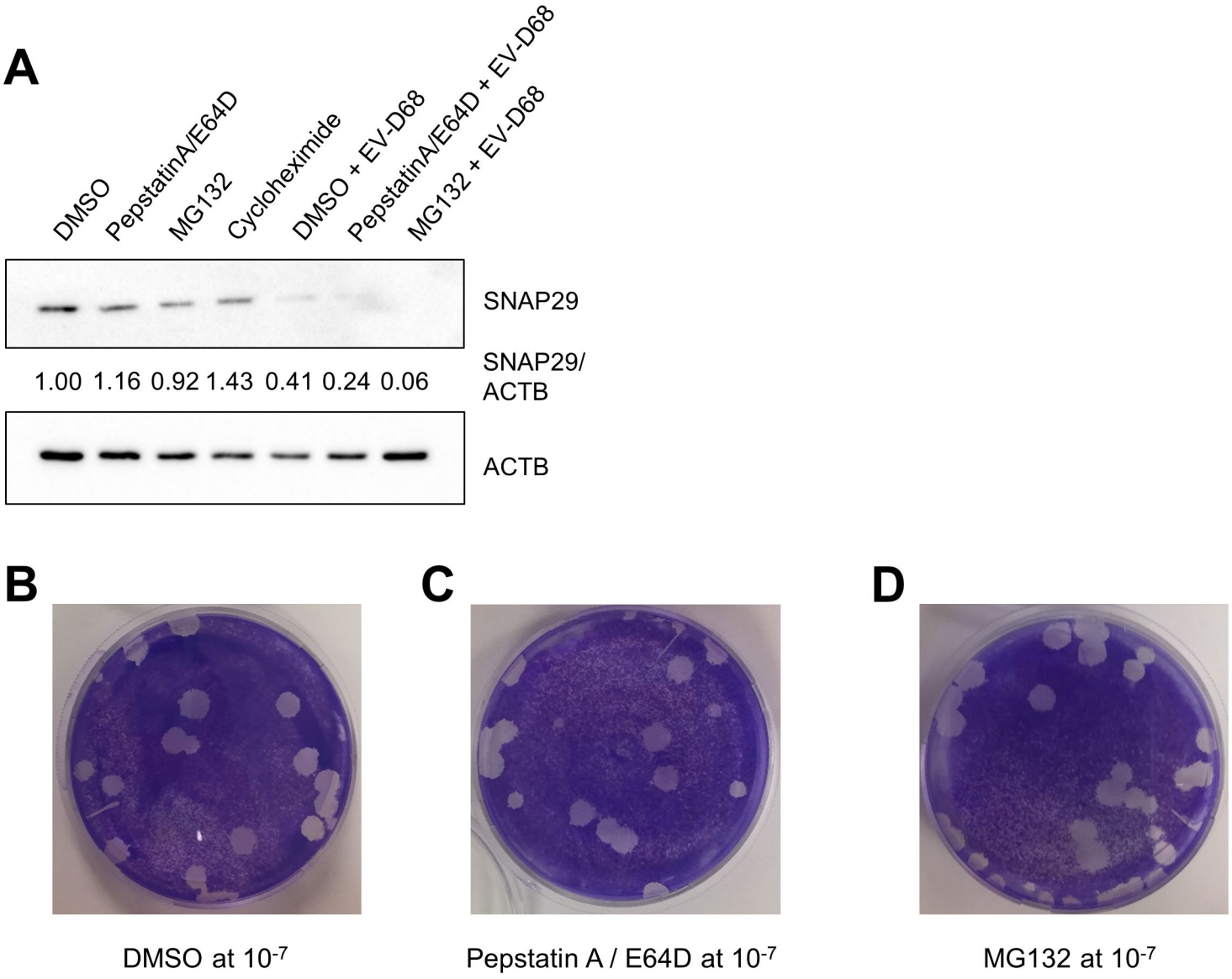

Figure S4: SNAP29 reduction is not due to regular protein turnover, lysosomal or proteasomal degradation, related to Figure 5. (A) H1HeLa cells were treated with 10µg/mL Pepstatin A and 100µM E64D, 20µM MG132, 100µM cycloheximide or vehicle. Cells were infected at an MOI of 25 with EV-D68 for 5 hr, then were collected. Samples were subjected to western blot analysis and immunoblotted for SNAP29 levels. **(B-D)** Parallel drug treatment plates were infected at an MOI of 0.1 for 5 hr. Cells were then collected and subjected to plaque assay analysis. Images of the 10^{-7} serial dilution plate are shown for each treatment.

