

Supplementary Methods

Cell Lines

PTR9 MDCK cells stably expressing the human TfR, rabbit pIgR, and the tetracycline transactivator were cultured in modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (FBS) and 0.05% hygromycin B (Sigma). MDCK cells stably expressing the GFP-tagged C-terminal domain of myosin Vb (MyoVbT cells) were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% FBS and 400 µg/ml G418. The MDCK cell line stably expressing GPI-anchored endolyn was previously described (Potter et al., 2004). The stable cell line expressing GFP-Sec15CT was created by transfecting MDCK-II cells using Lipofectamine 2000 reagent with pEGFP-C3 vector (Clontech, Palo Alto, CA) encoding the C-terminus 1296 bp fragment of Sec15. Transfected cells were selected with 500 µg/ml G418. The mutant GFP-Sec15CT(NA) construct was generated by mutating amino acid Asn₇₀₉ to alanine using the Quickchange Site-Directed Mutagenesis Kit (Stratagene) and a cell line stably expressing this construct was generated as described above. Parental MDCK II cells were used as controls where indicated.

Antibodies

Mouse monoclonal antibody 501 to rat endolyn has been described previously (Ihrke et al., 2001; Ihrke et al., 1998). Antibodies for VSV-G [8G5 from Dr. Douglas Lyles, Wake Forest University (Lefrancois and Lyles, 1982), HA (Fc125 from Dr. Thomas Braciale, University of Virginia), and p75 (MA 20.1 from Dr. Enrique Rodriguez-Boulán, Weill Medical College) were obtained from supernatants of cultured hybridomas. IgA was detected using goat anti-human IgA-Cy5 (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:500 dilution. Anti-Rab11a serum

was used at a dilution of 1:500. Rabbit anti-EEA1 antibody (Dr. Silvia Corvera, University of Massachusetts Medical School) and rabbit anti-furin antibody (Alexis Biochemicals, San Diego, CA) were used at 1:500 dilution. Affinity-purified rabbit polyclonal anti-cation independent mannose-6-phosphate receptor M6PR antibody (Dr. Linton Traub, University of Pittsburgh) was used at 1:100 and mouse anti-Lamp2 antibody (AC17, Dr. Enrique Rodriguez-Boulan) was used at a dilution of 1:500. Rat anti-ZO-1 hybridoma R40.76 culture supernatant (Dr. Daniel Goodenough, Harvard University,) was used at a dilution of 1:10. All Cy5 and FITC conjugated goat anti-mouse, rabbit and rat secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at 1:200 dilution. Cy3 conjugated secondary antibody (Jackson ImmunoResearch Laboratories) was used at 1:1000 dilution.

Adenovirus Infection

Generation of replication-defective recombinant adenoviruses expressing tetracycline-repressible HA (Japan serotype) and VSV-G (Indiana strain) has been previously described (Bruns et al., 2002; Henkel et al., 1998). A similar strategy was used to generate adenovirus encoding endolyn. Replication-defective recombinant adenovirus expressing tsVSV-G-YFP was a gift from Dr. Patrick Keller. For infection, cells grown on coverslips or transwells were first incubated in calcium-free PBS containing 1mM MgCl₂ (PBS-M) for 5 min. The PBS-M was replaced with PBS-M containing adenovirus at a multiplicity of infection of 50. Cells not stably expressing transactivator were coinfecting with adenovirus encoding this protein at a multiplicity of infection of 50. Cells were then returned to a 37°C incubator for 1 h, then washed in PBS-M and incubated in complete media overnight at 37°C or 40°C (for expression of tsVSV-G-YFP).

Immunofluorescence

Cells were washed twice in PBS and fixed with 4% paraformaldehyde in 100 mM sodium cacodylate buffer for 10 min at ambient temperature. Cells were washed three times in phosphate buffered saline (PBS) and remaining formaldehyde was quenched by incubating in PBS containing 20 mM glycine, pH 8.0 and 75 mM NH_4Cl for 10 min. After 3 washes in PBS, cells were blocked in block buffer (0.025% [w/v] saponin, and 8.5 mg/ml of fish skin gelatin in PBS) containing 10% (v/v) goat serum for 10 min. Cells were incubated with the appropriate primary antibodies diluted in the blocking solution for 1 h. After extensive washing with blocking solution, cells were incubated with secondary antibodies diluted in blocking solution for 1 h. This incubation was followed by three washes with blocking solution and 3 washes with PBS. Cells were then post-fixed in 4% paraformaldehyde for 10 min, quenched, and mounted as described previously (Apodaca et al., 1994). Imaging was performed on a TCS-SL confocal microscope (Leica, Dearfield, IL) equipped with argon, green helium-neon, and red-helium-neon lasers. Acquisition of images was performed with 100x plan-apochromat oil objective (NA 1.4) and the appropriate filter combination. Images were analyzed with Volocity software (Improvision, Lexington, MA).

