Supplementary Figure 1. TGN, early endosomes and late endosomes/ lysosomes remain distinct in NHE8-depleted cells. HeLa M cells were mock-transfected or transfected with a pool of 4 siRNA oligos targeted against NHE8. 48 h after the second transfection, cells were fixed, permeablised and stained using antibodies against the early endosome marker EEA1, the early/ recycling endosome marker TfR, the TGN marker TGN46 and the late endosome/ lysosome marker LAMP1. Cells were viewed using fluorescence microscopy. Scale bar=10 μm.

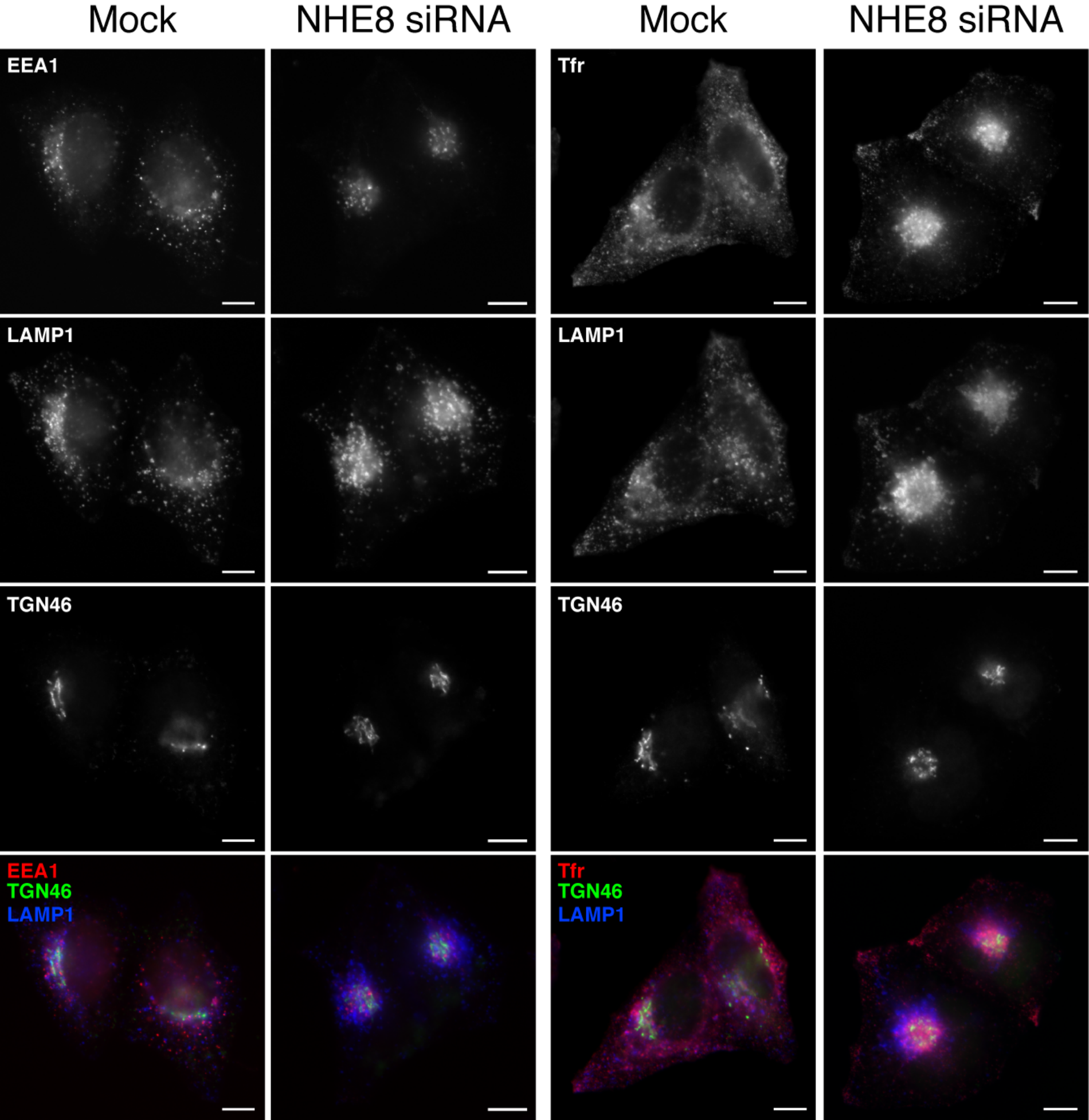
Supplementary Figure 2. Depletion of NHE8 by transfection with siRNA. HeLa M stably expressing NHE8-HA cells were transfected with either water (mock) or the pool of 4 NHE8 siRNA oligos at 100 nM (siRNA) using a double transfection protocol. Cells were fixed and stained with anti-HA antibodies (green) and nuclei were labeled with DAPI (blue). The upper panels show non-saturated images of cells imaged under identical settings and the lower panel the same images with the brightness/contrast increased by identical amounts. Scale bar=15 μm.