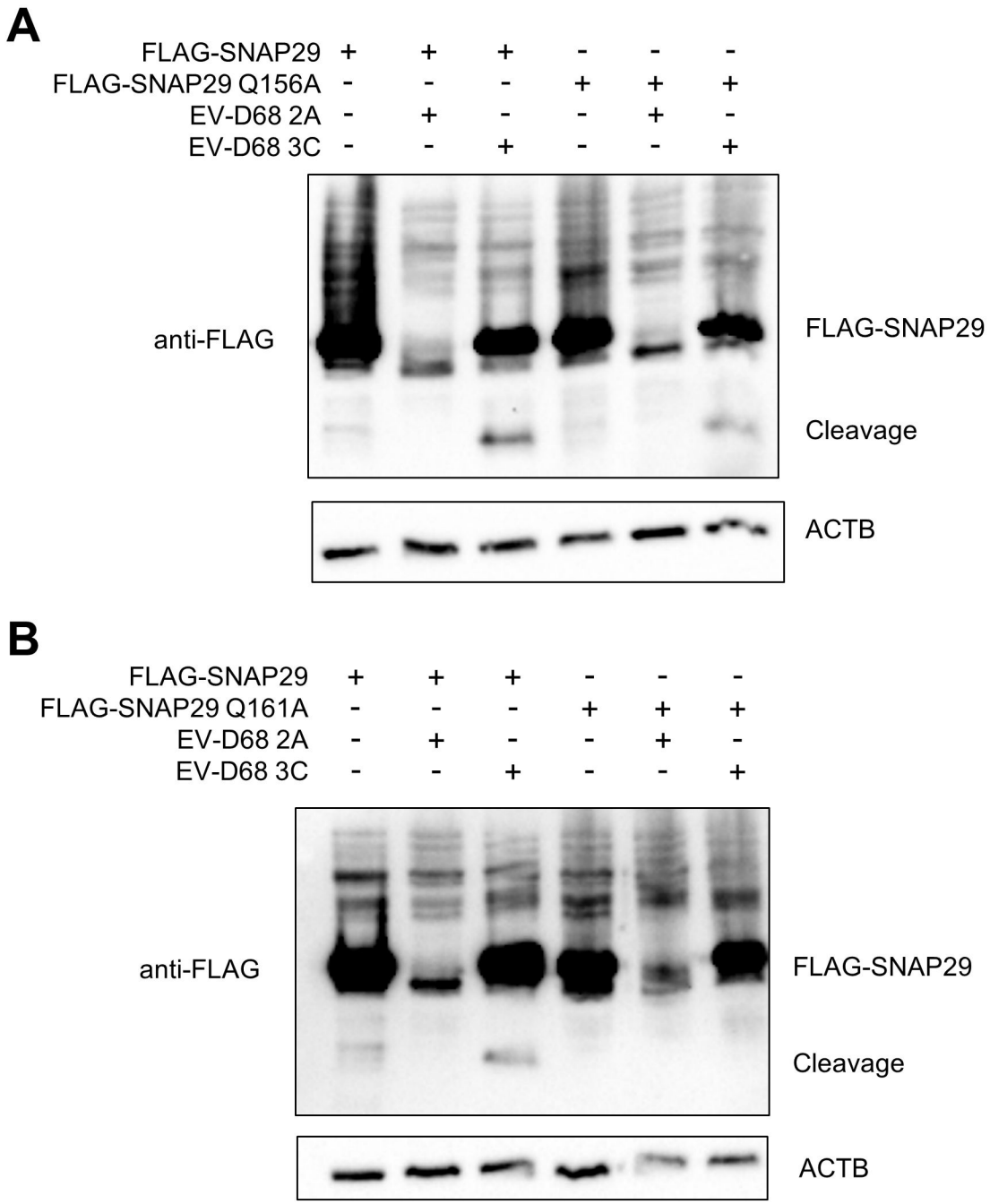

Figure S5: FLAG-SNAP29-Q161A is not cleaved by EV-D68 3C protease, related to Figure 5. 293T cells were transfected with FLAG-SNAP29, FLAG-SNAP29 Q156A **(A)**, or FLAG-SNAP29 Q161A **(B)**, and either EV-D68 2A or EV-D68 3C for 40 hr. Cells were collected and samples were immunoblotted for FLAG. Full length FLAG-SNAP29 and cleavage bands are marked.

