Supplementary Figure 1. EV internalization by electron microscopy.

Internalization of IHD-J F13-GFP EVs by HeLa cells was analyzed by transmission electron microscopy and immunogold labeling of GFP as described in figure 2E and F. Panels B, D, and F represent magnified views of virus particles in panels A, C, and E, respectively. Arrows in D highlight the two viral membranes of internalized EVs.

Supplementary Figure 2. MV internalization by flow cytometry.

IHD-J EGFP-A5 MVs (MOI 75) were bound to HeLa cells on ice for 1 h and cells subsequently incubated at 0° or 37°C for 30 min. To detect internalized MVs, bound virions were removed and cells detached with trypsin (int.); to quantify total cell-associated virions, cells were detached with EDTA (total). Cells were fixed, and green fluorescence quantified by flow cytometry. Representative histograms of untreated samples are shown in (A); green fluorescence intensity from three independent experiments was quantified and the average of measured geometric means of internalized (B) and total (C) MVs is displayed ±SEM.

Supplementary Figure 3. EV infection in presence of neutralized MVs.

WRΔA34R GFP EVs were incubated with 7D11 for 1 h at 37°C (MOI 2). EVs and increasing amounts of neutralized MVs of the same strain were added to HeLa cells and incubated at 37°C for 30 min. Cells were washed, incubated in full medium, and harvested 4 h p.i.; infection was quantified as in figure 4. Experiments were performed three times independently and normalized to untreated samples, mean ±SEM is shown.

Supplementary Figure 4. EVs in dextran-containing macropinosomes.

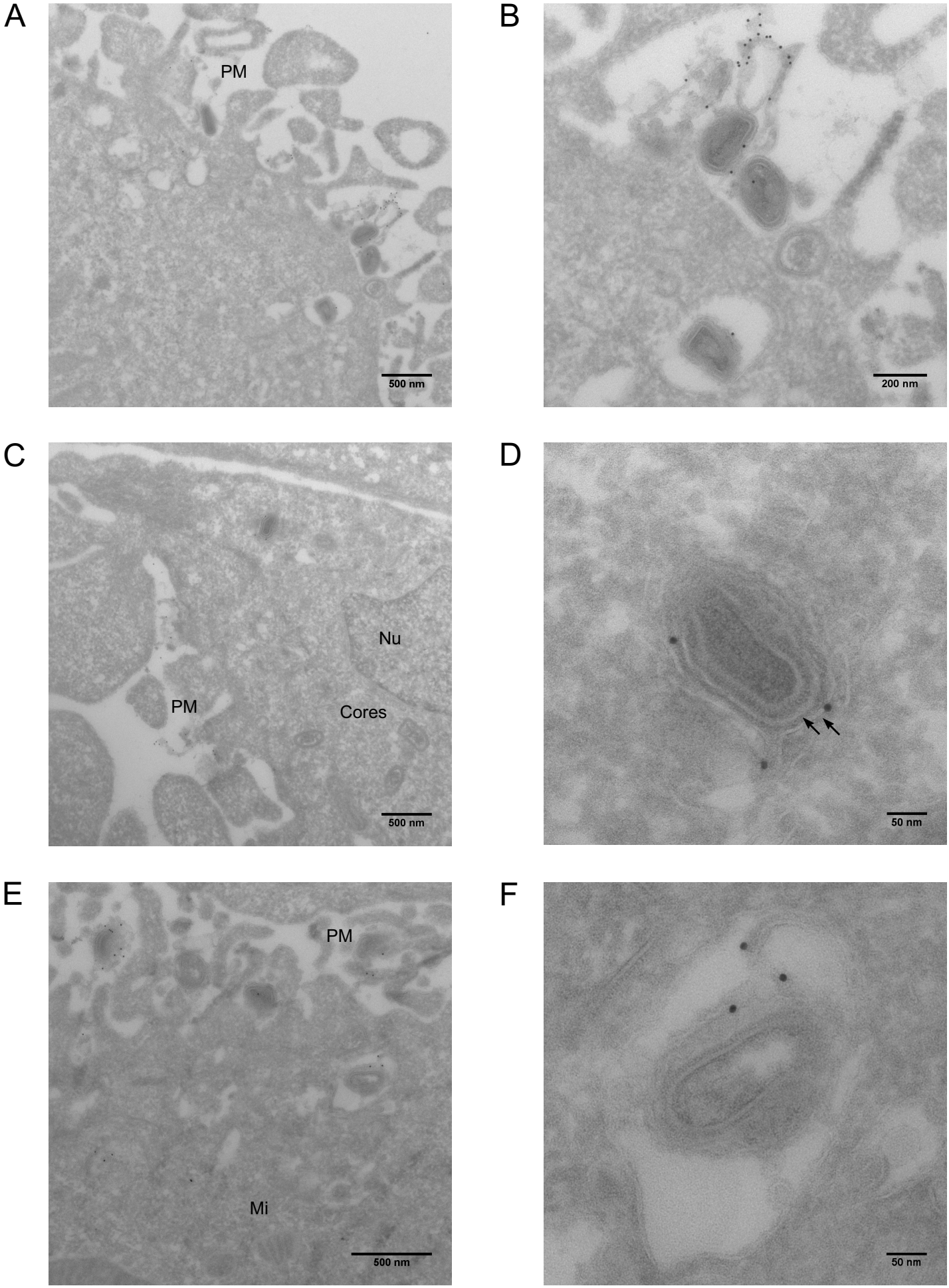
HeLa cells were preincubated with full (A) or serum-free medium (B) for 4 h. IHD-J F13-GFP EVs in 0.2% BSA/RPMI were bound to cells on ice for 1h (MOI 25) and incubated for 30 min at 37°C in presence of 0.5 mg/mL 10 kDa dextran AF 594 (A), or dextran and 25 μg/mL transferrin AF 647 (B). Images were recorded by confocal microscopy and representative maximum projections of Z-stacks are shown. Arrowheads highlight EV particles in dextran-containing vesicles. Scale bars = 5 μm.

