Supplemental Materials

Molecular Biology of the Cell

Scavone *et al*.

Supplemental Materials

Trafficking of K63-polyubiquitin modified membrane proteins in a macroautophagy-independent pathway is linked to ATG9A

Francesco Scavone[1,3,4], Sharon Lian[1], Eeva-Liisa Eskelinen[2], Robert E. Cohen[1,4] and Tingting Yao[1,4]

Affiliations:

[1]Department of Biochemistry and Molecular Biology, Colorado State University; Fort Collins, Colorado, USA

[2]Molecular and Integrative Biosciences Research Programme, University of Helsinki; Helsinki, Finland, and Institute of Biomedicine, University of Turku; Turku, Finland

[3]Present address: Department of Biology, Stanford University; Stanford, California, USA

[4]Corresponding authors

This file includes:

Figure EV1. Related to Figure 1: Vx3 is a sensor and an inhibitor of selective autophagy pathways

A: Formation of Vx3-EGFP foci requires binding to K63-polyUb. HeLa-Vx3NB (Vx3 non-binding mutant) and HeLa-Vx3^{SE} cells were treated with Dox for 24 h to induce expression of the EGFP-tagged sensors (*green*). Scale bar: 5 μm.

B: Vx3-EGFP does not colocalize with endogenous ULK1. HeLa-Vx3^{SE} cells were treated with Dox for 24 h to induce expression of Vx3 (*green*); cells were fixed and stained with anti-ULK1 (*magenta*). Magnified images (boxed area) show lack of colocalization between Vx3 (dotted circles) and ULK1. Scale bar: 10 μm.

C: Vx3-EGFP does not colocalize with endogenous FIP200. HeLa-Vx3^{SE} cells were treated with Dox for 24 h to induce expression of Vx3 (*green*). Cells were stained with anti-FIP200. No overlap between Vx3 (*green*) and endogenous FIP200 (*magenta*) is observed, as demonstrated in the zoomed inserts where dotted circles indicate the location of a Vx3 focus. Scale bar: 10 μm.

D: Vx3-EGFP does not colocalize with endogenous ATG16L1. HeLa cells expressing Vx3^{SE} (*green*) for 24 h were stained with anti-ATG16L1 (*magenta*). Individual and merge channels for Vx3 (*green*) and ATG16L1 (*magenta*) are shown. Magnified images (boxed area) show no overlap between Vx3 focus (dotted circles) and ATG16L1 signals. Scale bar: 10 μm.

E: Vx3-EGFP does not colocalize with endogenous WIPI2b. HeLa-Vx3^{SE} cells were treated with Dox for 24 h to induce expression of Vx3 (*green*). After fixation, cells were stained with anti-WIPI2b (*magenta*). Magnified images (boxed area) show no colocalization between Vx3 foci (dotted circles) and WIPi2b foci. Scale bar: 10 μm.

F: Vx3-EGFP partially colocalizes with endogenous p62/SQSTM1. Single z-section image showing colocalization of Vx3 (*green*) and p62/SQSTM1 (*magenta*). HeLa-Vx3^{SE} cells were cultured with Dox for 24 h before staining with anti-p62/SQSTM1 (*magenta*). Magnified images (boxed area) illustrate colocalization of the two proteins indicated by the dotted circles. Scale bar: 10 μm.

G: Addition of the cationic lipid Effectene triggers Vx3-EGFP and LC3 colocalization. HeLa cells were treated with Dox for 24 h to induce expression of Vx3 (*green*) before addition of Effectene reagent for 4 h. Cells were stained with anti-LC3 (*magenta*). Zoomed panels show partial overlap between Vx3 and endogenous LC3. Scale bar: 10 μm.

H: Zoomed panels from HeLa-Vx3^{SE} cells treated as in (**G**) and immunostained with anti-ATG9A and anti-LC3. Vx3 signals (*green*) partially overlap with both ATG9A (red) and LC3 (*blue*) signals.

I: Two zoomed areas (indicated as panels “I” and “II”) from HeLa-Vx3^{SE} cells treated as in Fig 1C and immunostained with anti-Galectin-3. Shown is partial overlap between

Vx3 (*green*) and Galectin-3 (*magenta*) upon Lipofectamine 2000 treatment or Salmonella-RFP (*light blue*) infection.

Figure EV2. Related to Figure 2: Vx3-EGFP foci are clusters of vesicles containing ATG9A and K63-polyUb

A: Vx3-EGFP colocalizes with VMP1-iRFP and NBR1. HeLa-Vx3^{SE}-VMP1 cells stably transduced to constitutively express VMP1-iRFP were treated with Dox for 24 h to induce Vx3 expression and then stained with an antibody against NBR1. Individual and merged channels for Vx3 (*green*), NBR1 (*red*), and VMP1-iRFP (*blue*) are shown. Scale bar: 10 μm.

B: HeLa cells were transiently transfected with VMP1-iRFP and stained with an antibody specific for K63-polyUb. Individual and merged channels for K63-polyUb (*green*) and VMP1-iRFFP (*magenta*) are shown. Magnified images (boxed area) show colocalization between VMP1-iRFP foci and K63-polyUb. Scale bars: 5 μm.

C: Vx3^{SE} and VMP1 exhibit a greater overlap than Vx3^{SE} and ATG9A. Left panel: individual and merged Airyscan superresolution images of four representative planes from a z-stack (depth of 8.5 μm with 180 nm step size) of structures containing Vx3-EGFP (green), VMP1-RFP (magenta), and mCherry-ATG9A (light blue). Right panel: line profile plots indicate the normalized fluorescence intensity distribution of each channel throughout the dashed yellow line. Scale bar: 1 μm.

Figure EV3. Related to Figure 3: Vx3-EGFP expression accumulates K63-polyubiquitylated cargo of plasma membrane origin in close proximity to endolysosomes

A: Vx3-EGFP foci contain endogenous MHC-I. Single z-section image of HeLa-Vx3^{SE} cells (Vx3, *green*) treated with Dox for 24 h and stained with anti-MHC-I (*red*) and anti-ATG9A (*light blue*) antibodies. Vx3 foci (dotted circles) are projected onto the MHC-I and ATG9A channels to visualize colocalization. Scale bar: 10 μm.

B: Vx3-EGFP partially colocalizes with CAV-1. Single z-section image of HeLa-Vx3^{SE} cells (Vx3, *green*) treated with Dox for 24 h, fixed and stained with anti-CAV-1 (*magenta*). Magnified images of the boxed area show partial overlap between Vx3 and CAV-1 signals. Scale bar: 10 μm.