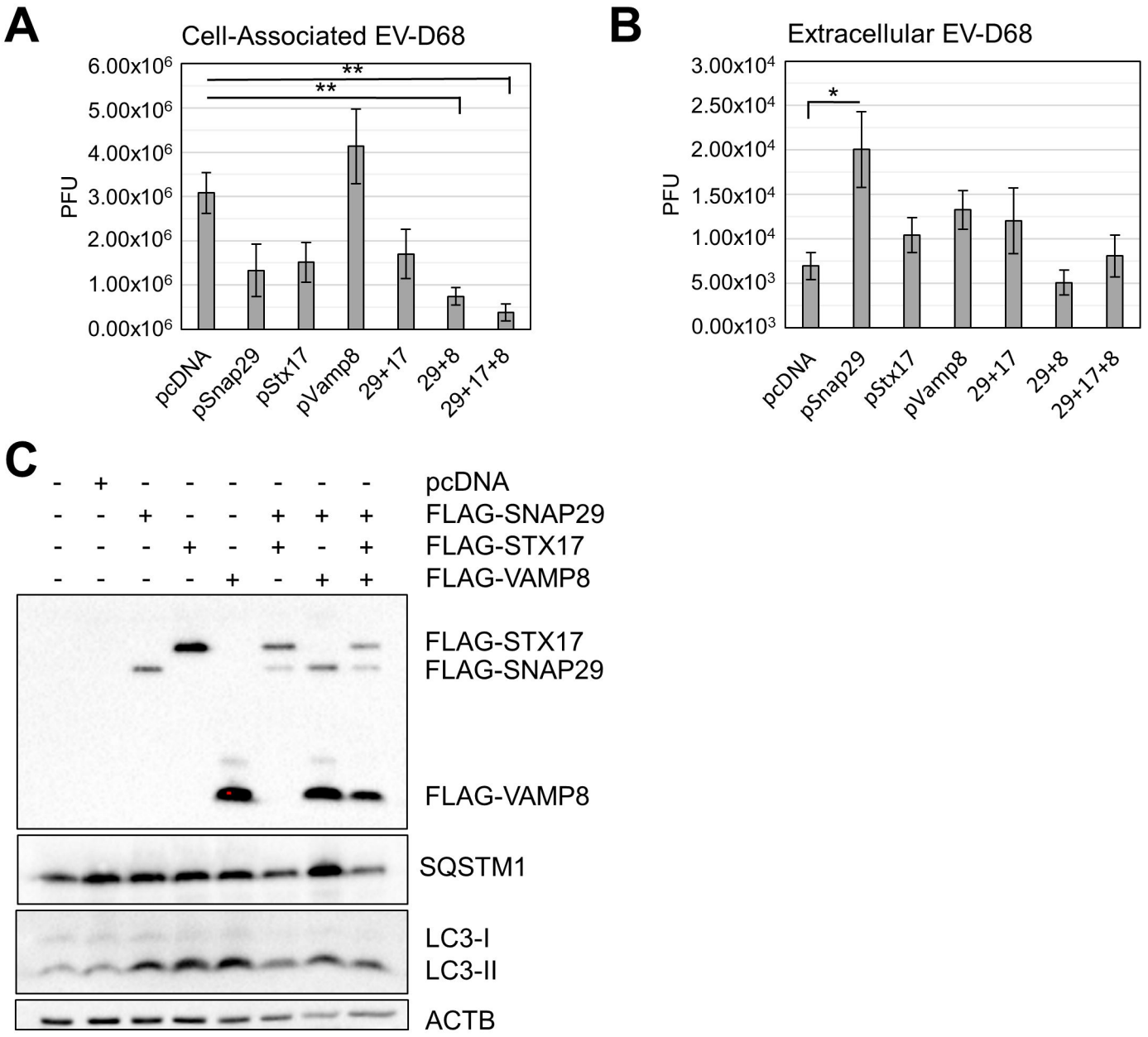

Figure S6: Overexpression of SNARE proteins involved in autophagosome-lysosome fusion and EV-D68 viral titers, related to Figure 6. H1HeLa cells were transfected with FLAG-SNAP29, FLAG-STX17, FLAG-VAMP8 or pcDNA control 40 hr prior to infection. **(A, B)** Transfected cells were infected at an MOI of 25 with EV-D68 for 5 hr and then collected. Viral titers were determined by plaque assay for both cell-associated (A, p=0.0031, p=0.0011) and extracellular virus (B, p=0.043). **(C)** Transfected cells were collected and immunoblotted for FLAG, SQSTM1, and LC3. Statistical tests were done using an unpaired Student's t test with statistical significance set at *$p \leq 0.05$, **$p \leq 0.01$.

