

Figure s1

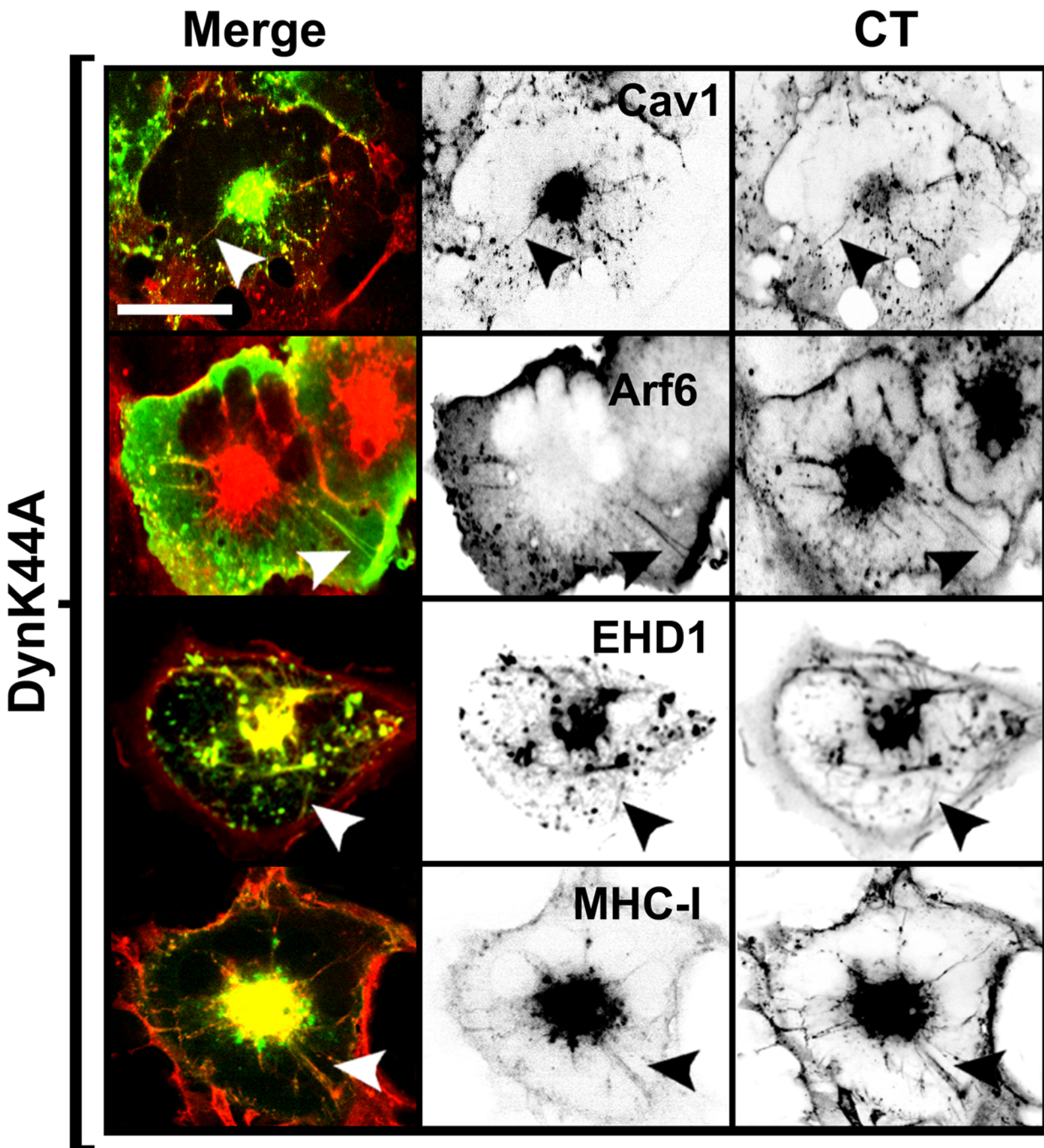
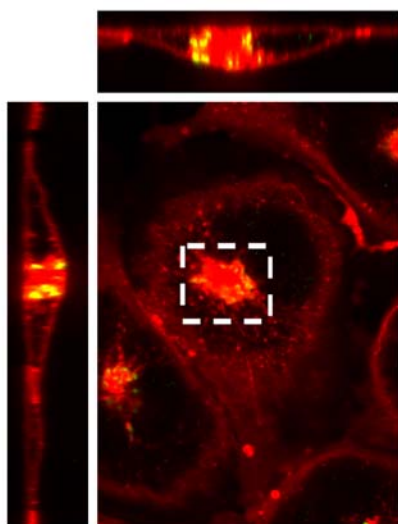