Supplementary Figure 5. Intracellular accumulation of EVs in presence of BafA.

HeLa cells were infected with IHD-J mCherry-A5 F13-GFP EVs (MOI 25) in the presence of 5 μg/mL ActD without (A) or with (B) 25 nM BafA. Particles were bound to pretreated cells on ice for 1 h, washed with PBS, and incubated in full medium with drugs for 3h. Bound EV particles were stained with VMC-20 (anti-B5) under non-permeabilizing conditions. Images were recorded by confocal microscopy and representative maximum projections of Z-stacks are shown. Arrows in the inset of B highlight internalized EV particles not accessible to VMC-20 staining. A bound EV (open arrowhead), and a free membrane (closed arrowhead) are visualized as well. Scale bars = 10 μm.

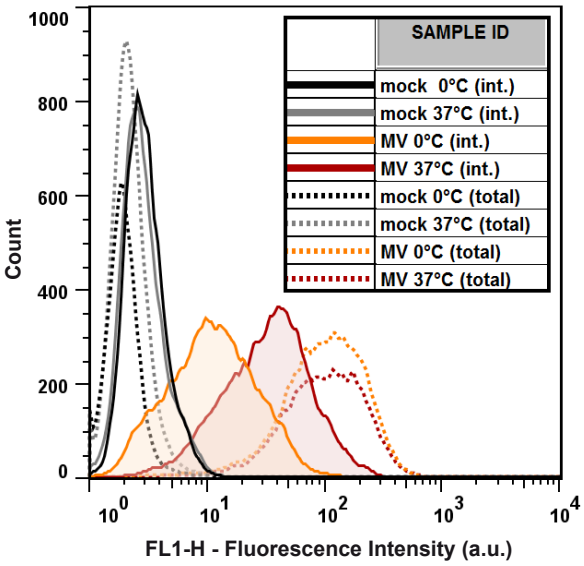
Supplementary Figure 6. Examplary images of core release assay.

Additional examplary images of core release assay quantification in figure 8E. HeLa cells were infected with IHD-J EGFP-A5 7D11-treated EVs (MOI 8 after 7D11 incubation) (A), MVs (MOI 45) (B-D), or 7D11-treated MVs (equivalent to MOI 45 before neutralization) (E) in the presence of 5 μg/mL ActD and 25 nM BafA where indicated. Particles were bound to pretreated cells on ice for 1 h, washed with PBS, and fixed after binding (A-B) or after incubation in full medium with drugs for 3h (C-E). L1 (Mab 7D11) and actin were stained and images were recorded by confocal microscopy. Representative maximum projections of Z-stacks are shown. Arrows in insets highlight released viral cores, closed arrowheads mark viral particles stained for the MV membrane marker L1. Scale bars = 10 μm.