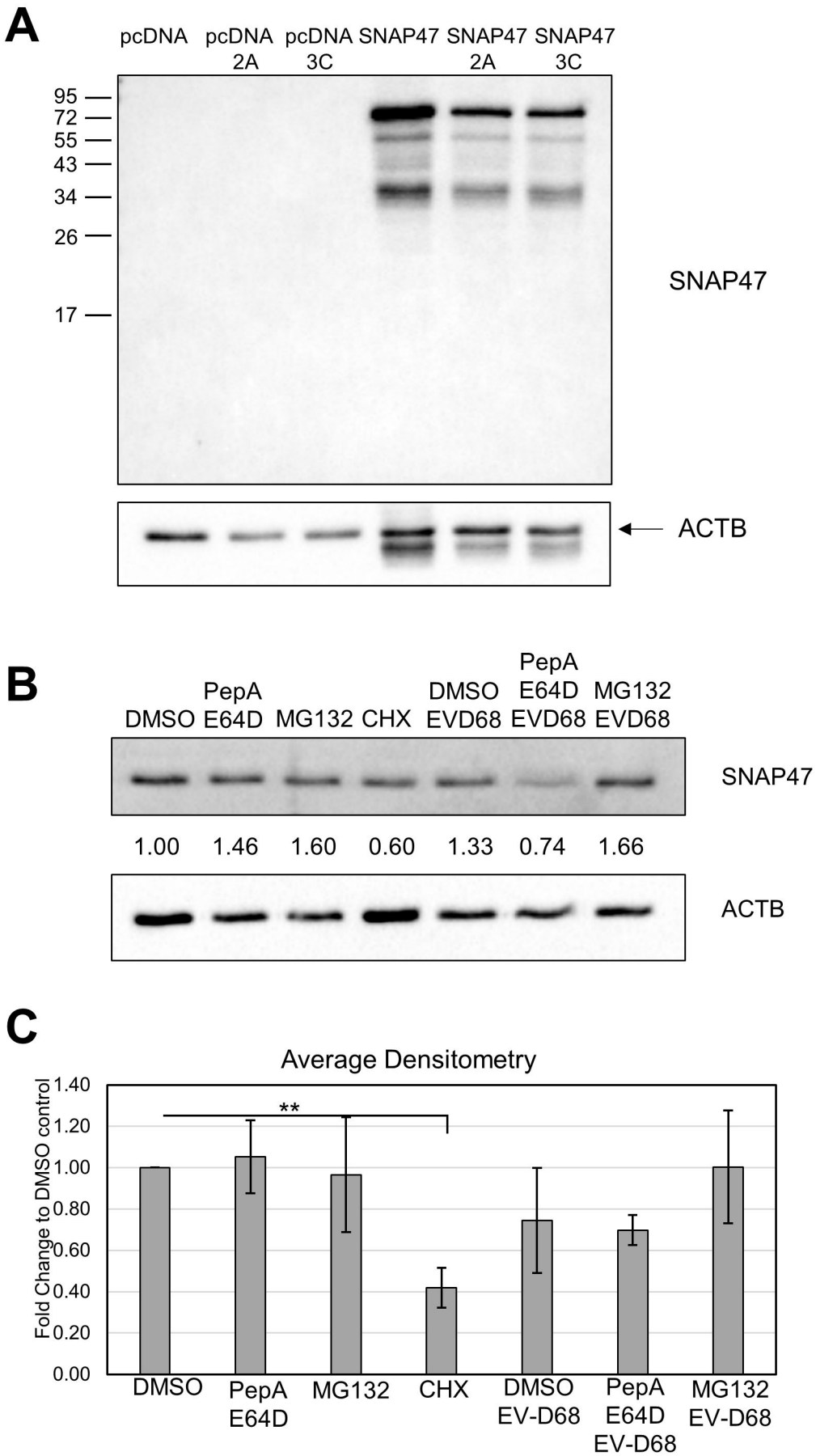

Figure S7: SNAP47 reduction is not due to EV-D68 protease cleavage, but may be due to protein turnover, related to Figure 7. (A) 293T cells were transfected with pcDNA or SNAP47 for 24 hr, then transfected again with EV-D68 2A or EV-D68 3C for 24 hr, a total of 48 hr. Cells were collected and samples were immunoblotted for SNAP47. **(B)** 293T cells were treated with 10μg/mL Pepstatin A and 100μM E64D, 20μM MG132, 100μM cycloheximide or vehicle. Cells were infected at an MOI of 25 with EV-D68 for 5 hr, then were collected. Samples were subjected to western blot analysis and immunoblotted for SNAP47. **(C)** Densitometry analysis of B, from 3 independent experiments. P=0.0078. Statistical tests were done using an unpaired Student's t test with statistical significance set at *p ≤ 0.05, **p ≤0.01.

Supplemental Experimental Procedures

Cell Culture

H1HeLa cells were obtained from ATCC (ATCC Cat# CRL-1958, RRID:CVCL_3334) and have been tested for mycoplasma contamination. Cells were maintained at 37°C in 5% CO_2 in MEM media (Gibco), supplemented with 10% heat inactivated FBS (Gibco), 1x penicillin/streptomycin (Gibco) and 1x sodium pyruvate (Gibco). Media were changed every 2 days and subculture splits were 1:5. 293T cells were obtained from ATCC (ATCC cat# CRL-3216, RRID:CVCL_0063). Cells were maintained at 37°C in 5% CO_2 in DMEM media (Gibco), supplemented with 10% heat-inactivated FBS (Gibco), and 1x sodium pyruvate (Gibco). 293T cells were subcultured at 1:5-1:10 splits. Subculture procedure for both cell lines was as follows: cell plate was aspirated of used media, cells were washed once in 1x PBS if needed, 2mL of trypsin was added, and cells were pipetted to dissociate, then re-suspended in complete medium to 10mL. Plating for transfections took place the day before in media without antibiotics.