Figure s2

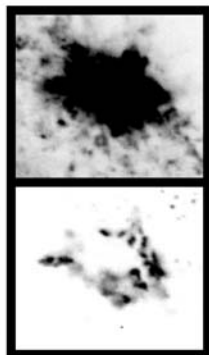
methyl- β -cyclodextrin

-

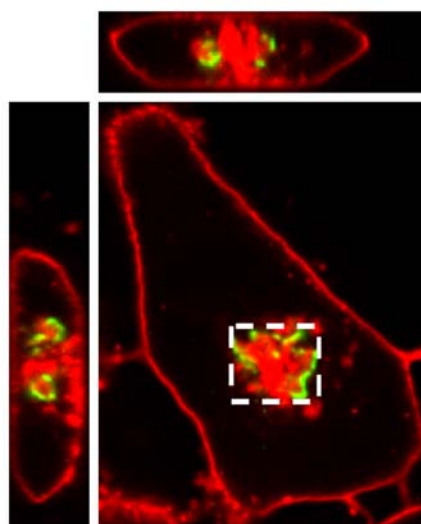
Golgi



GM130 CT



+



GM130 CT

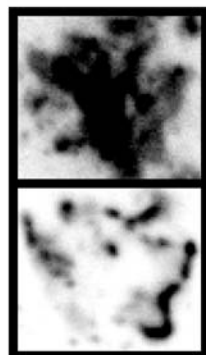


Figure s3

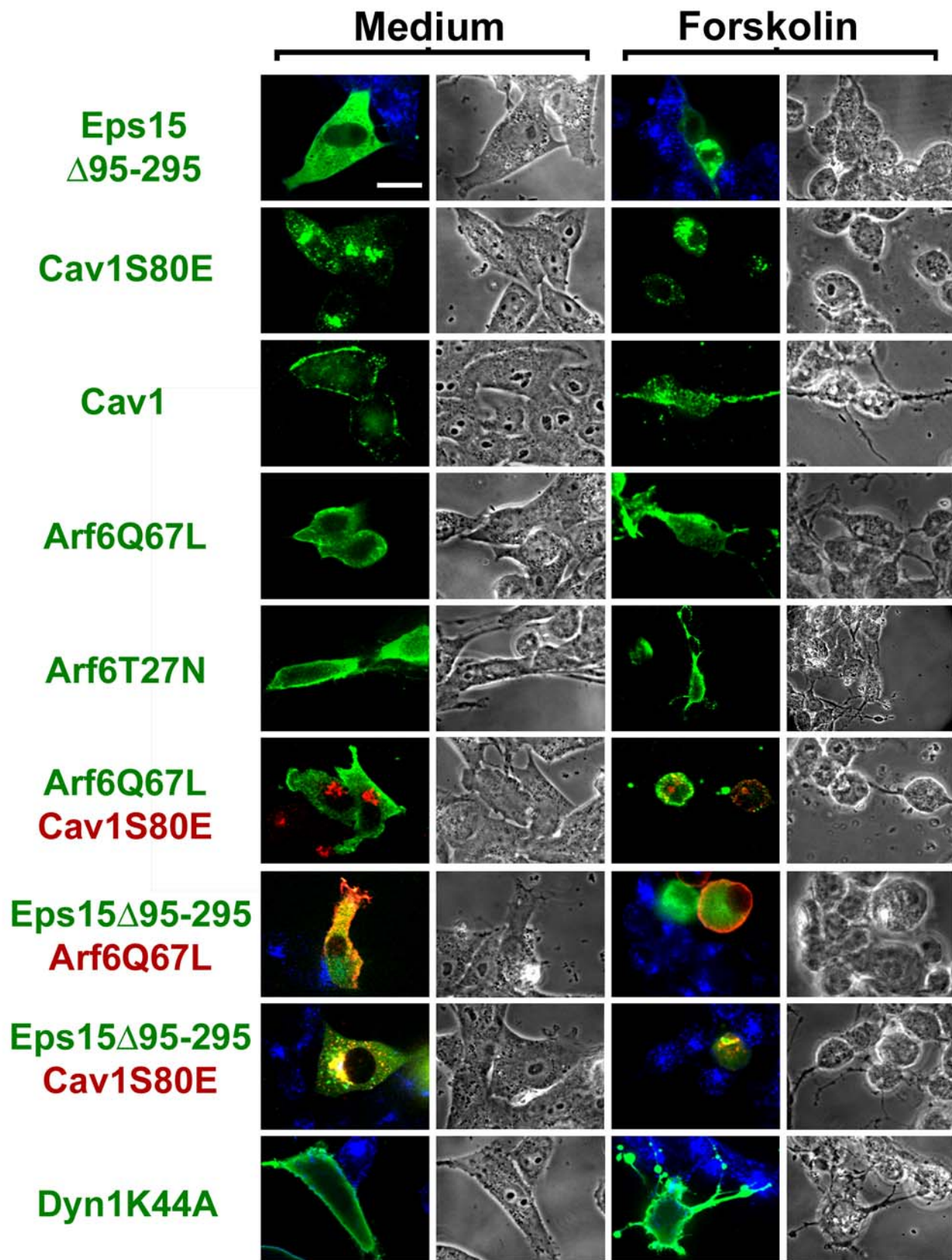


Figure s4

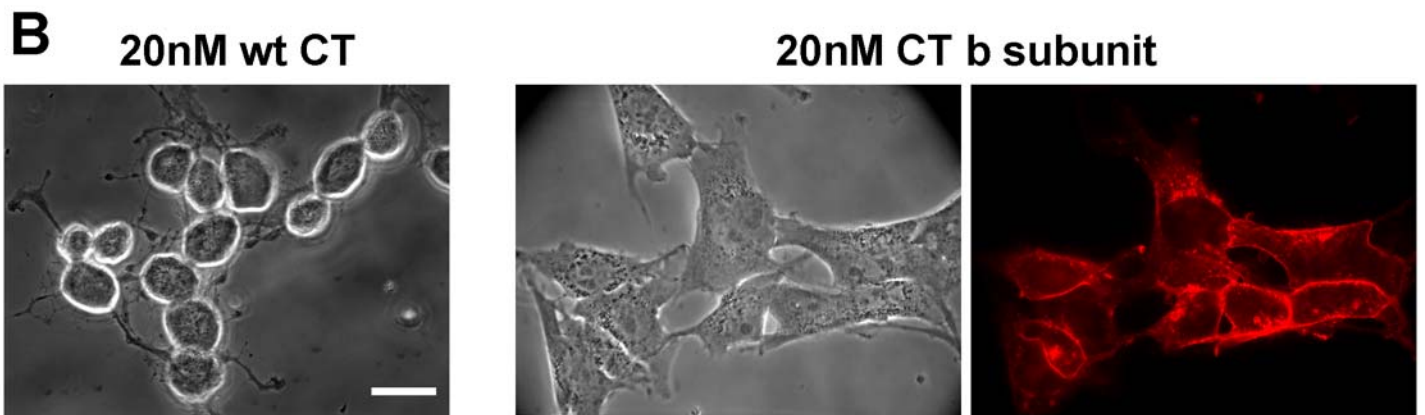
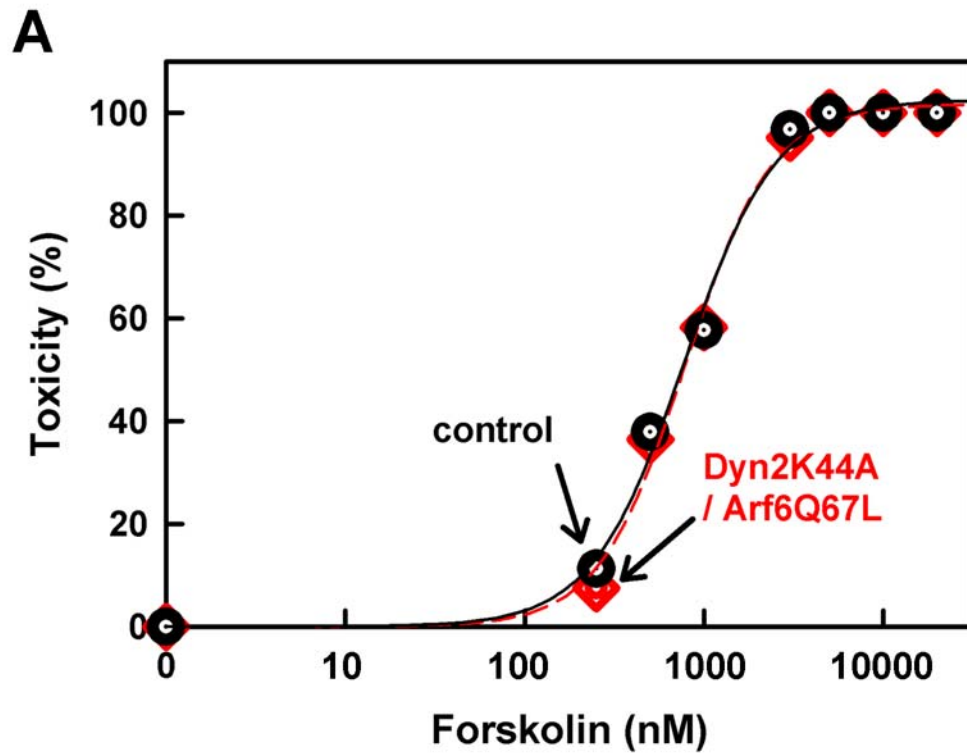