C-D: HeLa-Vx3^{SE}-VMP1 cells were treated with Dox for 24 h to express Vx3 (*green*) and stained with an antibody against TfR (**C**, *red*) or MHC-I (**D**, *red*). VMP1-iRFP foci are shown in *blue*. Magnified images (boxed area) illustrate overlap of the three proteins. Scale bars: 10 μm.

E-F: Vx3-EGFP does not colocalize with early or recycling endosomes. HeLa-Vx3^{SE} cells were treated with Dox for 24 h to induce expression of Vx3 (*green*) and stained

with (**E**) EEA1 (*magenta*) or (**F**) Rab11a (*magenta*) antibodies. Magnified images (right panels; merged channels at bottom) of boxed areas show no overlap between Vx3 foci and early (EEA1) or recycling (Rab11a) endosome markers. Scale bars: 5 μm.
G-H: Vx3-EGFP does not colocalize with Golgi markers. HeLa-Vx3^{SE} cells were treated with Dox for 24 h to induce expression of Vx3 (*green*) and stained with (**G**) anti-GM130 (*magenta*) or (**H**) anti-TGN38 (*magenta*) antibodies. Magnified images (right panels; merged channels at bottom) of boxed areas show no overlap between Vx3 foci and Golgi membranes positive for GM130 (**G**) or TGN38 (**H**). Scale bars: 10 μm.
I-K: Vx3-EGFP foci are in close proximity to ER tubules. HeLa-Vx3^{SE}-VMP1 cells were treated with Dox for 24 h to induce Vx3 localization to VMP1-iRFP (*light blue*) foci and then stained with (**I**) Sec31A or (**K**) SEC61β antibodies (*magenta*). (**J**) HeLa-Vx3^{SE} cells were treated with Dox for 24 h to induce expression of Vx3 (*green*) and stained with anti-CNX antibody (*magenta*). Magnified images (boxed area) show that Vx3 foci (*green*) are in contact with ER tubules (*magenta*).
L-M: Immunoelectron microscopy showing distribution of immunogold-labeled Vx3-EGFP. HeLa-Vx3^{SE} cells transiently transfected with mCherry-Rab5Q79L and treated with Dox for 24 h to express Vx3. (**L**) Three images (*top*, *middle*, *bottom*) showing Vx3-EGFP vesicular clusters in close proximity to enlarged MVB. Magnified images (right panels) show anti-GFP immunogold particles at the cytoplasmic side of the Vx3-containing membranes. Scale bars: 0.1 μm (*top* panel); 0.5 μm (*middle and bottom* panels). (**M**) Image showing localization of immunogold-labeled Vx3 (indicated by the *arrows*) in the lumen of MVB. Scale bar: 0.1 μm.

Figure EV4. Related to Figure 4: Vx3-EGFP expression inhibits delivery of K63-polyubiquitylated transmembrane protein cargo to endolysosomes
A: HeLa FRT cells were co-transfected with DD-Vx3^{TT} (*green*) and TfR-HALO (*red*) and treated with Dox and Shield1 as in Fig 4C-D. After 24 h, cells were rinsed three times in PBS for drug washout and cultured in medium containing 100 nM PA-JF646 Halo ligand. Sites of photoactivation are indicated by the white dots around DD-Vx3^{TT} foci (*green*) and projected into the TfR-HALO (*red*) channel. Representative images of pre-activation (i.e., no visible signal) and post-activation (*red*) TfR-HALO are shown. Scale bar: 5 μm.
B-C: Live-cell imaging of HeLa FRT cells co-transfected with DD-Vx3^{TT} and HALO-ATG9A and treated as described in Figure 4C-D. Representative montages of single z-section time-lapse images show the release of photoactivated HALO-ATG9A (*white)* independently of Lysotracker-positive signals (*red*) during a 20 s interval starting 2 h 54 min 53 s (B) or 6 h 48 min 33 s (C) after drug washout. A post time-lapse frame including the EGFP channel for DD-Vx3^{TT} is shown to indicate the site of

photoactivation (B). *Yellow arrowheads* indicate the appearance of a small ATG9A punctum that moves away from the site of photoactivation. Scale bar: 1 μm.
D: Vx3-EGFP foci colocalize with internalized ligand-bound EGF. HeLa cells expressing Vx3 (*green*) were stimulated with EGF-AlexaFluor647 (*magenta*) conjugate and imaged 1 h after treatment. Scale bar: 5 μm.
E: Knockdown of USP8 increases Vx3-EGFP foci. HeLa-Vx3^{SE} cells were treated with either control siRNA or siRNA against USP8 for 72 h. Vx3 expression was then induced by treatment with Dox for 24 h and cells were stained with anti-ATG9A antibody. Individual and merged channels for Vx3 (*green*) and ATG9A (*magenta*) are shown. Scale bar: 10 μm.

Figure EV5. Related to Figure 5: Among core components of the machinery needed for autophagosome formation, only ATG9A is required for Vx3-EGFP foci formation
A: Knockdown of ATG9A reduces Vx3-EGFP foci. HeLa-Vx3^{SE} cells were treated with either control siRNA or siRNA against ATG9A for 72 h. Post transfection 24 h, Vx3 expression was then induced by treatment with Dox for 48 h and cells were stained with anti-ATG9A antibody and DAPI. *Left* panel: individual and merged channels for tile scan images (5 x 5) showing ~ 250 cells for siRNA control and ~ 150 cells for siRNA ATG9A. *Right* panel: histogram showing quantification of cells with ≥2 Vx3 foci from tile images for siRNA control vs siRNA ATG9A. Scale bar: 50 μm.
B: Knockdown of ATG9A does not affect Vx3 levels. *Left* panel: HeLa-Vx3NB or HeLa-Vx3^{SE} cells were treated with either control siRNA or siRNA against ATG9A for 72 h. Post transfection 24 h, Vx3-EGFP or Vx3NB-EGFP expression was then induced by treatment with Dox for 48 h; cells were also subjected to treatment with 200 nM bafilomycin A1 (BafA1) for 5 h. Whole-cell lysates were analyzed by immunoblotting with anti-ATG9A and anti-Flag antibodies to monitor efficiency of ATG9A knockdown and Vx3 expression levels, respectively. Bands corresponding to ATG9A (*green*), Vx3NB-EGFP (*red*), Vx3-EGFP (*red*) and multi-ubiquitylated Vx3-EGFP (red) are indicated. Immunoblotting with anti-Rab11 was used as loading control. *Right* panel: histogram showing quantification of Vx3NB and Vx3 levels (including multi-ubiquitylated species) normalized against Rab 11 levels from the immunoblot shown in the left panel.
C: ATG9A but not VMP1 depletion reduces Vx3-EGFP foci. *Left* panel: HeLa-Vx3^{SE}-VMP1 cells were treated with either control siRNA or siRNA against ATG9A for 72 h. Vx3 expression was induced by treatment with Dox in the last 24 h. Scale bar: 10 μm. Right panel: HeLa-Vx3^{SE}-VMP1 cells were treated with either control siRNA or siRNA against ATG9A or VMP1 for 72 h. Quantification of Vx3 foci mean intensity in HeLa-Vx3^{SE}-VMP1 cells was performed using VMP1 foci or Vx3-EGFP foci as masks for the siRNA ATG9A or siRNA VMP1 conditions, respectively. Analysis of 20 cells per knockdown condition shows that Vx3-EGFP foci average intensity in HeLa-Vx3^{SE}-VMP1