Supplementary Figure Legends

Supplementary Figure 1. Newly synthesized HA does not colocalize with GFP-MyoVbT

Polarized MyoVbT cells were infected with adenovirus encoding HA. At 6 h postinfection, cells were incubated at 19°C for 2 h to accumulate newly synthesized biosynthetic cargo in the TGN. Cells were then warmed to 37°C for 20 min, fixed, and processed for immunofluorescence to detect HA and GFP-MyoVbT. Individual confocal sections and a merged image taken at the level of peak GFP-MyoVbT fluorescence are shown. Scale bar = 10 μ m.

Supplementary Figure 2. Newly synthesized endolyn colocalizes with Rab11a in MDCK cells.

Polarized MDCK cells infected with adenovirus encoding DOX-repressible endolyn were incubated overnight with 1 ng/ml DOX, then washed extensively and incubated in DOX-free medium for 6 h to initiate endolyn synthesis. Cells were then incubated at 19°C for 2 h to accumulate newly synthesized biosynthetic cargo in the TGN. During the last hour at 19°C, monoclonal anti-endolyn antibody was included in the apical medium of the sample in panel C. Cells were then warmed to 37°C for 0 (panel A) or 15 min (panels B and C), fixed, and processed for indirect immunofluorescence to detect endolyn (panels A and B) or internalized anti-endolyn antibody (panel C) and Rab11a. Individual confocal sections and merged images are shown in each panel. Scale bar = 10 μ m.

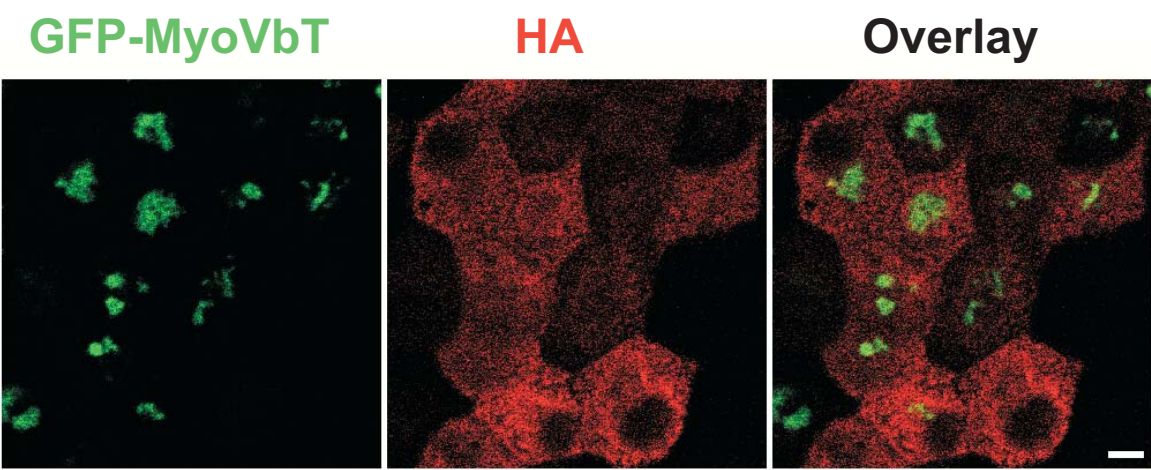
Supplementary Figure 3. Surface delivery of a non-raft associated mutant of HA is insensitive to inactivation of HRP-WGA-containing compartments. Butyrate-induced, filter grown MDCK cells stably expressing the Triton X-100 soluble HA mutant A517 (provided by Michael Roth, U.T. Southwestern) were radiolabeled for 15 min, then incubated for 2 h at 19°C.