Figure s5

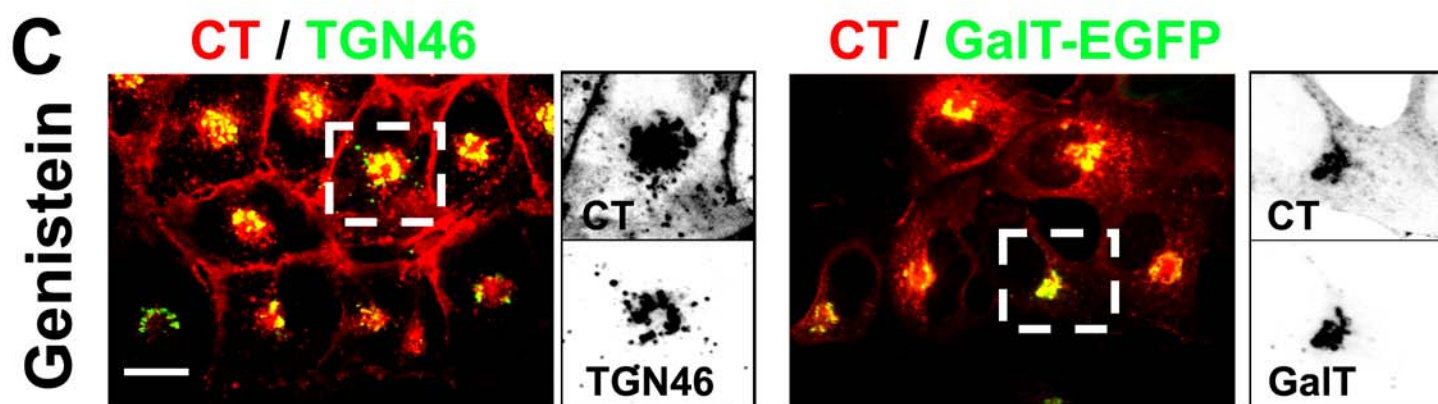
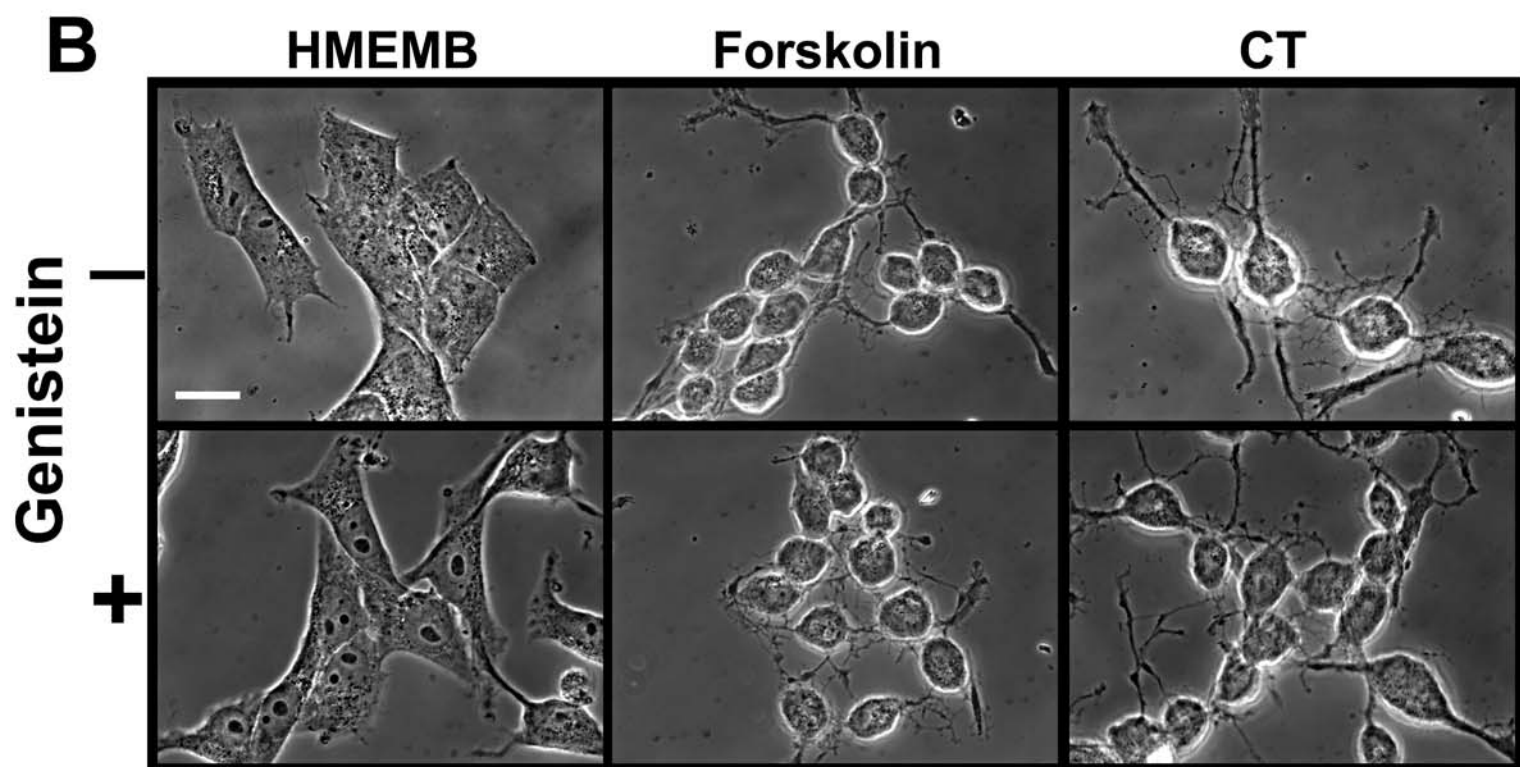
