

## **SUPPLEMENTARY INFORMATION**

for

### **Endocytic tubules regulated by Rab GTPases 5 and 11 are used for envelopment of herpes simplex virus**

**Hollinshead *et al.***

#### **SUPPLEMENTARY MATERIALS AND METHODS**

##### ***Antibodies and reagents***

Monoclonal antibodies to gD and gH were kindly provided by Helena Browne. Monoclonal antibodies to gE and ICP27 were kindly provided by David Johnson and Steve Rice respectively. Anti-ICP0 (11060) and LAMP2 antibodies were obtained from Santa Cruz. Giantin, EEA1, CD63, Rab1, Rab7 and Rab9 antibodies were obtained from Abcam. Antibody to transferrin receptor was from Invitrogen Zymed. Sheep anti-TGN46 was from Serotec. Antibodies to Rab5, Rab11 and Rab24 were from BD Biosciences. Alpha-tubulin antibody was obtained from Sigma. All Alexa fluor conjugated secondary antibodies, and the F(ab')<sub>2</sub> fragment of goat anti-mouse IgG HRP conjugate were obtained from Invitrogen. Cy3 conjugated transferrin (Jackson Immunoresearch) was used at a concentration of 200 ng/ml to label early and recycling endosomes. To disrupt cellular microtubules, nocodazole (Sigma) was added to media at a concentration of 2 µg/ml for 30 minutes. The inhibitor of dynamin Dynasore (Sigma) was added to cells at a concentration of 25 µg/ml.

##### ***Cells and viruses***

Vero cells were grown in DMEM supplemented with 10% NCS and antibiotics. HFFF-2 and HeLa cells were grown in DMEM supplemented with 10% FBS and antibiotics. HSV1 strains s17, sc16 and HFEM were routinely propagated and titrated in Vero cells in DMEM supplemented with 2% NCS and antibiotics. All plaque assays contained 1% human serum (Harlan Sera-Lab). HSV1 (s17) expressing the capsid protein VP26 or the glycoprotein gD as GFP fusion proteins were constructed by cotransfecting infectious s17 genomic DNA with plasmids for GFP-VP26 (kindly provided by Prashant Desai) or gD-GFP (kindly provided by Helena Browne). Recombinant virus was plaque purified three times and characterized by Southern and Western blotting before use.

### ***SDS-PAGE and western blots***

Protein samples were analysed by electrophoresis on polyacrylamide gels in Tris- glycine buffer followed by transfer to nitrocellulose membrane. Western blots were developed using an enhanced chemiluminescence kit (Pierce).

### ***Immunofluorescence***

Cells were grown on coverslips and fixed with 4% paraformaldehyde in PBS for 20 minutes followed by permeabilisation with 0.5% triton-X100 for 10 minutes. The fixed cells were blocked by incubation for 20 mins in PBS containing 10% newborn calf serum, and primary antibody added for 30 mins in the same solution. Following extensive washing in PBS, the appropriate Alexa fluor conjugated secondary antibody (Invitrogen) was added in block solution and incubated for a further 20 mins. The coverslips were then washed extensively in PBS and mounted in Vectashield containing DAPI (Vector Labs). Images were acquired using a Zeiss LSM510 Meta confocal microscope, and processed using Adobe Photoshop software. Transferrin uptake was measured by incubating cells in serum free medium containing 200 ng/ml Cy3-transferrin for times up to 60 min. Relative amounts of TGN46 and transferrin positive membranes in individual cells were compared by first measuring the area of the cell in question, then counting positive pixels for TGN46 and transferrin fluorescence respectively. Suitable thresholds were set as background for each channel.

**siRNA duplexes**

	<b>Duplex 1</b>	<b>Duplex 2</b>
Rab1A	gggaacaaaugugaucuga	caaagaaaguaguagacua
Rab1B	gcaccagccuuaaccuca	gcugaaaucaaaaagcgga
Rab5A	ggaagaggaguagaccuua	caagccuagugcuucguuu
Rab5B	ggagcgauaucacagcuua	cgacauuacuaaucaggaa
Rab5C	ggacaggagcgguaucaca	gcaaugaacgugaacgaaa
Rab7A	gcuagucacaaugcagaua	gcugcguucugguauuuga
Rab7B	gcucugucgagguaccaga	agauuaucauauuggguga
Rab9A	ccagcucuuccauacaaua	ggucagaucauuugauuca
Rab9B	gcuuaugaaccguuacgua	ggguaacaagguagacaaa
Rab11A	caacaaugugguuccuauu	gagauuuaccgcauuguuu
Rab11B	gcuucaagaacauccuca	cuaacguagaggaagcauu
Rab24	gcuuugagcgagcaaaguu	cgagcaaaguucuggguga

Sequence of siRNA duplexes used for Rab depletion.