HRP-WGA was included in the apical medium during the last hour of the 19°C stage. Cell surface HRP-WGA was stripped and WGA-containing compartments were inactivated on ice as described in Materials and Methods. The cells were warmed to 37°C for 0 or 90 min, and apical delivery was quantitated by cell surface trypsinization. The mean \pm S.E. of three independent experiments is plotted. In these studies, we consistently observed that treatment of this stable cell line with H₂O₂ alone resulted in significantly reduced surface delivery compared with other control conditions. Because inclusion of HRP-WGA did not further reduce surface delivery compared with H₂O₂ alone, we conclude that A517 delivery does not require passage through an HRP-WGA-containing compartment. * $p < 0.05$ as compared to no add'n and no H₂O₂ controls by paired t-test.

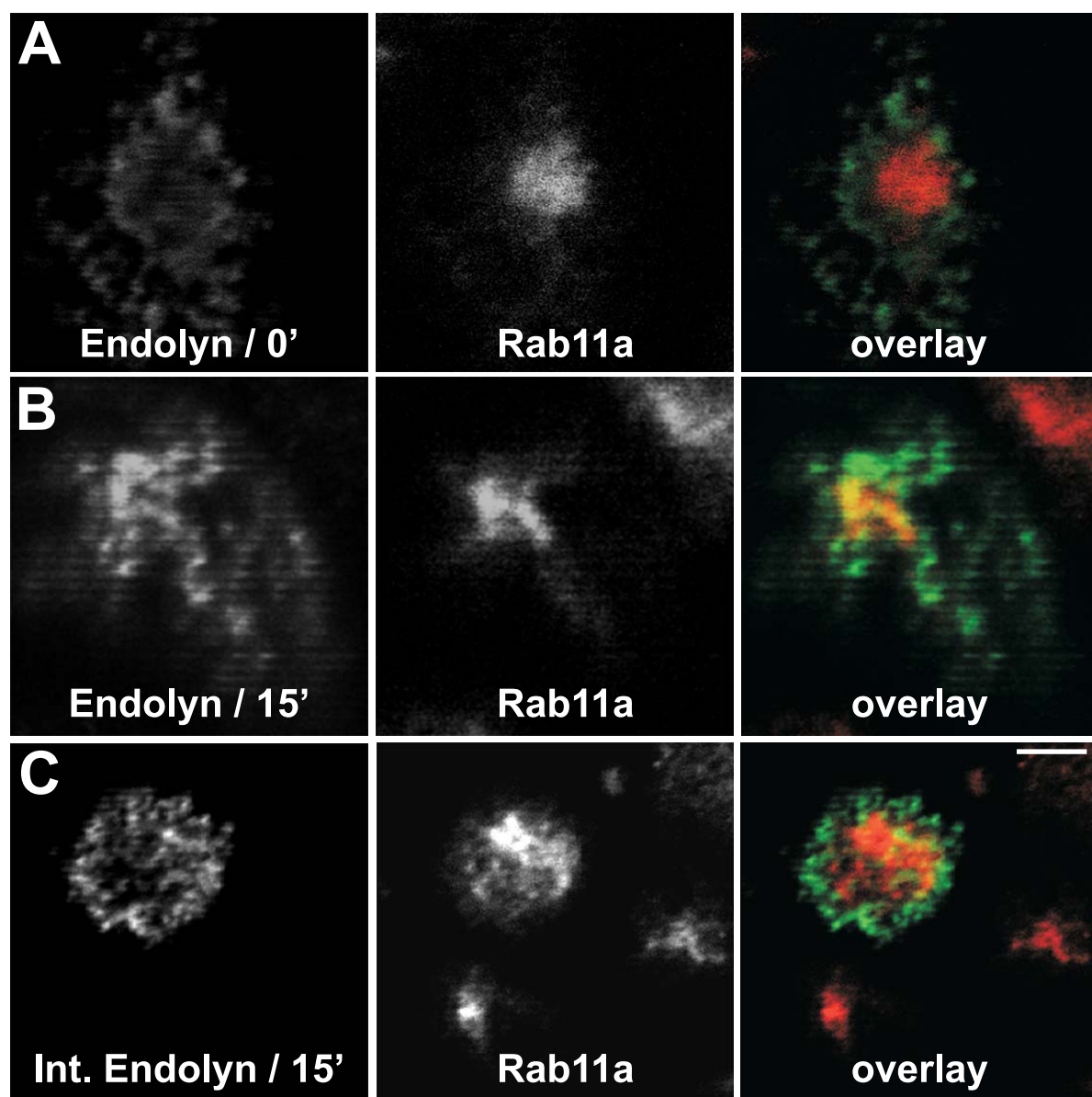
Supplementary Figure 4. Inactivation of HRP-WGA-containing compartments in MyoVbT cells does not further inhibit apical delivery of endolyn or HA. (Panel A) Control or MyoVbT cells expressing HA were radiolabeled for 15 min at 37°C in the presence or absence of apically added HRP-WGA. After inactivation, surface delivery was quantitated by cell surface trypsinization. The bars show the average % apical delivery after a 90 min chase in two independent experiments; the actual values obtained in each experiment are shown in circles and triangles. Although the total % of HA reaching the surface varied between experiments as noted in the Methods section, HRP-WGA inactivation inhibited delivery in both cases, and HRP-WGA did not further inhibit HA delivery in MyoVbT cells relative to control cells. (Panel B) Inactivation of HRP-WGA containing compartments in MyoVb cells does not affect delivery of endolyn. MyoVbT cells expressing endolyn were radiolabeled for 15 min at 37°C in the presence or absence of apically added HRP-WGA. After endosome inactivation, surface delivery was