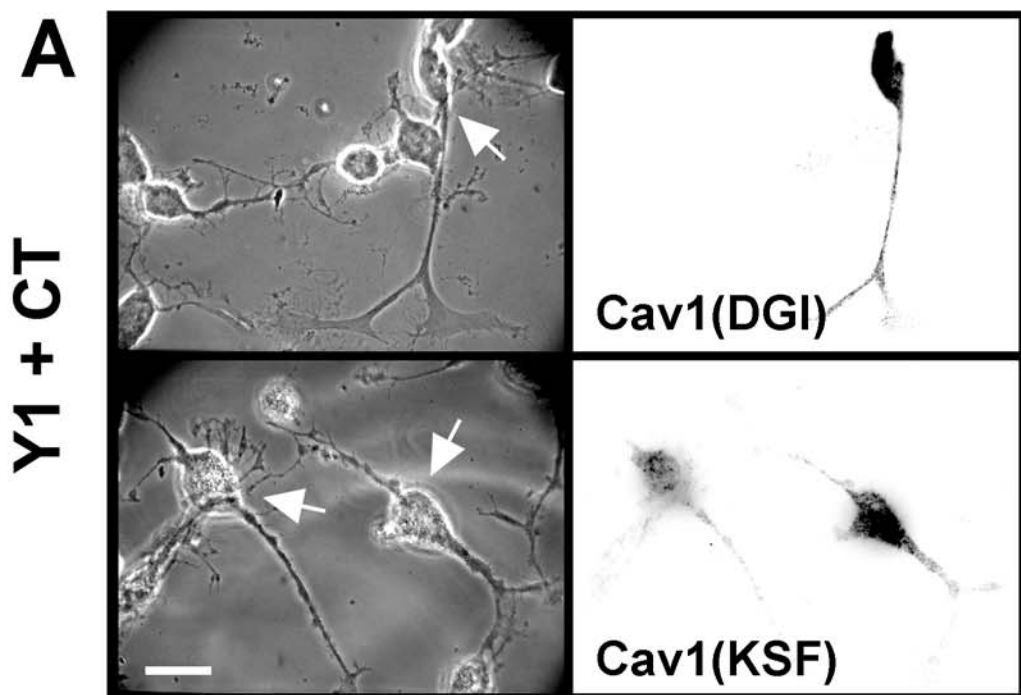
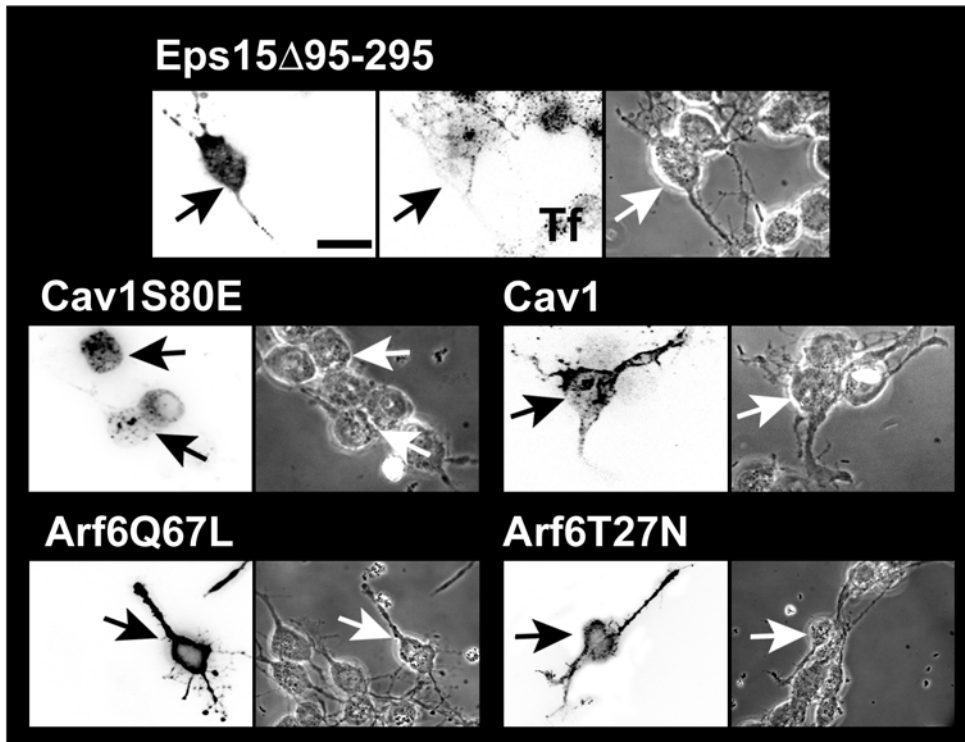