is significantly reduced by knockdown of ATG9 when compared to control cells, whereas VMP1 knockdown does not reduce the Vx3 foci. Unpaired two-tailed *t*-test: non-significant, $p > 0.05$ ($p= 0.1585$ for siRNA Control vs siRNA VMP1, in *black*); significant, $p < 0.05$ ($p= 0.0001$ for siRNA Control vs siRNA ATG9A, or siRNA ATG9A vs siRNA VMP1, in *red*).

D: Vx3-EGFP foci formation does not require early autophagic machinery or LC3. Maximum projection images of HeLa ATG5-KO, HeLa FIP200-KO, or HeLa hATG8s-KO cells transiently transfected with DD-Vx3-EGFP and treated with 1 μM Shield1 for 4 h. The absence of ATG5, FIP200, or the six human ATG8 proteins (LC3A, LC3B, LC3C, GABARAP, GABARAPL1, GABARAPL2) did not affect formation of Vx3 foci. Scale bar: 10 μm.

E: Knockdown of early autophagy genes does not reduce Vx3 foci formation. HeLa-Vx3^{SE} cells expressing Vx3 were treated with either control siRNA or siRNA against ULK1, ATG14, Beclin1, VMP1, or ATG16L1 for 48 h and then with Dox for an additional 24 h to induce expression of Vx3 (72 h total). Scale bar: 10 μm.

F: Depletion of ATG9A does not generally perturb intracellular K63-polyUb signals. HeLa cells were treated with either control siRNA or siRNA against ATG9A for 72 h. After fixation, cells were stained with an anti-Ub K63-specific monoclonal antibody. Intracellular K63-polyUb signal is shown. Scale bar: 20 μm.

G: Loss of the retromer–WASH supercomplex perturbs ATG9A localization. HeLa cells expressing Vx3NB were treated with control siRNA or siRNA against VPS35 or WASH1 for 72 h and then stained with anti-ATG9 antibody as in Fig 5C. Magnified images (boxed area) show that, unlike in the siRNA control cells, in both the siRNA VPS35 and siRNA WASH1 cells, ATG9A vesicles form a tight cluster in the perinuclear region of the cells. Scale bar: 10 μm.

H: Exocyst is required for Vx3 foci formation. HeLa-Vx3^{SE}-VMP1 cells were treated with either control siRNA or siRNA against EXOC8 for 72 h. Vx3 expression was induced with Dox for 24 h and cells were stained with anti-ATG9A antibody. Individual and merged channels for Vx3 (*green*), ATG9A (*red*), and VMP1-iRFP (*blue*) are shown. Scale bar: 10 μm.

I: Loss of TRAPPIII perturbs ATG9A trafficking. HeLa cells expressing Vx3NB were treated with control siRNA or siRNA against TRAPPC8 for 72 h and stained with anti-ATG9 antibody. Representative images show dispersal of ATG9A signal throughout the cytoplasm. Scale bar: 10 μm.

J: Loss of TRAPPIII promotes formation of larger Vx3 and VMP1 foci. HeLa-Vx3^{SE}-VMP1 cells were treated with either control siRNA or siRNA against TRAPPC8 for 72 h. Vx3 expression was induced with Dox for 24 h and cells were stained with anti-ATG9A antibody. Individual and merged channels for Vx3 (*green*), ATG9A (*red*), and VMP1-iRFP (*blue*) are shown. Scale bar: 10 μm.

K: Accumulation of ATG9A into Vx3 foci does not depend on autophagy cargo receptors. PENTA-KO and WT HeLa cells were transiently transfected with $Vx3^{TT}$ (*green*) for 24 h and stained with anti-ATG9A antibody (*magenta*). The absence of five autophagy cargo receptors (i.e., p62, NBR1, OPTN, TAX1BP1, NDP52) does not affect Vx3 (*green*) and ATG9A (*magenta*) colocalization (magnified boxes). Dashed lines mark the cell contours. Scale bar: 20 μm.
L: Loss of NEDD4L but not of NEDD4 reduces Vx3 foci formation. HeLa-$Vx3^{SE}$ cells were treated with either control siRNA or siRNA against NEDD4 or NEDD4L for 48 h and treated with Dox for additional 24 h to induce expression of Vx3. Cells were stained with antibodies against TfR. Representative maximum projection images show that loss of NEDD4L results in the decrease of Vx3 (*green*) but not TfR signals.

Supplemental Dataset 1: Quantification of colocalization between Vx3-EGFP and autophagic or endocytic markers. Time for Dox treatment to induce expression of Vx3 is indicated (24 or 48 h). Quantifications were performed using Fiji (Image J) and Mander's coefficients (M2) were calculated using the JaCoP colocalization plug-in. Total cell numbers analyzed for each immunostaining experiment are reported.

Supplemental Dataset 2: Mass spectrometry analysis of Vx3-associated proteins. Lysates from HeLa-Vx3NB (non-binding control), HeLa-$Vx3^{SE}$ (stably expressed), or HeLa-$Vx3^{SE}$ (stably expressed)-VMP1 cells were immunoprecipated with GFP-Trap and captured proteins were identified after trypsin digestion and LC-MS/MS. Shown are proteins identified by at least 2 unique peptides and their corresponding peptide counts from the analysis of two sequential elutions (Elution 1 and Elution 2). Proteins captured by Vx3 are potentially modified by K63-polyUb. Proteins highlighted in *red* have membrane localization identified by UniProt (https://www.uniprot.org).