Supplementary Figure 1

A



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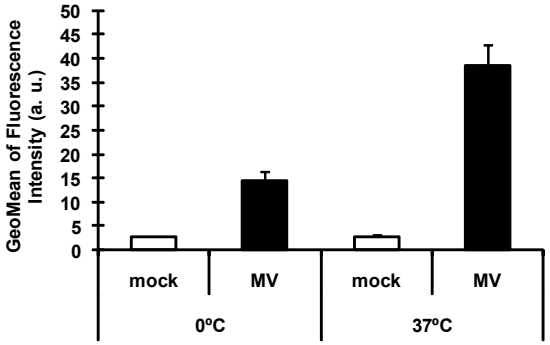
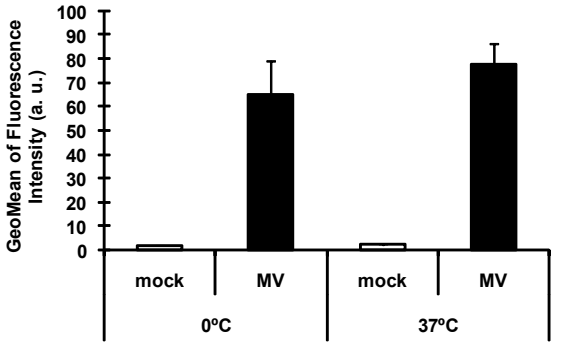
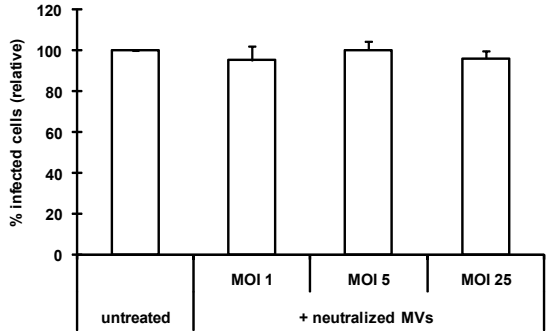