## SUPPLEMENTARY FIGURE LEGENDS

**Figure S1.** Virus replication in Vero, HeLa and HFFF-2 cells. (A) One-step growth curves of HSV1 (s17) were carried out by infecting HFFF-2, Vero or HeLa cells at a multiplicity of 2, harvesting at a range of times after infection and titrating progeny virus on Vero cells. (B) One-step growth curves of 3 strains of HSV1 – s17, sc16 or HFEM – on HFFF-2 cells were carried out as described for (A).

**Figure S2.** HSV1 wrapping membranes derive from the plasma membrane in Vero and HeLa cells. Vero or HeLa cells were infected at a multiplicity of 2 with HSV1 and labeled 11 h after infection with 10mg/ml HRP for 30 min. After fixation cells were stained with DAB and processed for imaging by EM. PM, plasma membrane. tu, HRP positive tubule. Ca, capsid. Cc, clathrin coat.

**Figure S3.** HSV1 wrapping membranes do not contain markers for membranes of the late endosomal pathway. Ultrathin cryosections of HFFF-2 cells infected with HSV1 at a multiplicity of 2 and fixed at 14 h were labeled with anti-LAMP2 monoclonal antibody followed by Alexa 546 anti-mouse (red) and DAPI (blue) for immunofluorescence (A) or rabbit anti-mouse and protein A conjugated to 9 nm gold (B to G). Labelling was detected in late endosomal structures (B, C & D), but not in the Golgi, wrapping virions (D & E), or extracellular virions (F). GA – Golgi apparatus. LE – late endosomal structures. PM – plasma membrane. Ca – capsid. Scale bar = 200 nm.

**Figure S4.** HSV1 wrapping membranes do not contain TGN46. Ultrathin cryosections of HFFF-2 cells infected with HSV1 at a multiplicity of 2 and fixed at 14 h were labeled with anti-TGN46 polyclonal antibody followed by donkey anti-sheep conjugated to 6 nm gold. Labelling was detected on the trans side of Golgi stacks in uninfected cells (A), and less frequently on the trans side of Golgi stacks in HSV infected cells (B). TGN46 was not detected at the plasma membrane of infected cells (B), on capsids in the process of wrapping (C) or extracellular virions (D). GA – Golgi apparatus. PM – plasma membrane. Ca – capsid. For (A) scale bar = 10  $\mu$ m. For (B to F), scale bar = 200 nm.

**Figure S5.** Glycoprotein D localises to multiple membranes in HSV1 infected cells. Ultrathin cryosections of HFFF-2 cells infected with HSV1 at a multiplicity of 2 and fixed at 14 h were labeled with anti-gD monoclonal antibody followed by rabbit anti-mouse and protein A conjugated to 6 nm gold. Labelling was detected in the

Golgi (A), plasma membrane (A, B & C), extracellular virions (C) and intracellular wrapping particles (D). GA – Golgi apparatus. PM – plasma membrane. Ca – capsid. Scale bar = 200 nm.

**Figure S6.** Relative localisation of virus capsids and glycoprotein E in HSV1 infected Vero, HeLa and HFFF-2 cells. Cells grown on coverslips were infected with HSV1 expressing the capsid protein VP26 as a GFP fusion protein (green) at a multiplicity of 2, and fixed at 5, 7, 9 or 11 hours after infection. Cells were processed for IF with antibody against gE (red) and nuclei stained with DAPI (blue) before imaging with a Zeiss LSM510 Meta confocal microscope. Scale bar = 10  $\mu$ m.