Figure s6

Single Interference



Multiple Interferences

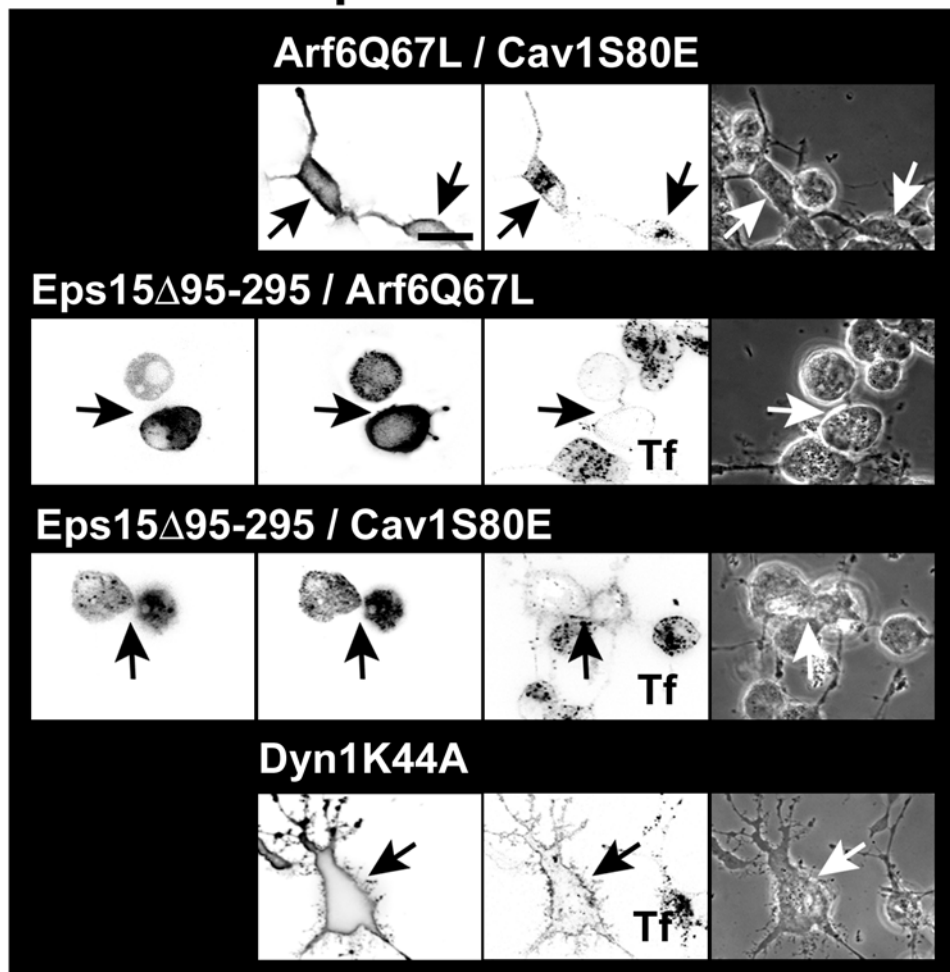
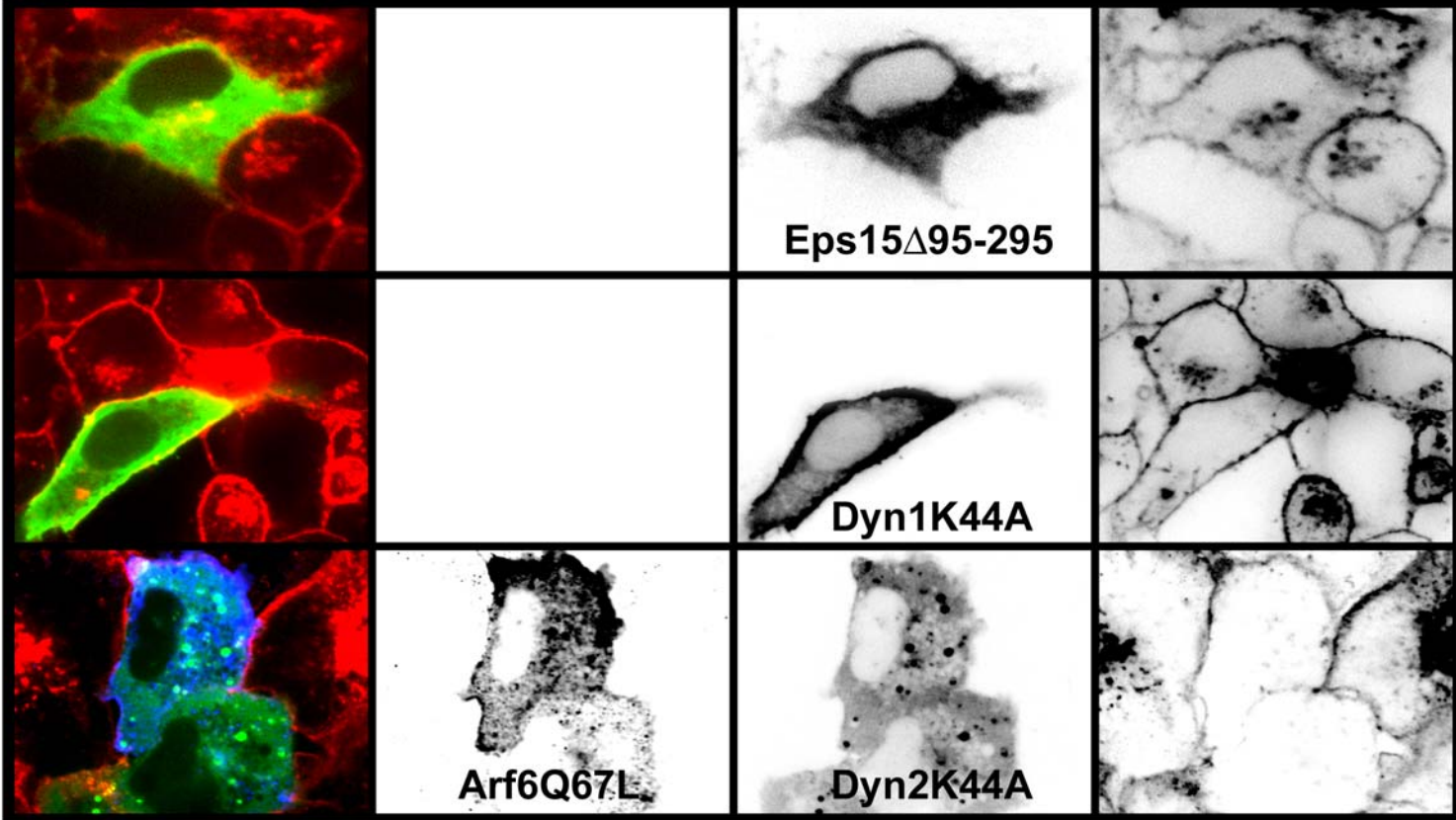