Viral Plasmids

Viral plasmids were as described for Mahoney Poliovirus 1, and Coxsackievirus B3 (Feuer et al., 2002; Maynell et al., 1992). Fermon EV-D68 sequence was generated in pBluescript with the addition of a 3' MluI restriction site (Bio Basic, inc.).

Viral Stock Growth Method

Viral plasmids were obtained and transformed into subcloning efficiency DH5α *Escherichia coli* (Invitrogen, Cat # 18258012) and grown in selective LB agar with ampicillin. Individual colonies were selected, grown up and the plasmids purified by phenol-chloroform extraction. Viral plasmids were digested with restriction enzyme to release insert, RNA synthesized, and then transfected into H1HeLa cells using DMRIE-C transfection reagent (Invitrogen) and overlaid with 2x MEM/2% agar mixture. Plaques were isolated 48 hours post-transfection. Plaque-picks were freeze-thawed 3x in a dry ice/ ethanol bath and a 37° water bath. Plaque-picks were then expanded on a plate of H1HeLa cells for 5-6 hours and cells were harvested in 1mL of PBS^+ (PBS with 10μg/mL of both calcium chloride and magnesium chloride). These midi-stocks were freeze-thawed 3x and expanded once more. The final stocks were titered via plaque assay.

Viral Infections

Viral infections were carried out for 5 hours for EV-D68 and CVB3, and 6 hours for PV, except where noted. Cells were counted in a parallel plate by hemacytometer and virus needed for the appropriate MOI was calculated. The 2 MOIs used within, with exceptions were noted, are 0.1 for low MOI infections for growth curves and an MOI of 25 as a high MOI used in fluorescent microscopy, electron microscopy and western blotting methods. Virus was diluted in PBS^+. Cell media was aspirated from plates and virus mixture added. Plates were incubated for 30min at 37°C. Plates were then rinsed in PBS 1x to remove any unbound virus, and complete media was added for duration of the infection time point. Collection of samples post infection was done by scraping cells into 1mL of PBS and either frozen at -20C immediately, or spun down at 18600xg for 5 minutes, PBS supernatant was aspirated, and just the pellet stored at -20°C for further sample processing.

Drug Treatments

Bafilomycin A1 (BafA1) (Santa Cruz Biotech, Cat# SC-201550; CAS# 88899-55-2) was prepared into a stock solution of 100μM in ethanol and used at a concentration of 0.1μM. Baf A1 was added to fresh culture media and pretreated for 18 hours prior to infection. BafA1 was added at its working concentration to the infection's adsorption PBS^+ and complete media during infection.

Starvation media was prepared, as described previously, with 140mM sodium chloride, 1mM calcium chloride, 1mM magnesium chloride, 5mM glucose, 20mM HEPES and 1% w/v bovine serum albumin in ddH_2O(Axe et al., 2008). Solution was sterile-filtered through a 0.22μm filter. Starvation media was used on H1HeLa cells, which

were starved for 4 hours prior to infection. Adsorption and the duration of the infection were completed as written in "viral infections."

Pepstatin A and E64D (also known as 2S,3S-*trans*-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester) treatments were completed using Pepstatin A (Invitrogen, Cat# 78436; CAS# 26305-03-3) prepared into a stock solution of 10mg/mL in DMSO, used at a working concentration of 10µg/mL. E64D (Sigma, Cat# E8640; CAS# 88321-09-9) was prepared into a stock solution of 10mM in DMSO, used at a working concentration of 100µM. This treatment was added to the experimental plates after the adsorption step of infections; added to the complete media for 5 hours concurrent with infection.
MG132 (Sigma Aldrich, Cat # M8699; CAS# 1211877-36-9) was purchased as a ready-made solution of 10mM in DMSO and was used at a working concentration of 20µM. This treatment was added concurrent with infection in the complete media.

Cycloheximide (Sigma Aldrich, Cat # C4859; CAS# 66-81-9) was purchased as a ready-made solution of 100 mg/mL in DMSO and was used at a working concentration of 100µM. This treatment was made up in complete media and added to a cell plate for 5 hours. The cells were then collected via scraping into 1 mL of PBS, spun down at 18600xg for 5 minutes, PBS supernatant aspirated, and the pellet stored at -20°C.

Trifluoperazine (Selleckt Chemicals, Cat # S3201; CAS# 440-17-5) was made up in ddH_2O and recommended to use 8.3µM as a working concentration(Zhang et al., 2007). This treatment was made up in complete media and pre-treated for 18 hours prior to infection. Adsorption and infection were also completed in the presence of this compound.