**Figure S7.** HSV1 does not wrap at EEA1 positive early endosomes. Ultrathin cryosections of HFFF-2 cells infected with HSV1 at a multiplicity of 2 and fixed at 14 h were labeled with anti-EEA1 monoclonal antibody followed by Alexa 546 anti-mouse (red) and DAPI (blue) for immunofluorescence (A) or rabbit anti-mouse and protein A conjugated to 6 nm gold (B to F). Immunofluorescence showed that very few EEA1 positive structures were present in these thin slices (A), a result backed up by immunogold labelling (B) where structures positive for EEA1 were detected rarely at the ultrastructural level. EEA1 labelling was also absent from Golgi (C), wrapping capsids (D & E) and extracellular virions (F). GA – Golgi apparatus. Ca – capsid. EEA1 – EEA1 positive structure. For (A) scale bar = 10  $\mu$ m. For (B to F), scale bar = 200 nm.

**Figure S8.** The effect of Rab depletion on the localization of CD63 and EEA1. HeLa cells transfected with siRNAs for Rabs 5, 7 or 11 were fixed 48 h later and immunofluorescence carried out for the early and late endosome markers EEA1 and CD63 (red). Cells were stained with DAPI to detect nuclei (blue). Scale bar = 10  $\mu$ m.

Figure S1

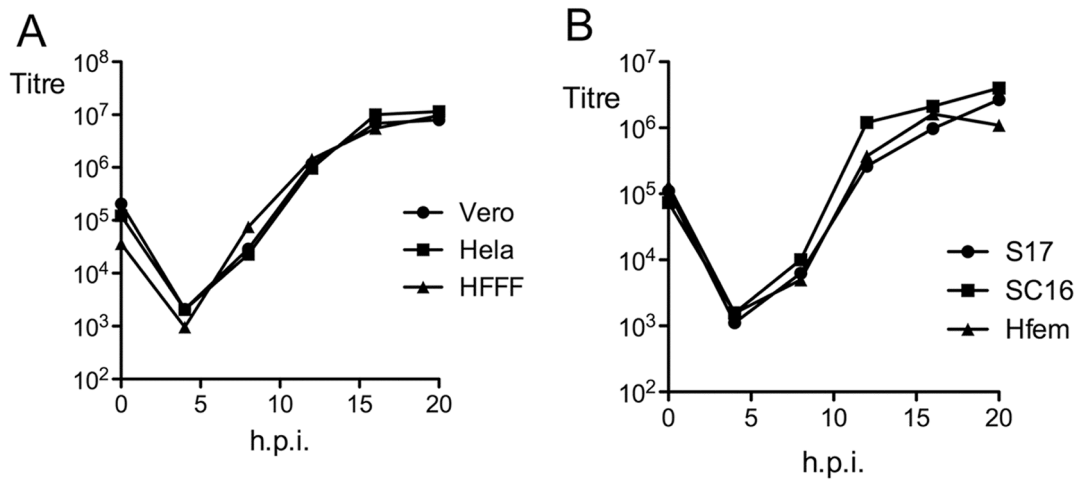


Figure S2

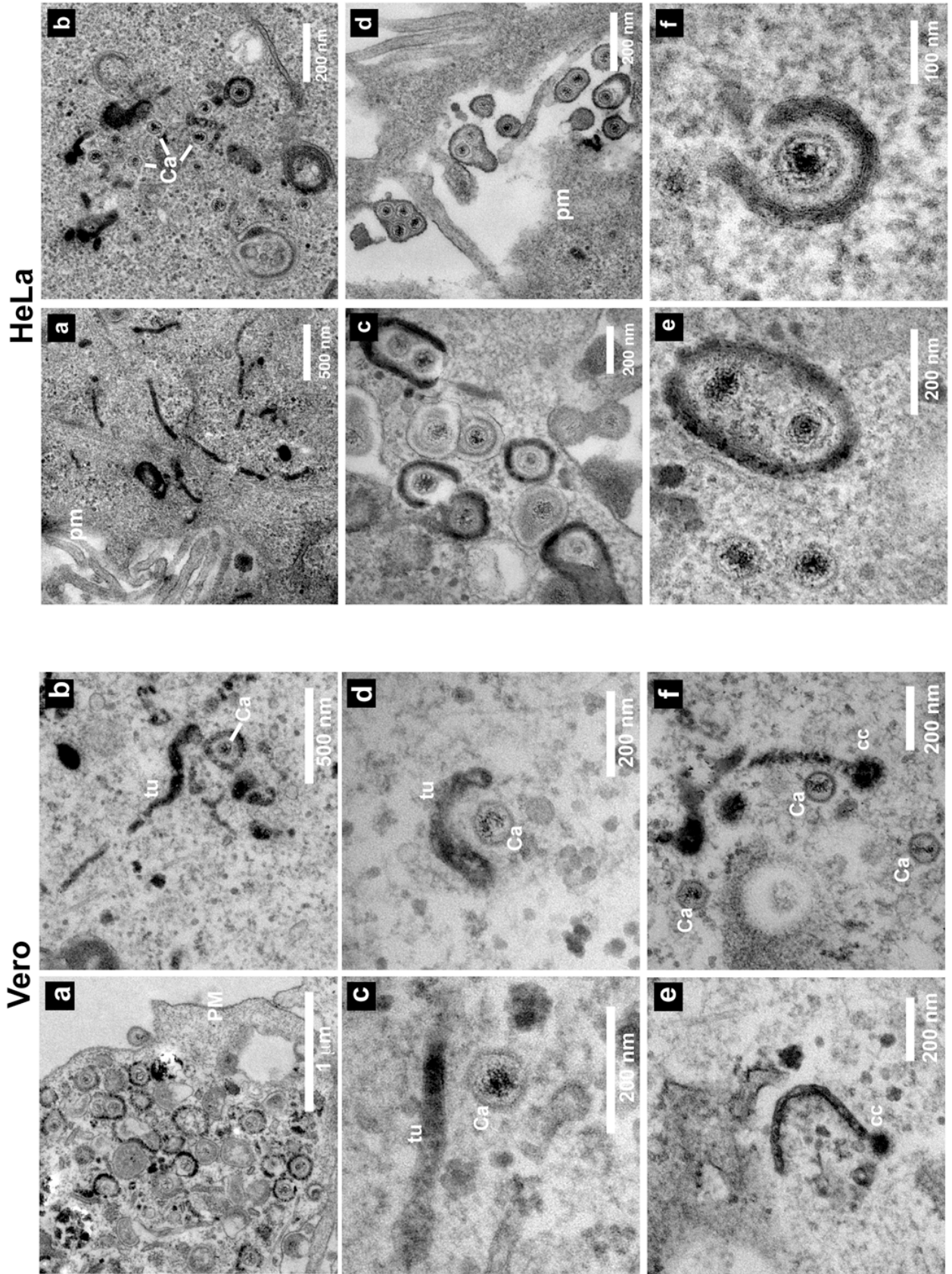


Figure S3