Figure s7

CT uptake in Y1 cells

Merge

CT



Eps15 Δ 95-295

Dyn1K44A

Arf6Q67L

Dyn2K44A

Figure s8

Figure s1. Vesicles and tubules containing CT align and move along microtubule tracts. BSC1 cells were pretreated with media in the absence (A) or presence (B) of 10 μ M nocodazole for 30min 37°C, followed by incubation with CT(E112D) in the absence or presence of nocodazole for 45min at 37°C. Cells were washed, fixed and then stained for tubulin as described. Scale bar, 20 μ m.

Figure s2. Expression of DynK44A induces formation of tubules containing CT and markers of the clathrin, caveolin and Arf6-dependent pathways. BSC1 and Y-1 (data not shown) cells transiently co-expressing DynK44A and Cav1-EGFP, Arf6-HA, EGFP-EHD1 or EGFP-MHC-I for 36-48h, were incubated with 20nM CT(E112D) for 45min at 37°C. For some conditions, we identified transfected cells by staining for the HA epitope. In the case of co-expression of Arf6-HA with DynK44A, we used Dyn2K44A-EGFP. Representative confocal images from middle sections of cells were captured and displayed using an inverted monochrome scale to aid visualization. Scale bar, 20 μ m.

Figure s3. Cholesterol depletion strongly decreases CT transport to the Golgi complex. BSC1 cells were pre-treated with 10mM methyl- β -cyclodextrin or with HMEMB medium for 30min at 37°C, followed by incubation with CT(E112D) for 45min at 37°C in the absence or presence of methyl- β -cyclodextrin. Cells were washed, fixed and stained for the Golgi matrix (GM130). Orthogonal cross-sections from 3D confocal images stacks (step size 0.25 μ m) are shown. Selected Golgi areas (stippled-line boxes) are displayed

using an inverted monochrome scale to aid visualization. Under conditions of cholesterol depletion, approximately 12-fold less CT reaches Golgi complex.