Supplementary Figure 3. Cells transfected with different NHE8 siRNA oligos have perinuclear, clustered endosomes and an accumulation of dense MVB's. (A) HeLa M cells stably expressing NHE8-HA were transfected with water (mock) or 100 nM of siRNA oligos 1-4. Depletion using the oligo pool (oligos 1-4 mixed at 25 nM each) is also shown. Post-nuclear supernatants were loaded at 50 μg total protein per lane and the western blots developed using anti-HA antibodies. Levels of calreticulin were also assessed in the same samples as a loading control. (B) HeLa M cells were transfected with either water (mock) or 100 nM of siRNA oligos 1-4. All depletions resulted in similar phenotypes and here we present representative results for oligos 3 and 4. Left panels: Cells were fixed and endosomes were labeled with antibodies to EEA1, TfR, LAMP1 and nuclei were labeled with DAPI (blue). Scale bar=10 μm. Right panels: cells were fixed and processed for transmission EM as described in materials and methods. Scale bar=500 nm.

Supplementary Figure 4. ts045 VSV-G-GFP transport to the cell surface is unaffected in NHE8-depleted cells. On day 3 of the siRNA transfection protocol HeLa M cells were seeded onto glass coverslips and transiently transfected with ts045 VSV-G-GFP using TransIt-HelaMONSTER (Mirus) following the manufacturer's instructions. The cells were incubated at 40°C overnight. All subsequent incubations were done at 32°C for 0, 30 or 180 min in the presence of 50 μg/ml cycloheximide (Sigma). Each time point was processed by rinsing the cells in ice-cold PBS, incubating with 10 μg/ml rhodamine-labelled wheat germ agglutinin (WGA) (Vector Laboratories) for 10 min on ice followed by a further wash with ice-cold PBS before fixation and antibody staining. As shown in the figure, ts045 VSV-G-GFP in mock or NHE8-depleted cells showed a reticular staining pattern after incubation at 40˚C (0 min time point). This reticular staining colocalised with antibodies against the ER marker calreticulin (not shown), demonstrating that ts045 VSV-G-GFP was effectively held in the ER after incubation at 40˚C. After 30 min incubation at 32˚C in the presence of cycloheximide, ts045 VSV-G-GFP had moved to the TGN where

it overlapped with TGN46. After 180 min at 32˚C the majority of the ts045 VSV-G-GFP was at the cell surface where it colocalised with wheat germ agglutinin (WGA). Incubation of HeLa M cells overnight at 40˚C and then for 180 min at 32˚C in the presence of 20 μM CCCP or 25 μM monensin inhibited ts045 VSV-G-GFP transport to the cell surface.