C

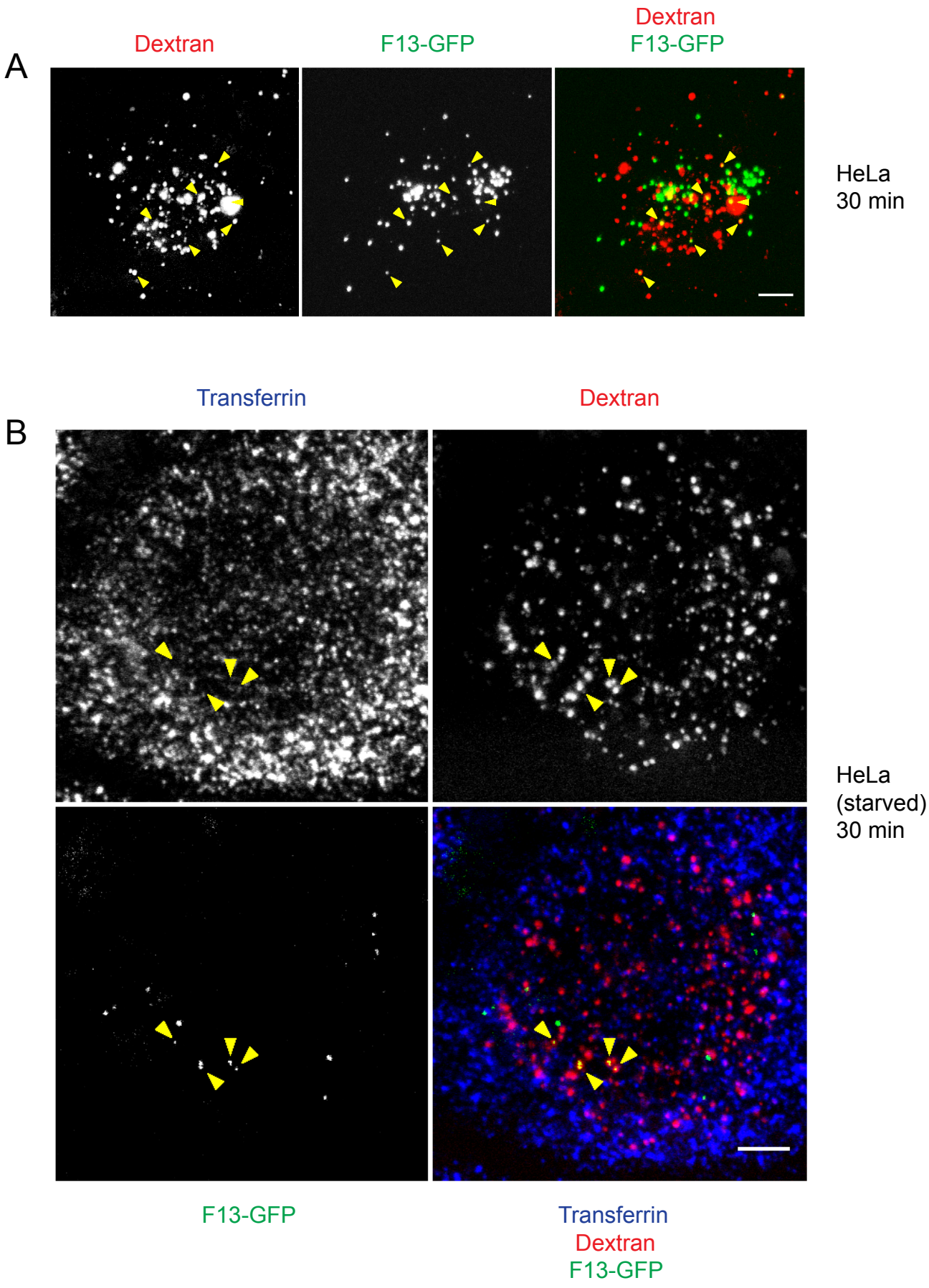


Supplementary Figure 2

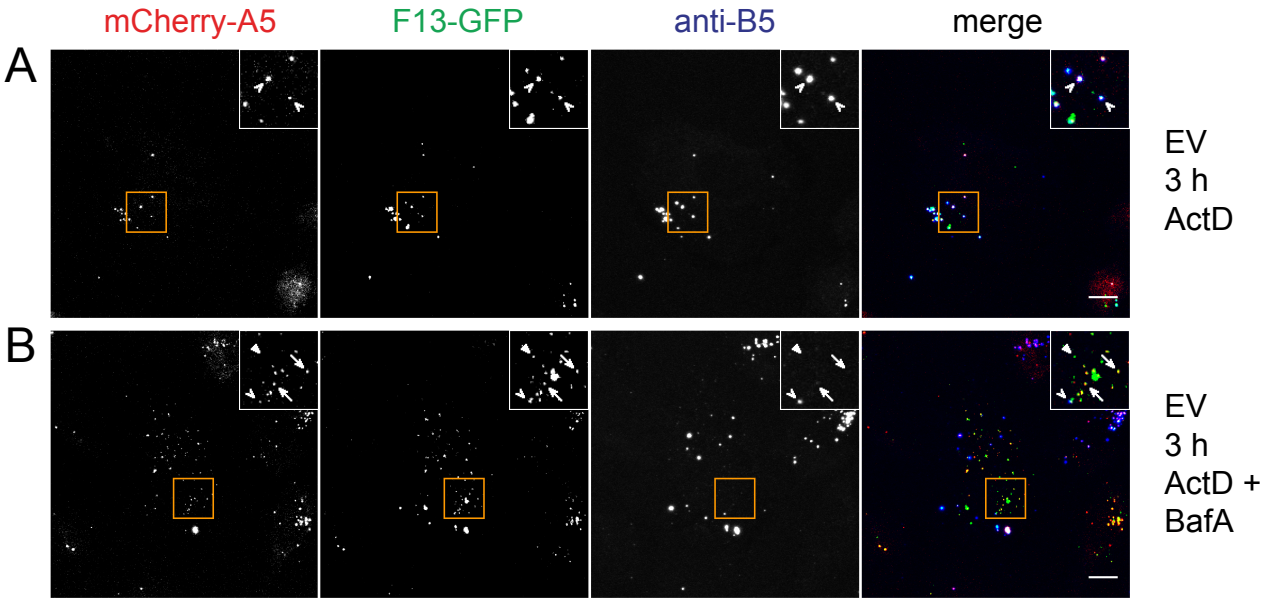
EV Infection in presence of neutralized MVs