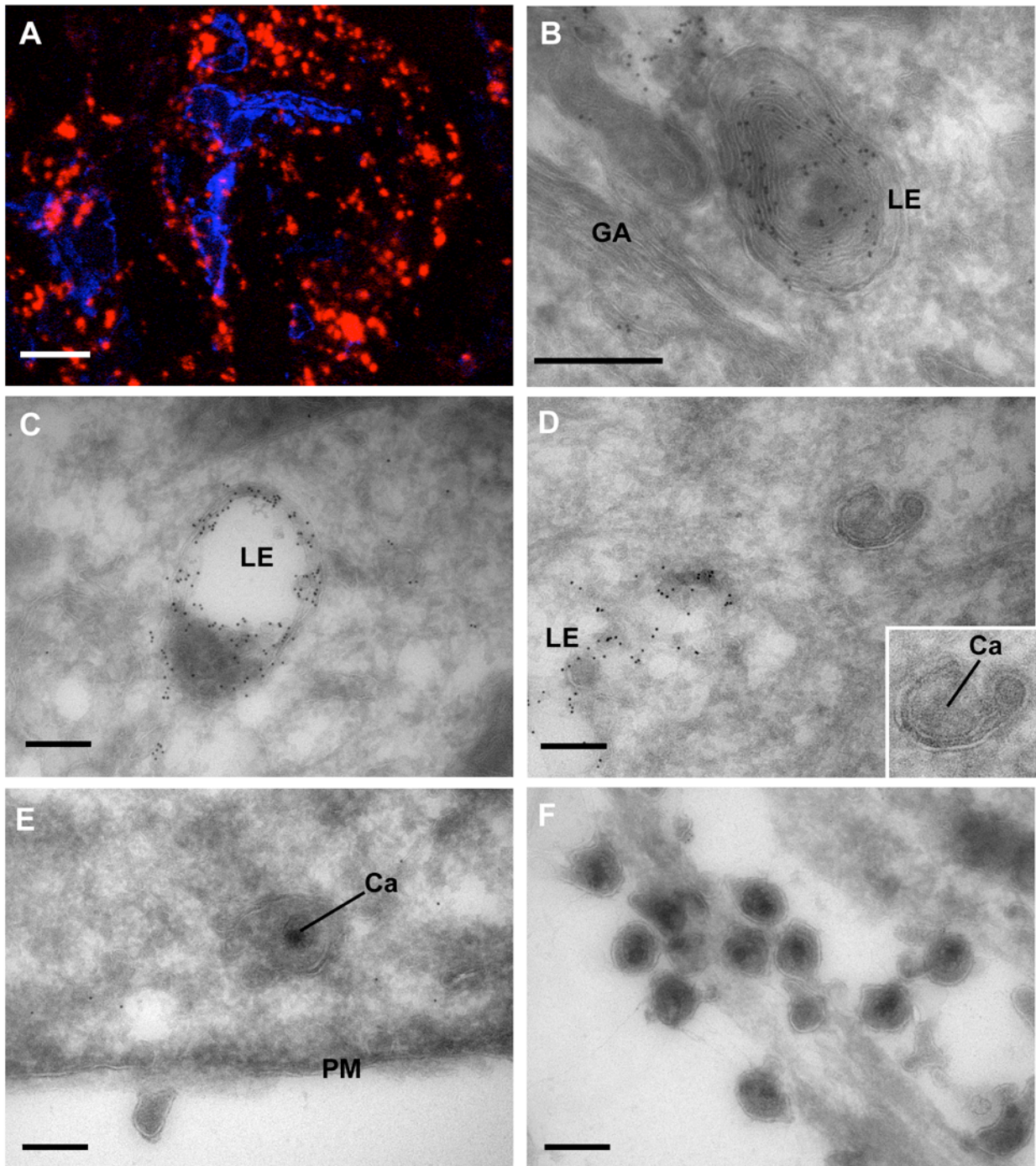




Figure S4

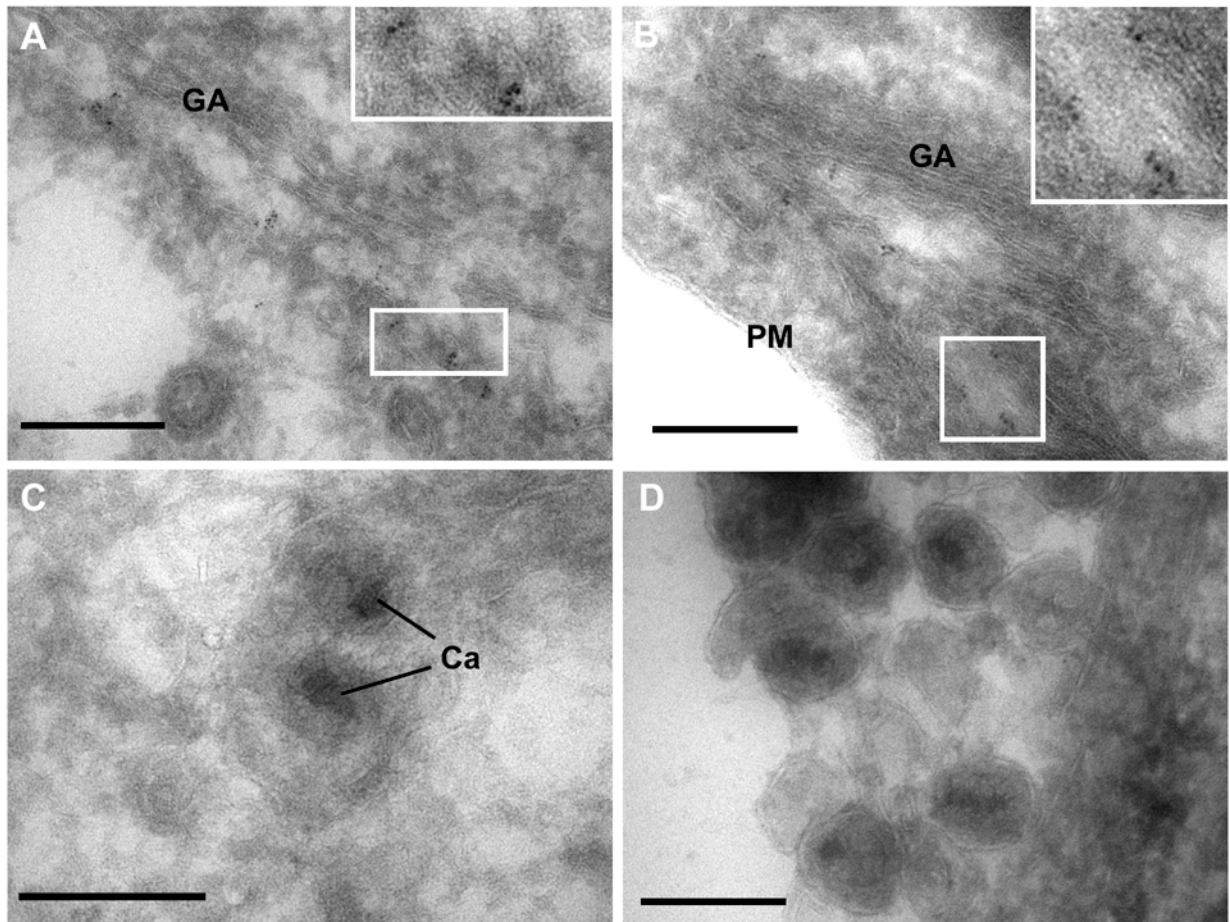


Figure S5

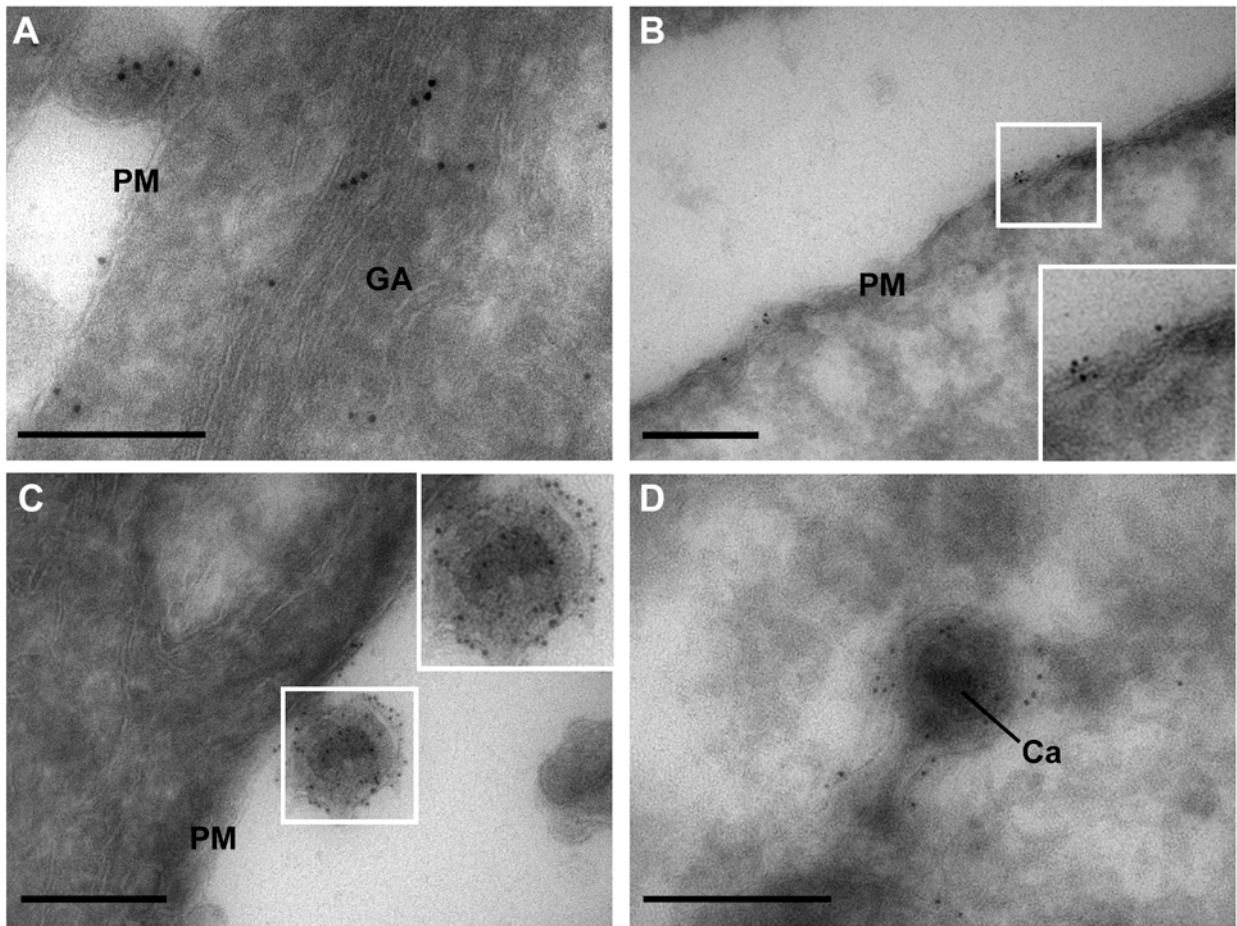


Figure S6

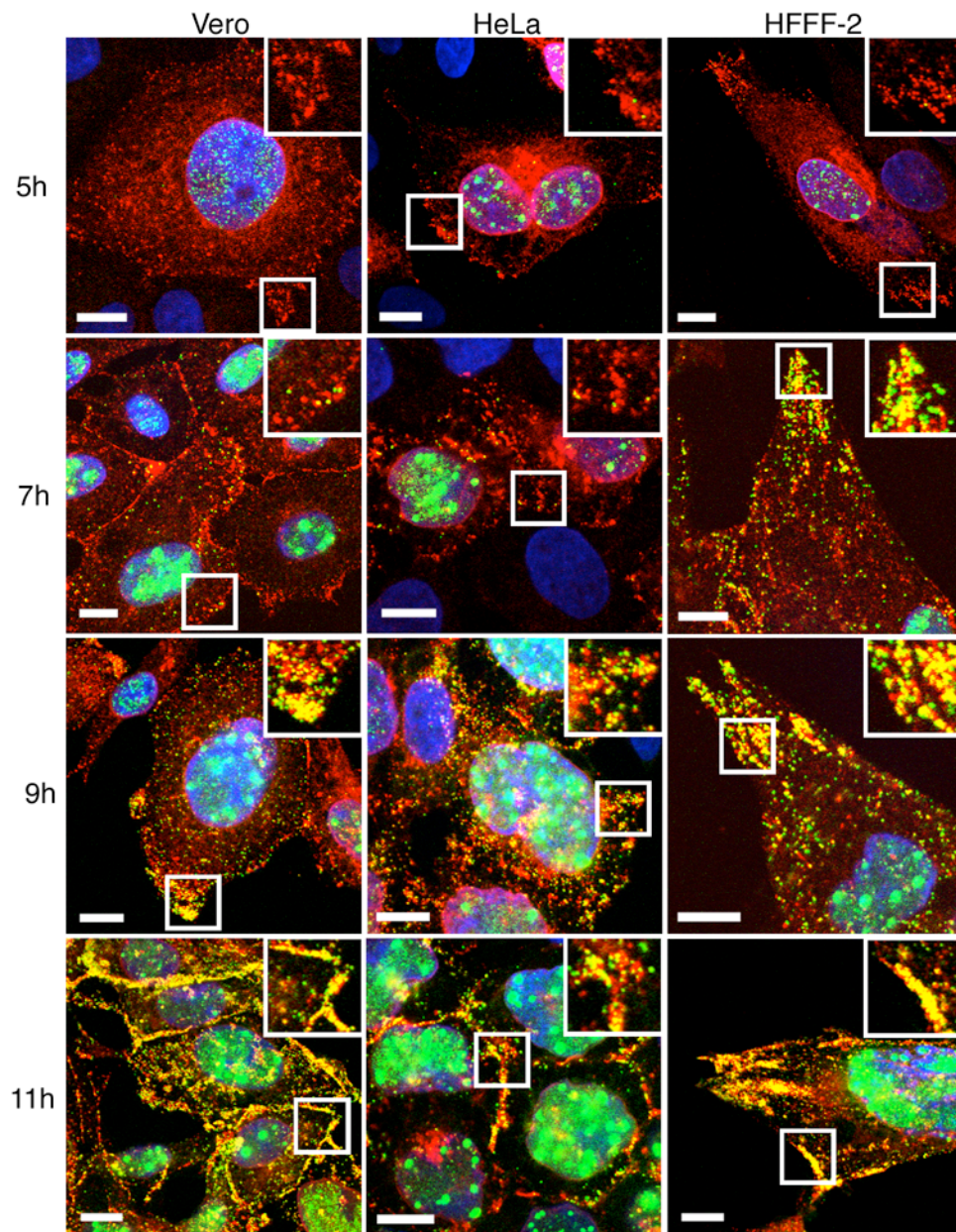


Figure S7

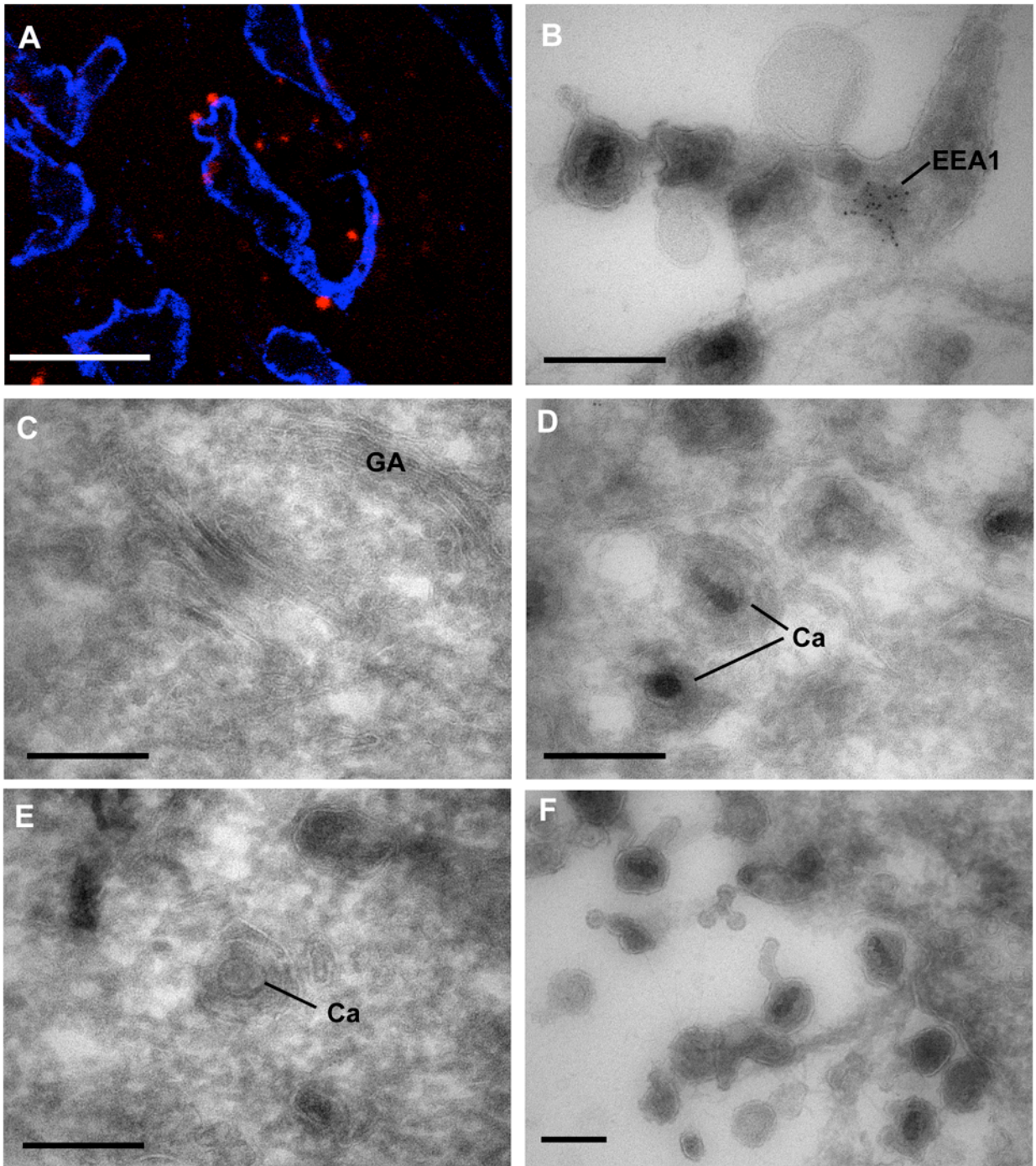


Figure S8