Supplemental Dataset 3: RNAi screen for cellular components that affect Vx3 foci formation. List of siRNA targets used to examine effects of their knockdown on Vx3 foci formation. The knockdown experiments were performed at least twice and the effects reported here are based on qualitative assessment of images containing >50 cells. Autophagy factors, SNAREs, and trafficking machinery were chosen as targets because of their roles during autophagosome formation/maturation and general membrane fusion or transport, respectively. E2 Ub conjugating enzymes (E2s), E3 Ub ligases (E3s), and deubiquitinating enzymes (DUBs) chosen were previously reported to regulate K63-polyUb conjugation/deconjugation. General protein descriptions are based on information available in UniProt (https://www.uniprot.org).

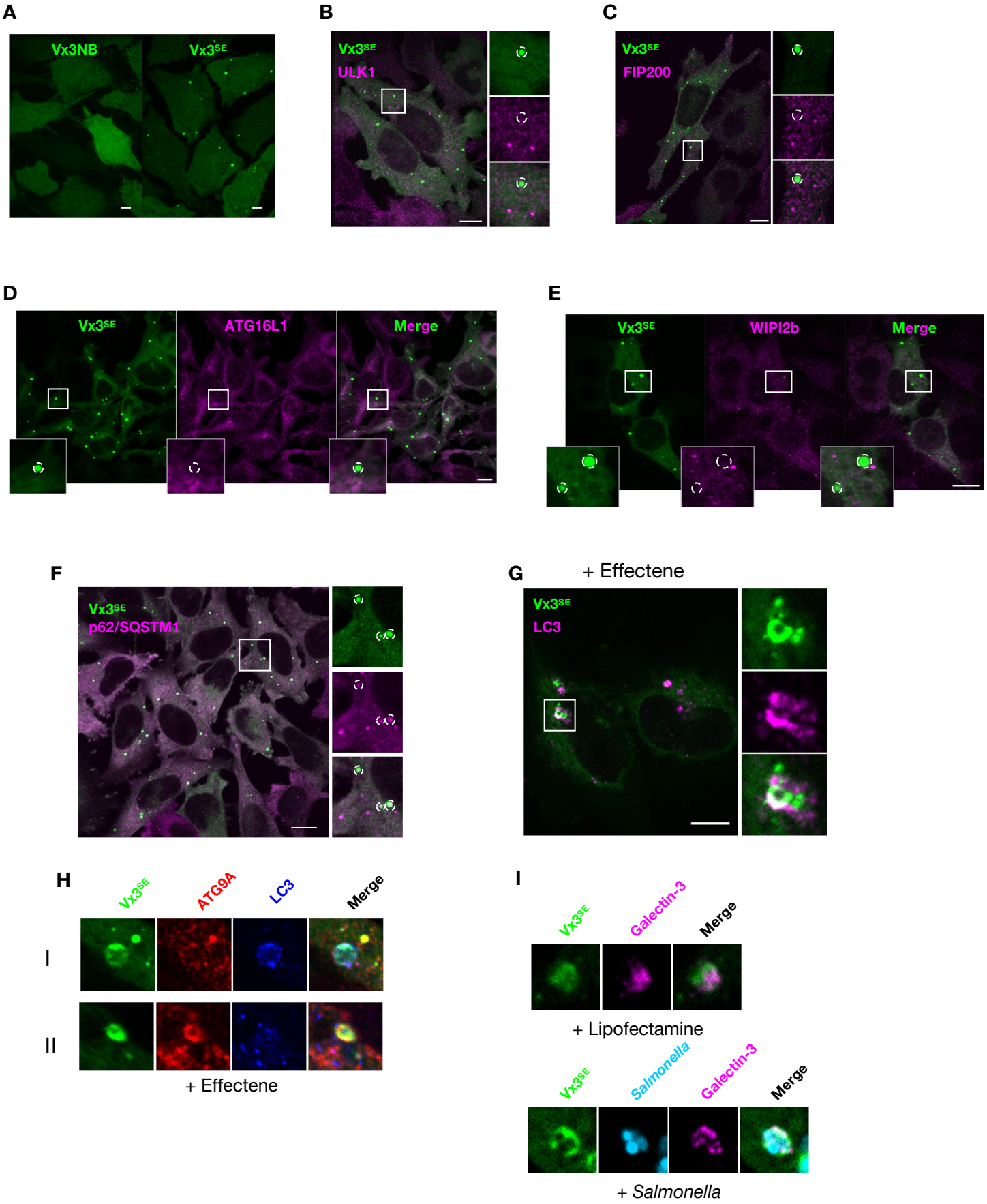
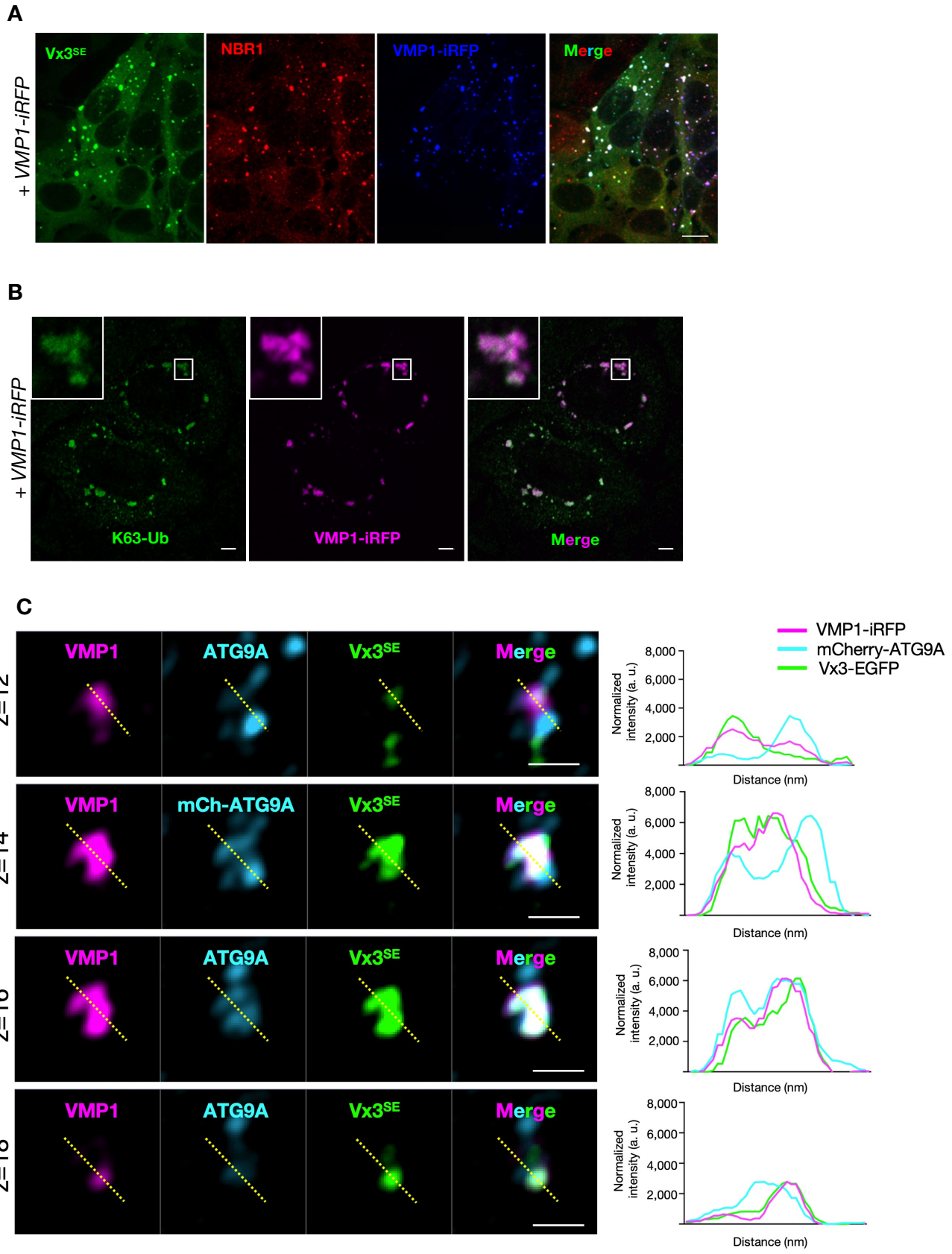