Plaque Assays
Plaque assays were used to determine viral growth titers in this work. Samples for assay were freeze-thawed 3x between an ethanol-dry ice bath and 37°C water bath. These samples were then serially diluted either 1:10 or 1:100 into PBS^+ containing tubes. H1HeLa cells were plated to approximately 95% confluence on 6cm plates the day before assays were to begin. Media was aspirated from these plates. 250µL of the serially diluted sample was added to a plate, and plates were incubated for 30 minutes at 37°C. After the incubation, 4mLs of a 1:1 2x MEM/2% agar mixture was added to each plate. Plates were incubated for 32 hours at 37°C. Agar top was removed from each plate, and cells were fixed with a 20% ethanol/ 0.01% crystal violet solution and rinsed in water. Plaques were counted and titers were back-calculated to plaque forming units per sample (PFU).

Western Blotting
Sample pellets collected for western blot were removed from the -20°C freezer and thawed on ice. Pellets were lysed in a 10mM sodium chloride, 1.5mM magnesium chloride, 1% NP-40/Tergitol, 10mM Tris-HCl (pH 7.5), and 1x protease inhibitor cocktail (Thermo) for 20 minutes on ice. Protein content was quantified via Bradford assays, using 0-1mg/mL BSA standards for comparison. Polyacrylamide gels were poured for Tris-Glycine SDS-PAGE and samples were loaded with Pageruler dyed protein ladder (Invitrogen). Gels were run at 90V through the stacking gel, and 125V through the resolving gel. Gels were transferred using an iBlot 2, on pre-installed program 0, using PVDF cassettes (Invitrogen).

Blots were blocked in 5% milk/TBST for 30 minutes at room temperature. Primary antibodies were either made in 1% milk/TBST or 2.5% BSA/TBST, as per experimental optimization dictated, and stored at -20° between uses. Blots were incubated in primary antibody overnight at 4°C. Blots were washed 3x in TBST for 10 minutes each at room temperature. Secondary antibodies of either goat anti rabbit (1:2000) or goat anti- mouse (1:10,000) (Biorad) were diluted into 5% milk/TBST and made fresh each week. Blots were incubated in secondary antibody for 1 hour at room temperature. Blots were washed 3x in TBST for 10 minutes each, at room temperature. Western blots were visualized by a short incubation in Western Lightning ECL (Perkin Elmer) and use of a Biorad ChemiDoc

instrument. If needed, blots were stripped for 10 minutes at room temperature using Restore stripping buffer (Invitrogen), rinsed in TBST to remove buffer, then re-blocked for 30 minutes using 5% milk/TBST.

siRNA Knockdowns

This work uses siRNA knockdown technology to silence genes of interest prior to an infection. Mission Sirna Universal Negative Control 1 from Sigma, Cat # SIC001, was used as a negative control in all cases. The sequence for ATG7_1 and siATG7_2 were generated from Sigma's Mission predesigned siRNA generated by Rosetta siRNA Design Algorithm and sequences are provided in the oligonucleotide table. The sequence for siSNAP47 was from Sigma's Mission predesigned siRNA library generated by Rosetta siRNA Design Algorithm and sequence is provided in the oligonucleotide table. The SNAP29 siRNA sequence is provided in the oligonucleotide table. The siRNAs were generated by Sigma and were resuspended from their lyophilized form in a buffer comprised of 100mM potassium acetate, 2mM magnesium acetate, and 30mM HEPES-KOH (pH7.2), as suggested by Sigma, to a concentration of 100µM. The tubes were boiled at 100°C for 5 minutes, and incubated at 37°C for 1 hour to anneal. siRNAs were aliquoted and frozen at -20°C. A total of 75 pmol per 6-well was transfected into H1HeLa cells at a ~40% confluence density using 5µL per well of Lipofectamine 2000 (Invitrogen) in antibiotic-free media conditions. siRNA transfected cells were incubated at 37°C for 40 hours before continuing with the experiment. Knockdown efficacy was assessed by western blot in each experiment.

Overexpression transfections

This work uses gene-of-interest containing plasmids for ectopic expression. pcDNA was used a vector control for most overexpression experiments. FLAG-SNAP29, FLAG-STX17 and FLAG-VAMP8 plasmid constructs were obtained through Addgene as a gift from Noboru Mizushima (Addgene plasmids 45915, 45911, and 45912, respectively). GFP-SNAP47 was purchased from OriGene Technologies (cat # RG205867). SQSTM1-WT and SQSTM1-MT/SQSTM1-G241E were obtained from Honglin Luo (Shi et al., 2013). FLAG-SNAP29-Q156A, FLAG-SNAP29-Q161A, EV-D68 2A, and EV-D68 3C were generated by this lab for this work, as described below. For H1HeLa cell transfections, 0.4µg of plasmid was transfected into 60% confluent cells using Effectene transfection reagent's protocol (Qiagen). Cells were incubated at 37°C for 24 hours post transfection before continuing with the experiment. For 293T cell transfections, 2.5µg of each plasmid was transfected into 80% confluent cells using Mirus TransIT-293 and the reagent's protocol (Mirus Bio cat# MIR 2700) for 24-48 hours prior to continuing the experiment. Ectopic expression of the proteins was assessed by western blot in each experiment.