Supplementary Fig. 5. Depletion of retromer (Vps35) inhibits MVB to TGN transport of CIMPR; depletion of NHE8 does not. (A) HeLa M cells stably expressing a chimera consisting of the lumenal and transmembrane domains of CD8 and the cytosolic tail of the cation independent mannose 6-phosphate receptor (CD8-CIMPR) were transfected with NHE8 siRNA or VPS35 siRNA (ON-TARGET *plus*, Dharmacon) using a double knockdown protocol, and the experiment was performed 48 h after the second transfection. These CD8-CIMPR cells have been used previously to show that retromer, is required for the return of CD8-CIMPR to the TGN from MVB's (Seaman, 2004). Here, we deplete Vps35 (a subunit of the retromer complex as a control). Cells were incubated with anti-CD8 antibodies (clone UCHT4, Ancell) at 4°C, washed and then warmed to 37°C for 30 min before fixation. The cells were then labeled with antibodies to TGN46 followed by secondary antibodies. In the merged images CD8 is green and TGN46 is magenta. Untransfected HeLa M cells and HeLa M cells stably expressing CD8 with a cytosolic HA tag were used as controls to show that no staining is observed in the absence of CD8 and CD8 alone is held at the cell surface, respectively (upper panels). Scale bars = 10 μm. (B) Cells lysates from the experiment described in (A) were western blotted using antibodies to either Vps35 (to show depletion of Vps35; upper panel), Vps26 (protein levels of Vps26 and Vps35 are co-ordinately regulated (Seaman, 2007); lower panel) or calreticulin (loading control; middle panel). 