Figure EV1.

Related to Figure 1: Vx3-EGFP is a sensor and inhibitor of selective autophagy pathways



Related to Figure 2: Vx3-EGFP foci are clusters of vesicles containing ATG9A and K63-polyubiquitin

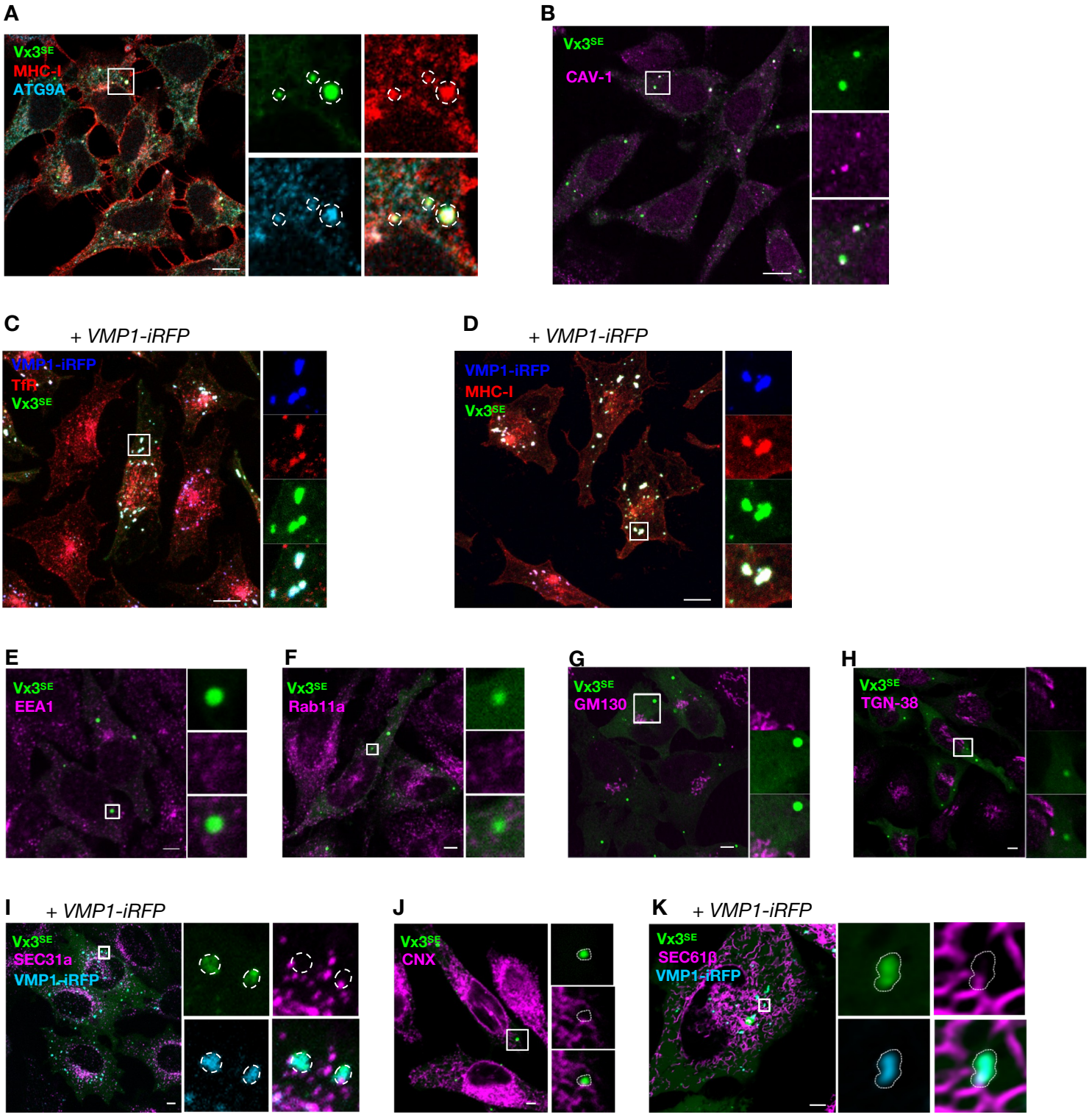


Figure EV3.