Viral protease EV-D68-2A and EV-D68-3C plasmids were constructed using the PHAGE system, which was a gift from Richard Mulligan (Murphy et al., 2006). Primers for the viral proteases were ordered from Sigma, and the Fermon strain viral plasmid was used as a template. Proteases were cloned out of the viral plasmid and into the PHAGE plasmid using restriction enzymes. PHAGE plasmid constructs were sequenced to confirm proper insertion. Plasmids were transformed into DH5α *E. coli,* grown up in cultured and a plasmid prep performed using Genelute midiprep kit (Sigma). PHAGE plasmid contains GFP, which can be used as a control for transfection. PHAGE plasmid was used as a vector control in these experiments only.

FLAG-SNAP29 point mutation plasmids for Q156 and Q161 were generated via PCR using the primers listed in the oligonucleotide table below. Plasmids were transformed into DH5α *E. coli*, per manufacturer's instructions. A series of colonies were picked, had minipreps performed on them, and sequenced to confirm the point mutation was the correct and the only mutation in the SNAP29 open reading frame.

Electron Microscopy

For electron microscopy analysis, cells were fixed with a solution of 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M PIPES buffer (pH 7.2), scrapped off the tissue culture vessel, washed in 0.1M PIPES buffer and collected by centrifugation. Cell pellets were enrobed in 2.5% low melting point agarose, trimmed into 1mm^3 blocks and post-

fixed with 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1M PIPES buffer for 1 hour at 4°C. After washing, agarose blocks containing cells were *en bloc* stained with 1% uranyl acetate in water and dehydrated using increasing concentration of ethanol from 30%; 50%; 70%; 90% and 100% for 10 min at each step. Specimen were then incubated with two changes of 100% acetone and infiltrated, in increasing concentration of Araldite-Epoxy resin (Araldite, EMbed 812; Electron Microscopy Sciences, PA), and embedded in pure resin at 60°C for 24 to 48 h. Ultrathin sections at ~70nm thickness were cut on Leica UC6 ultramicrotome (Leica Microsystems, Inc., Bannockburn, IL), and examined in a FEI Tecnai T12 electron microscope operated at 80 kV. Digital images were acquired by using a AMT bottom mount CCD camera and AMT600 software.

Fluorescence Microscopy

For fluorescent microscopy imaging, H1HeLa cells were plated in 12 wells on sterile poly-D-lysine coated coverslips. After experimental procedures, cells were fixed in ice-cold methanol for 15 minutes at -20°C. Coverslips were then blocked in either 1x PBS/ 5% normal goat serum/ 0.3% Triton X-100 or 1x PBS/ 5% normal goat serum/ 0.1% Saponin for 1 hour at room temperature. Coverslips were then incubated in primary antibody overnight at 4°C. CTSB primary antibody was used at 1:800, diluted in 1x PBS/ 1% BSA/ 0.1% Saponin antibody dilution buffer. LAMP1 primary antibody was used at 1:200, diluted in 1x PBS/ 1% BSA/ 0.3% Triton X-100 antibody dilution buffer. Coverslips were washed 3 times in 1x PBS for 5 minutes each. Secondary antibody Alexa fluor 568 was diluted in corresponding antibody dilution buffers for each primary at a concentration of 1:1000, and incubated for 1 hour at room temperature. Coverslips were washed 3 times in 1x PBS for 5 minutes each. Coverslips were then mounted with Vectashield with DAPI (H-1200) and slides sealed with nail polish. Images were acquired on a Zeiss LSM 710.

Autoradiography

H1HeLa cells were changed to a methionine, cysteine and glutamine-free MEM (Fisher ICN1641454) supplemented with 100µCi of 35-S Methionine (Perkin Elmer, Cat# NEG772007MC) for 1 hour during the course of an infection. Cells were collected at the end of that period in PBS, spun down and the supernatant was pulled off and discarded. Pellets were kept at -80°C until ready for processing. A 12% polyacrylamide gel was poured in large format gel plates. Lysates were loaded onto the gel and run at constant 35mA. When complete, gel was dried on Biorad Model #583 Gel Dryer for 1:50 hours at 60°C and exposed to X-ray film for 24 hours at room temperature. Film was developed using AFP Imaging Mini-MED/90 X-Ray Film Processor.