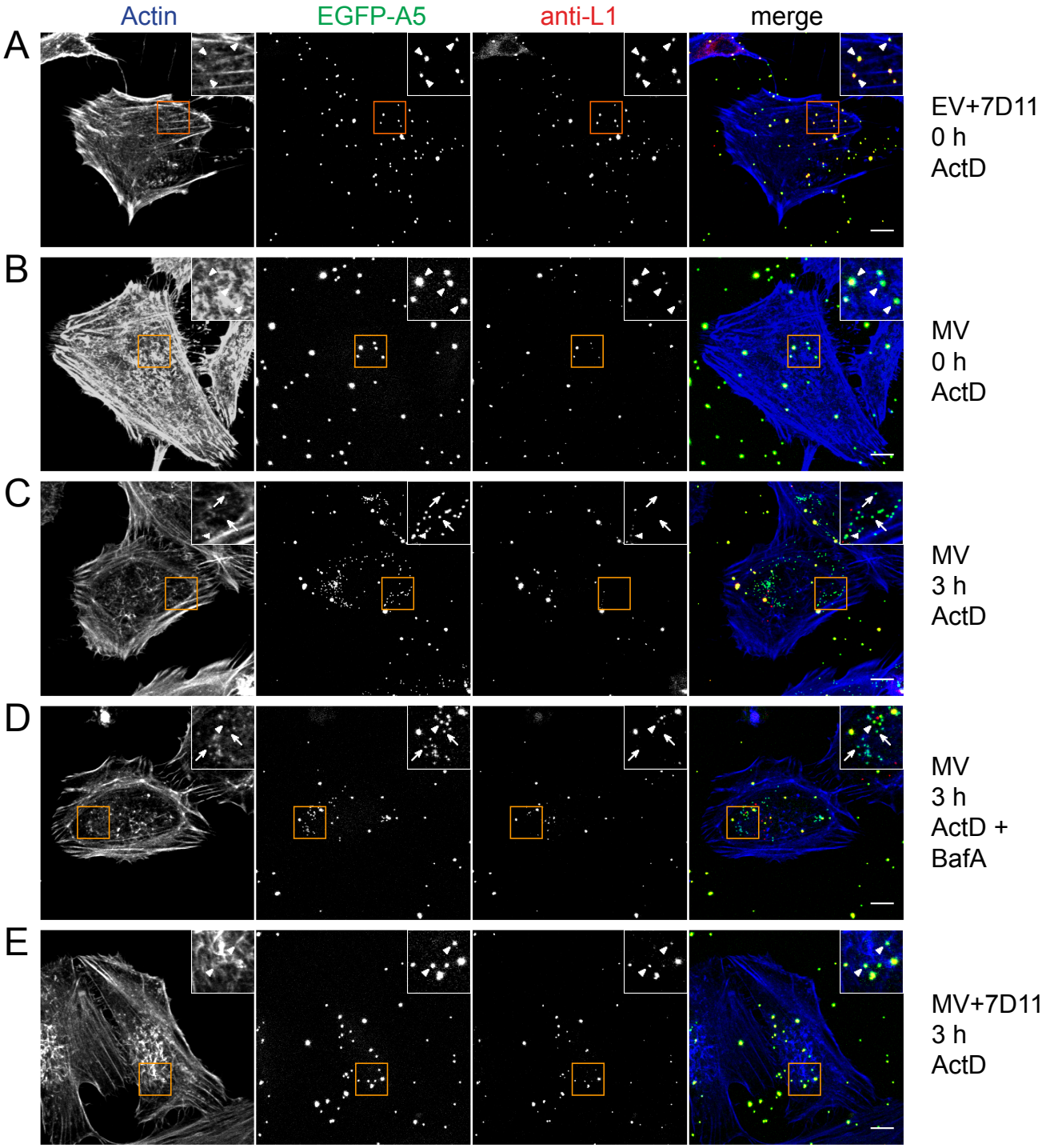
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6