Related to Figure 3: Vx3-EGFP expression accumulates K63-polyubiquitylated cargo of plasma membrane origin in close proximity to endolysosomes

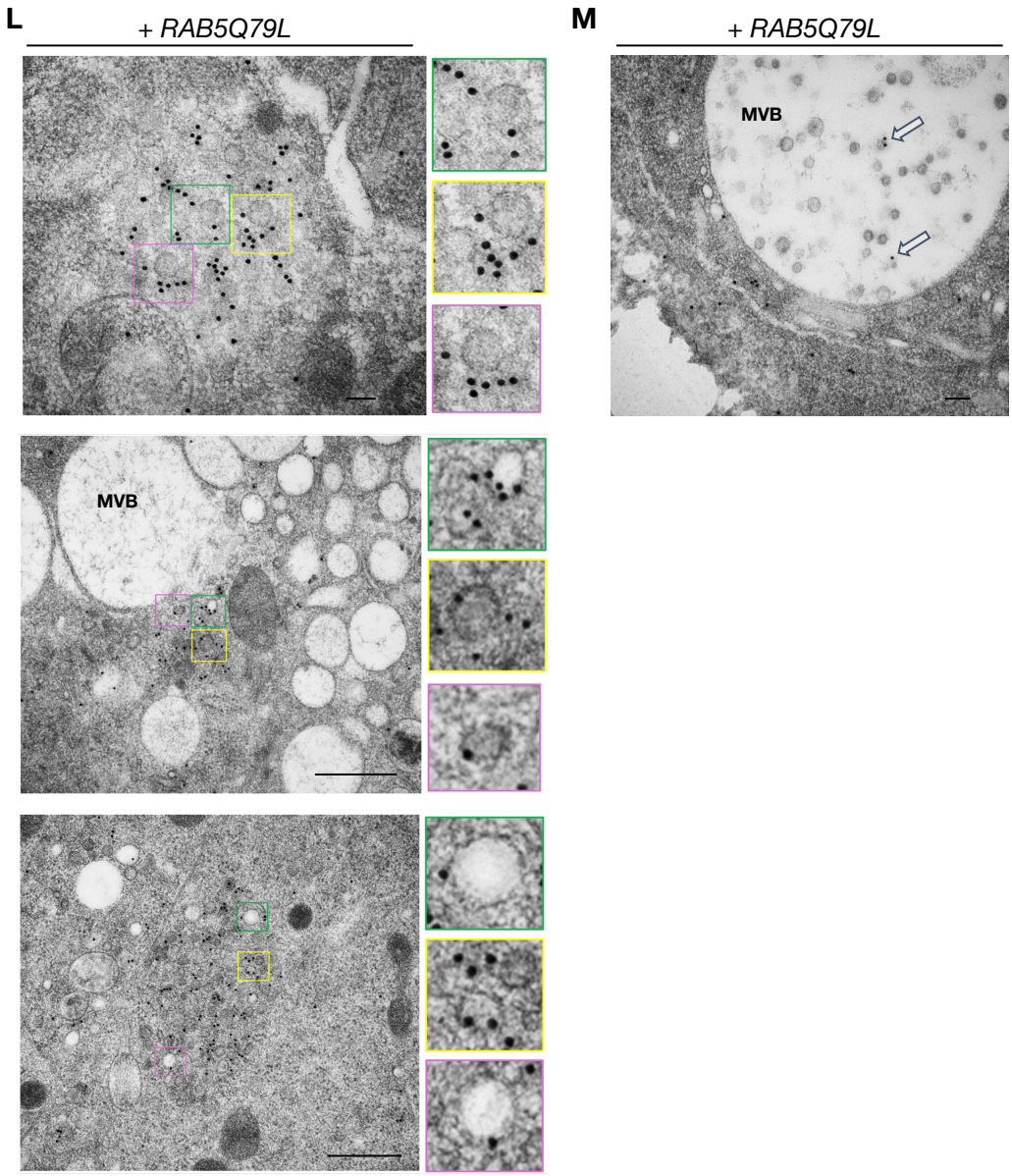


Figure EV3 (continued).

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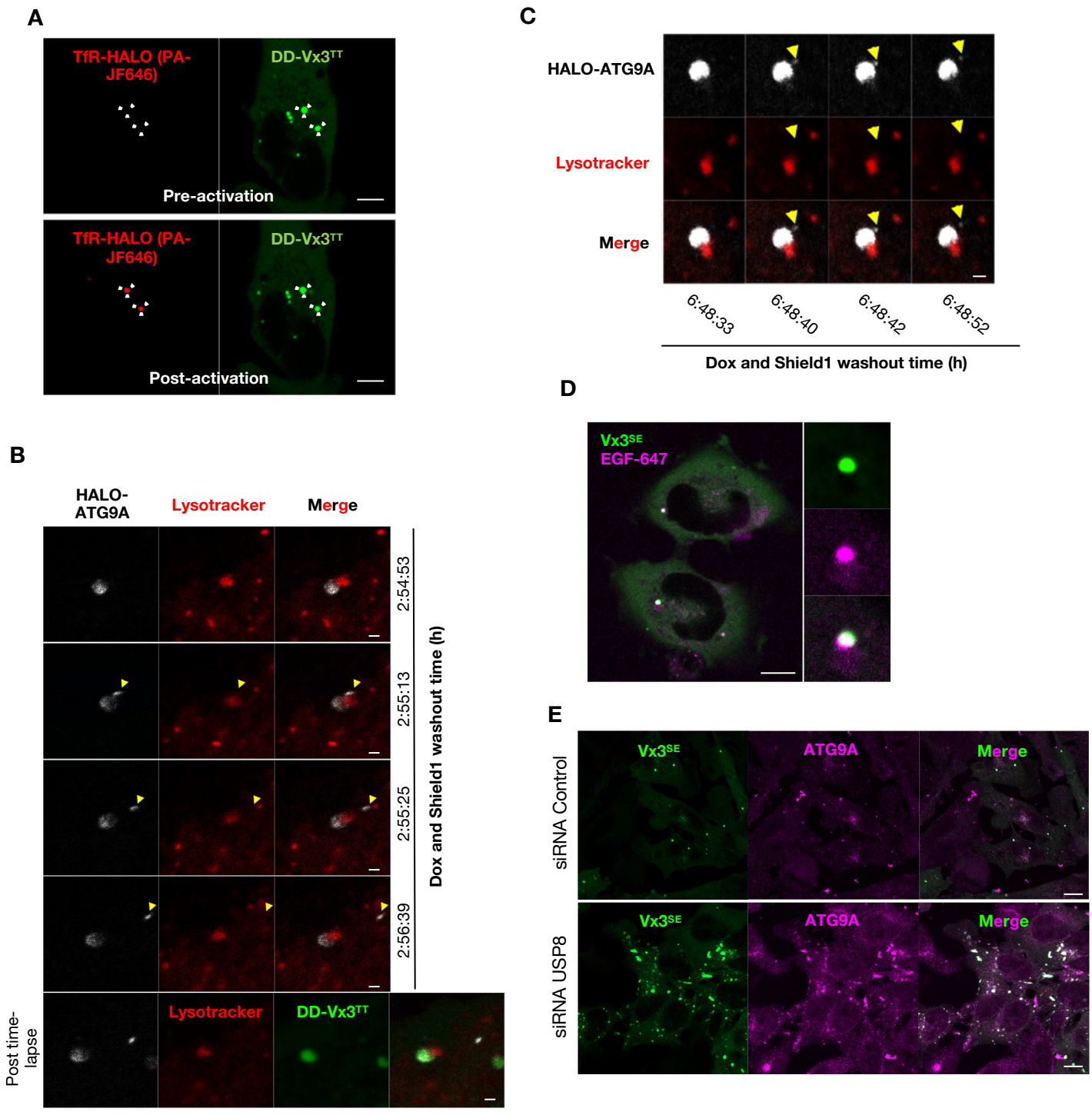