Antibody Table

Antibodies		
Mouse monoclonal anti-SQSTM1	Abnova	Abnova Corporation Cat# H00008878-M01 RRID:AB_437085
Rabbit polyclonal anti-LC3B	Novus	Novus Cat# NB600-1384 RRID:AB_669581
Rabbit polyclonal anti-ACTB	Novus	Novus Cat# NB600-532 RRID:AB_10002039
Rabbit polyclonal anti-STX17	Abcam	Abcam Cat# ab116113 RRID:AB_10903821
Rabbit monoclonal anti-SNAP29 C-terminal	Abcam	Abcam Cat # ab138500; RRID: AB_2687667
Rabbit polyclonal anti-TUBA	Cell Signaling Technology	Cell Signaling Technology Cat # 2144; RRID: AB_2210548
Mouse monoclonal anti-FLAG	Sigma Aldrich	Sigma-Aldrich Cat# F1804, RRID:AB_262044
Rabbit (monoclonal) anti-SNAP29 N-terminal	Abcam	Abcam Cat # ab181151; RRID: AB_2687668
Rabbit polyclonal anti-GFP	Cell Signaling Technology	Cell Signaling Technology Cat# 2555, RRID:AB_390710
Rabbit monoclonal anti-GAPDH	Cell Signaling Technology	Cell Signaling Technology Cat# 2118, RRID:AB_561053
Rabbit monoclonal anti-ATG7	Cell Signaling Technology	Cell Signaling Technology Cat#8558, RRID:AB_10831194
Rabbit monoclonal anti-Cathepsin B (CTSB)	Cell Signaling Technology	Cell Signaling Technology Cat# 31718, RRID:AB_2687580
Rabbit monoclonal anti-LAMP1	Cell Signaling Technology	Cell Signaling Technology Cat# 9091, RRID:AB_2687579
Goat anti-Rabbit	Bio-rad / AbD Serotec	Bio-Rad / AbD Serotec Cat# 170-6515, RRID:AB_11125142
Goat anti-Mouse	Bio-rad / AbD Serotec	Bio-Rad / AbD Serotec Cat# 170-6516, RRID:AB_11125547
Alexa Fluor 568 goat anti-rabbit	Invitrogen	Molecular Probes Cat# A-11036, RRID:AB_143011

Oligonucleotide Table

Oligonucleotides		
siRNA to SNAP29: Sense: 5'-GAAGCUAUAAGUACAAGUA-3' Antisense 5'-UACUUGUACUUAUAGCUUC-3'	This paper	N/A
siRNA to SNAP47: Sense: 5'-GAAAGAAGGGAUACUGAUA-3' Antisense: 5'-UAUCAGUAUCCCUUCUUUC-3'	Sigma Aldrich	Product # NM_053052
siRNA to ATG7_1: Sense: 5'-GAGAUAUGGGAAUCCAUAA-3' Antisense: 5'-UUAUGGAUUCCCAUAUCUC-3'	Sigma Aldrich	Product # NM_006395
siRNA to ATG7_2: Sense: 5'-CAGCUAUUGGAACACUGUA-3' Antisense: 5'-UACAGUGUUCCAAUAGCUG-3'	Sigma Aldrich	Product # NM_006395
Primer EV-D68 3C protease cloning primers: 5'end: 5'-ACGTGCGGCCGCATGGGACCAGGATTTGATTTTG-3' 3'end (positive strand): 5'-CTCTTACTTTACTGATACACAA-3' 3'end (negative strand): 5'- ACGTCTCGAGCTATTGTGTATCAGTAAAGTAAGAG-3'	This paper	N/A
EV-D68 2A protease cloning primers: 5' end: 5'-ACGTGCGGCCGCATGGGTCCAGGTTTTGG-3' 3'end: (positive strand): 5'-GATACTGATGTTATGGAACAA-3' 3'end (negative strand): 5'- ACGTCTCGAGCTATTGTTCCATAACATCAGTATC-3'	This paper	N/A
SNAP29 Mutagenesis primer set for Q156A: Positive strand: 5'-GAACTGGAAGCAAAGTACCAGGCCAGCC -3' Negative strand: 5'-GGCTGGCCTGGTACTTTGCTTCCAGTTC -3'	This paper	N/A
SNAP29 Mutagenesis primer set for Q161A: Positive strand: 5'-GAACAGGAAGCAAAGTACCTGGCCAGCC -3' Negative strand: 5'-GGCTGGCCAGGTACTTTGCTTCCTGTTC -3'	This paper	N/A
SNAP29 Sequencing primer set: Positive strand: 5'-GACGCAAATGGGCGGTAGGC-3' Negative strand: 5'-GCCACCCGGGATCCTCAG-3'	This paper	N/A