40 μg of protein was loaded per lane. HeLa M cells expressing CD8-CIMPR were a kind gift from Matthew Seaman, University of Cambridge.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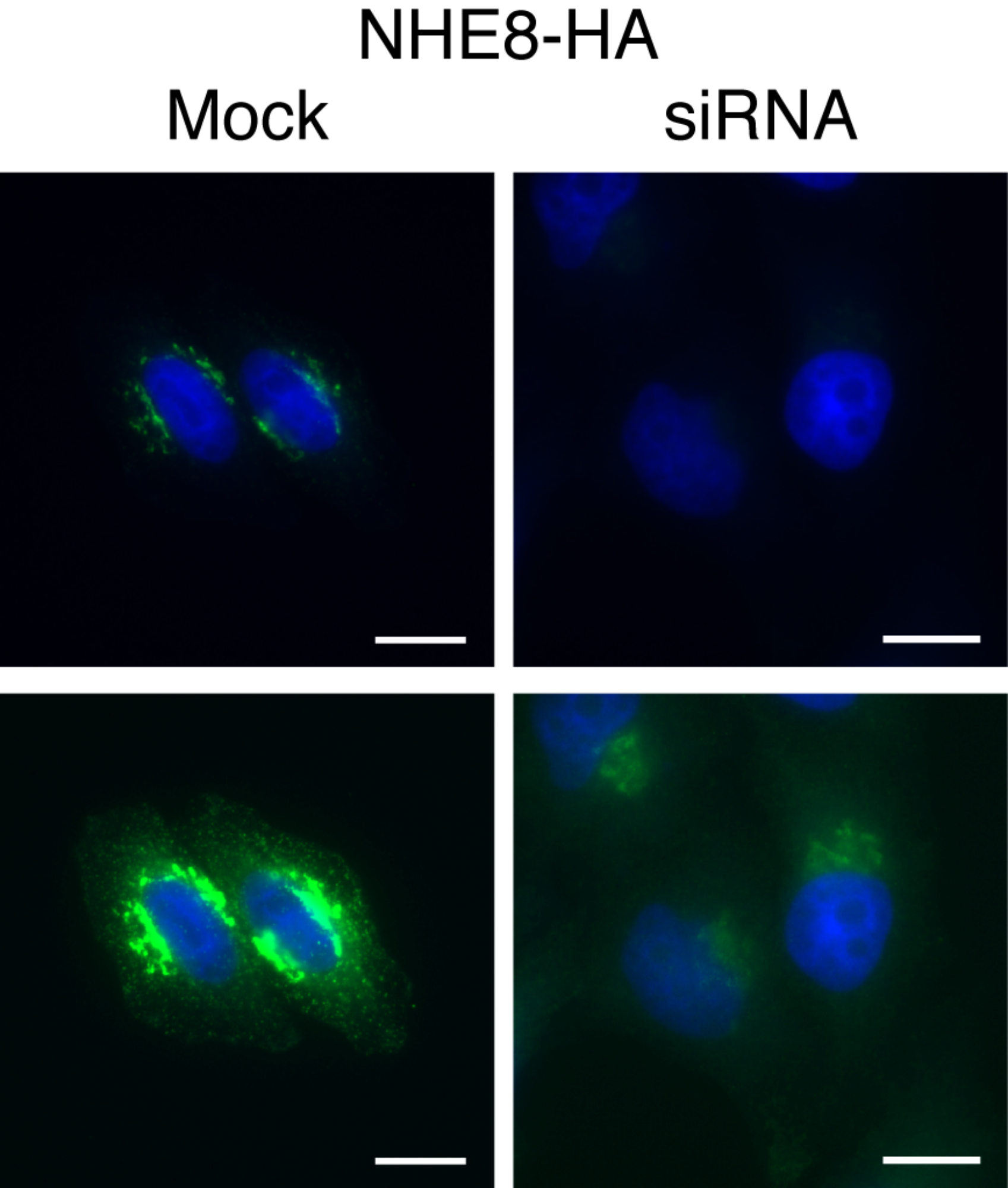
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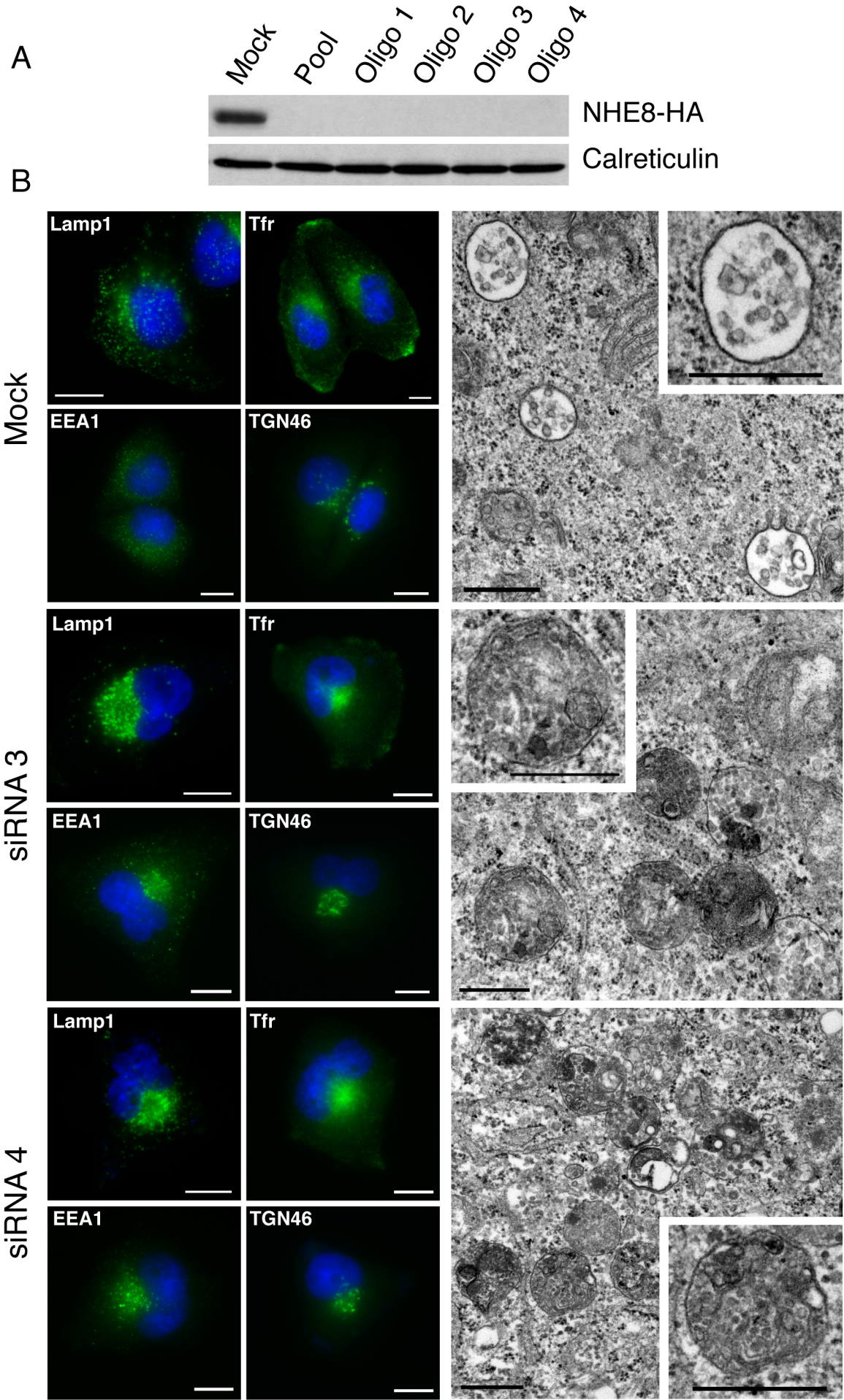
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B
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Lamp1
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EEA1
TGN46

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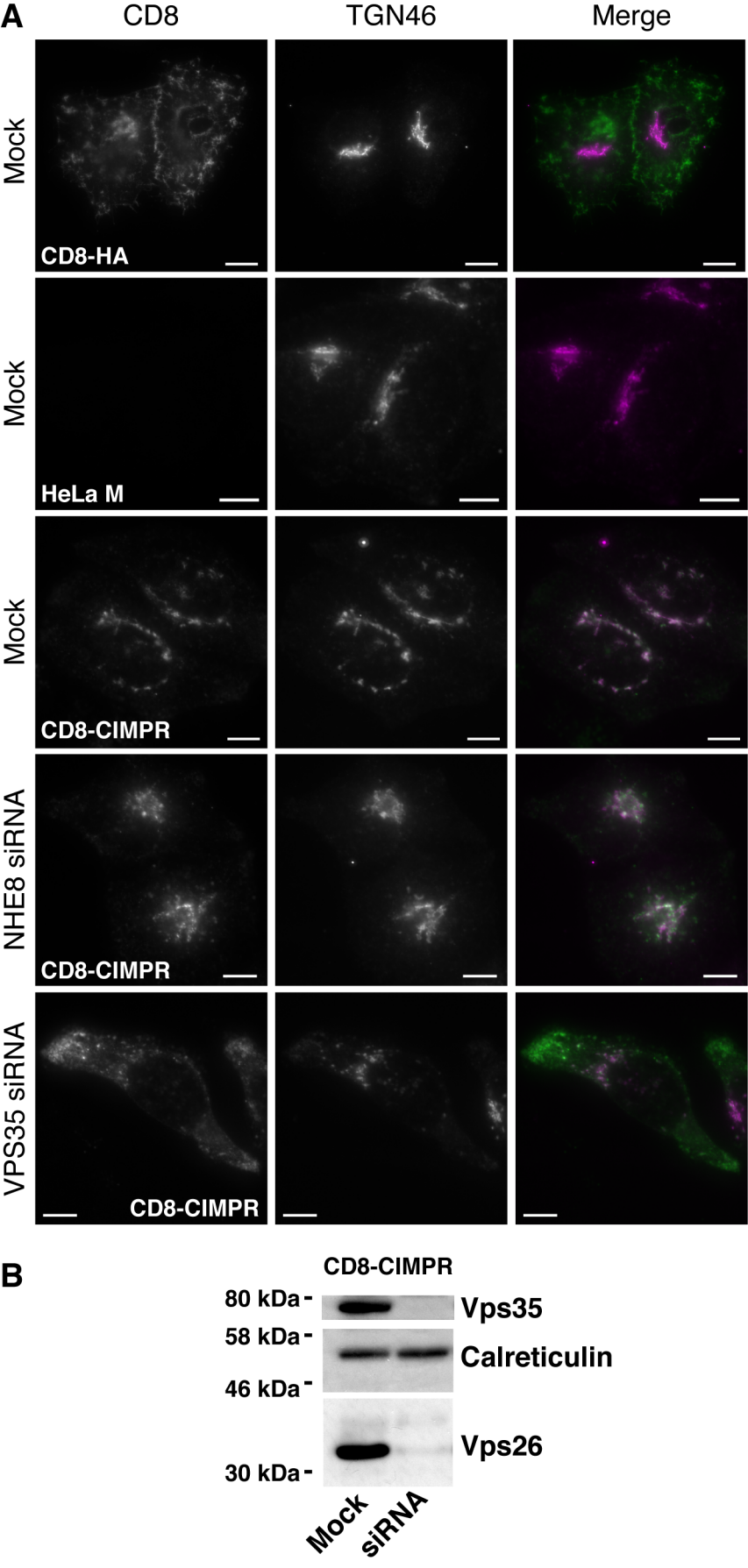
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siRNA