Figure EV4.

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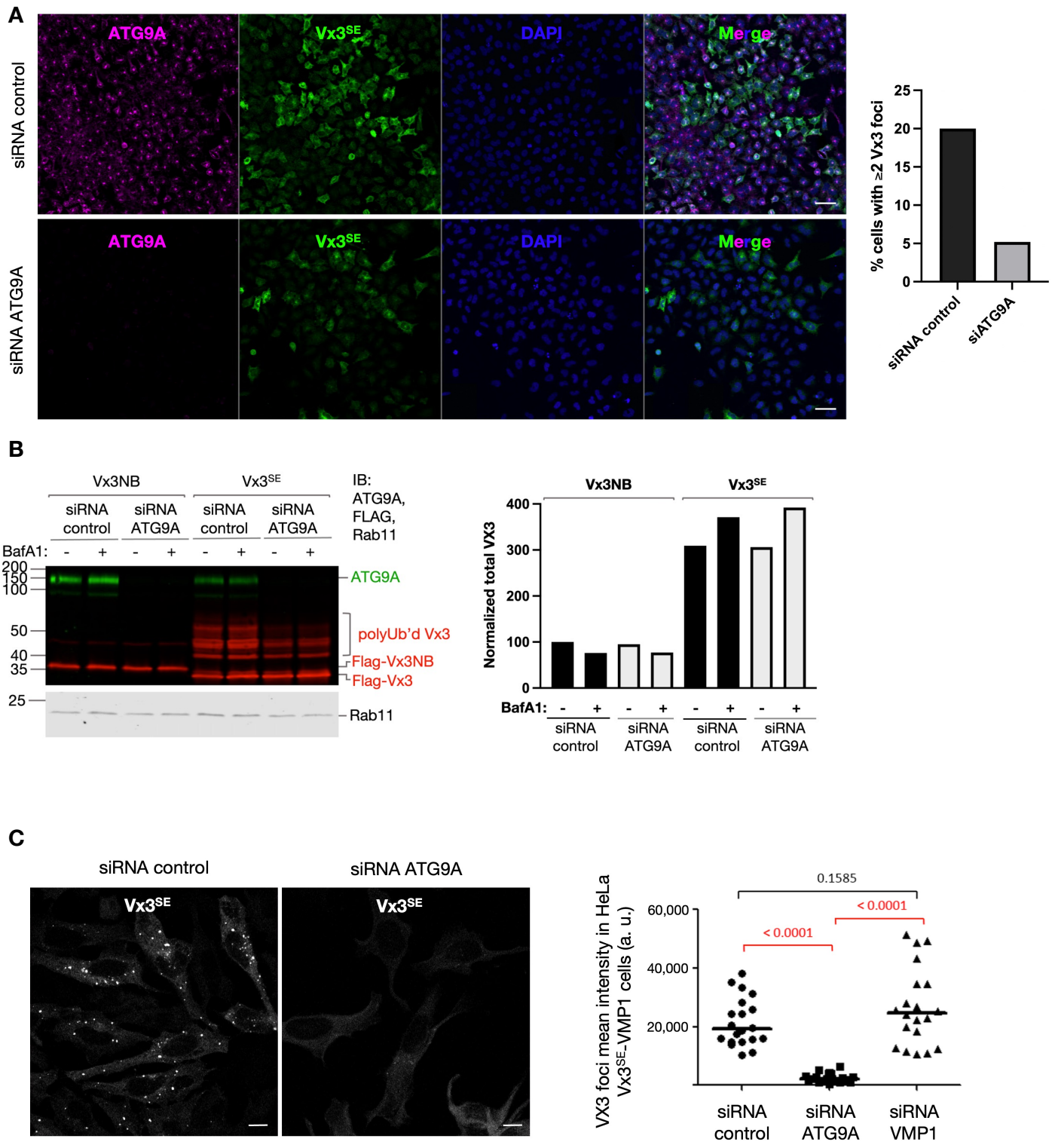

Figure EV5.

Related to Figure 5: Among core components of the machinery needed for macroautophagy, only ATG9A is required for Vx3-EGFP foci formation