monitored by domain selective biotinylation. A single experiment is shown in which the 60 min time points were performed in duplicate.

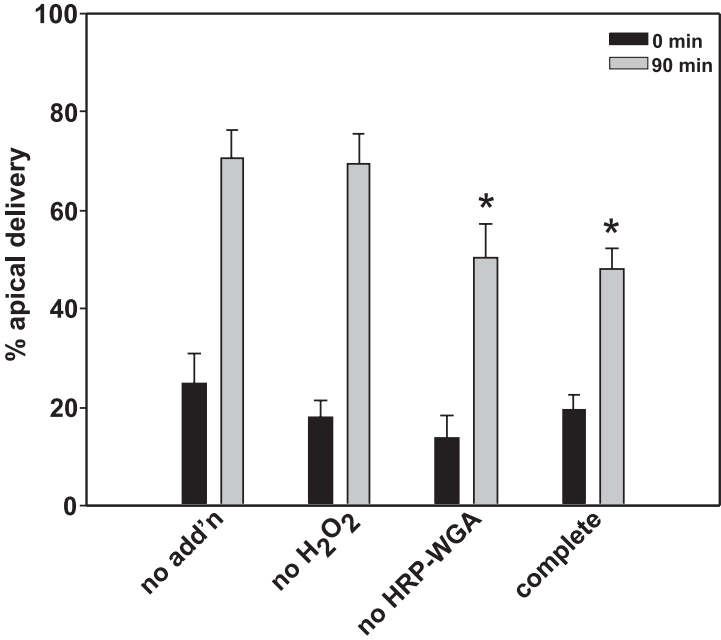
Supplementary Figure 1



Supplementary Figure 2

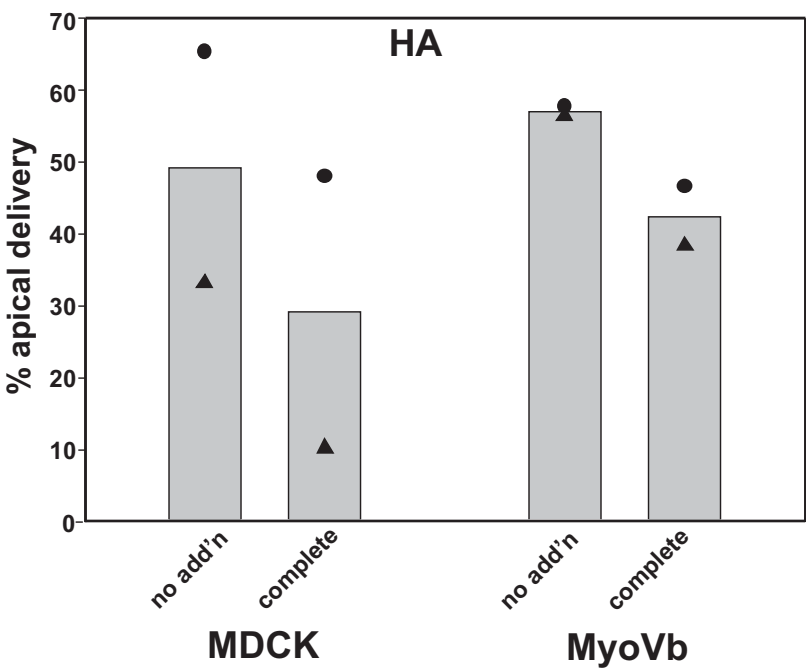


Supplementary Figure 3



Supplementary Figure 4

A



B