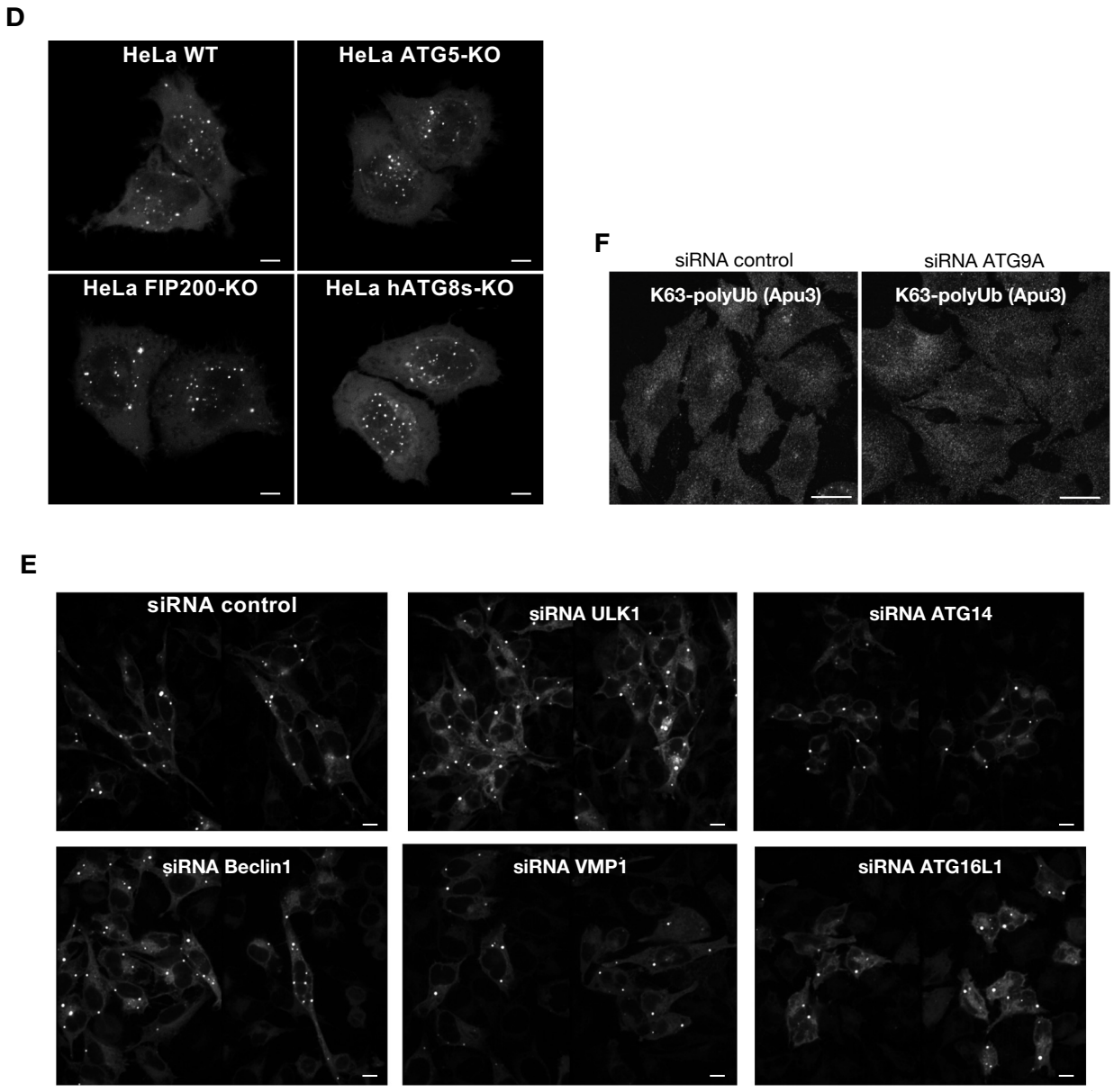

Figure EV5 (continued).

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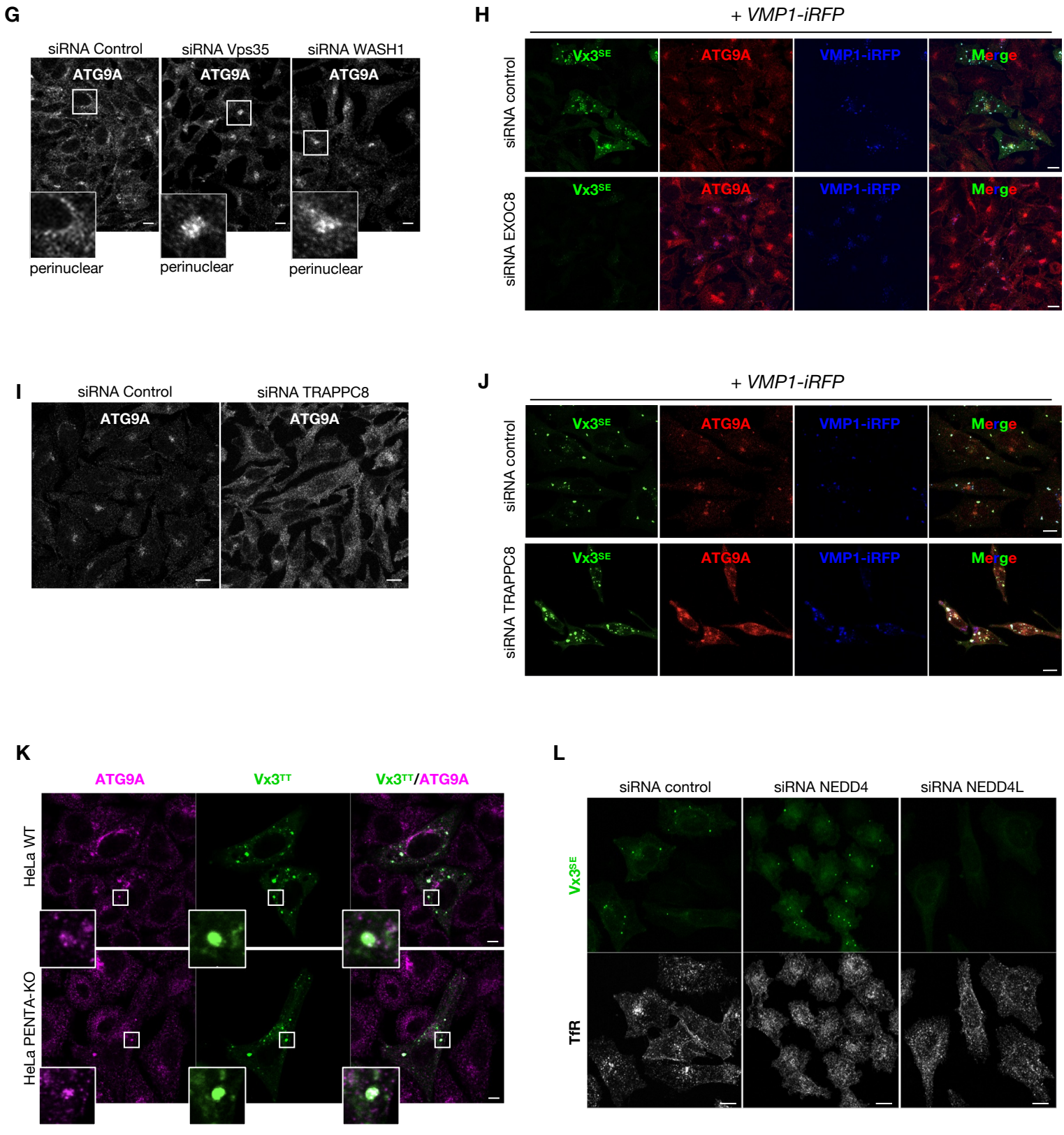

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