Figure s4. Expression of dominant mutants does not alter the morphology or the response of Y-1 cells to forskolin. Y-1 cells transiently expressing the indicated proteins for 24h, were incubated with HMEMB medium alone or supplemented with 10 μ M forskolin for 1.5h at 37°C. Cells expressing Eps15 Δ 95-295 or Dyn1K44A-HA were also incubated with Tf (blue) to verify the block in the clathrin pathway. Cells were washed, fixed and stained for the corresponding epitope tags. Representative confocal and phase-contrast images are displayed. Scale bar, 20 μ m.

Figure s5. Y-1 cell shape assay is not affected by overexpression of mutants of proteins involved in endocytosis nor by cross-linking of GM1 lipids. A) Dose-response analysis of forskolin-induced morphological changes show no difference in the response to this drug of transfected or untransfected cells, indicating that overexpression of the endocytic blockers does not alter the ability of cells to respond to increase cAMP levels. Assay was performed as described in material and methods. **B)** The CT-induced morphological change is due to increased production of cAMP and not to unspecific signaling by cross-linking GM1 lipids. Cells were incubated with high doses (30nM) of wild type CT (holotoxin; left), or with Alexa647-labelled CT B subunit (right) for 1.5h at 37°C. Representative confocal and phase-contrast images are displayed. Scale bar, 20 μ m.

Figure s6. Inhibition of caveolin-mediated endocytosis does not affect CT-induced toxicity. **A)** Y-1 cells transiently expressing the indicated caveolin1 mutants for 24h, were incubated with HMEMB medium alone or supplemented with 200pM CT for 1.5h at 37°C. Cells were washed, fixed and stained for myc epitope tag. Representative confocal (shown using an inverted monochrome scale) and phase-contrast images are shown. **B)** Y-1 cells were pre-incubated with HMEMB medium alone or supplemented with 100µM genistein for 1h at 37°C. Cells were incubated with HMEMB medium alone or supplemented with 10µM Forskolin, or 200pM CT for 1.5h at 37°C, and then washed and fixed. Representative phase-contrast images are shown. **C)** BSC1 and BSC1/GalT-EGFP cells were pre-treated with 100µM genistein for 1h at 37°C, followed by incubation with CT(E112D) for 45min at 37°C. Cells were washed, fixed and stained for the TGN (TGN46). Representative confocal are shown and selected TGN and Golgi (GalT-EGFP) areas (stippled-line boxes) are displayed using an inverted monochrome scale to aid visualization.

Figure s7. Inhibition of the clathrin, caveolin, or Arf6-mediated endocytic pathways does not importantly affect CT-induced toxicity. Wild type caveolin or dominant mutants (see also Fig.s6A), alone or in combination, were expressed in Y-1 cells and toxin function assessed by measurement of cell shape change. Representative fluorescence (displayed in inverted monochrome scale) and phase-contrast images of cells incubated with wild type CT are shown. Expression of these proteins did not alter the normal morphology of Y-1 cells or

their ability to change upon increased cAMP (Fig.s4 in Supplement), where cells were incubated with medium with or without forskolin. Cells transfected with Eps15 Δ 95-295 or Dyn1K44A were also incubated with Tf to verify the block in the clathrin pathway. For some conditions cells were stained with antibodies specific for HA or myc epitope tags to identify transfected cells. Scale bar, 20 μ m.

Figure s8. Combined inhibition of dynamin and Arf6-dependent pathways strongly inhibits CT entry in Y-1 cells.

Representative confocal images from middle sections of Y-1 cells transiently expressing EGFP-Eps15 Δ 95-295, Dyn1K44A-HA, or co-expressing Dyn2K44A and Arf6Q67L are displayed using an inverted monochrome scale to aid visualization. The block in Tf uptake assessed the inhibition of clathrin-mediated endocytosis (data not shown). Although combined inhibition of clathrin- and caveolin-dependent pathways slightly inhibits CT entry, only cells inhibition of dynamin and Arf6-dependent pathways strongly inhibits CT entry. Transfected cells were identified by imaging EGFP or by staining for the HA epitopes fused to the corresponding overexpressed proteins. In the case of co-expression of Arf6-HA mutants with DynK44A, we used Dyn2K44A-EGFP. Scale bars, 20 μ m.

Movie 1. CT traffics in vesicles and tubules. BSC1 cells were incubated with 20nM CT(E112D) at 37°C for 45min, and confocal images were captured every ~3sec for a total of 5min.

Movie 2. Vesicles and tubules carrying CT move along microtubules. BSC1 cells transiently expressing tubulin-EGFP for 20h, were incubated with 20nM

CT(E112D) at 37°C for 45min, and images were captured every 2sec for a total of ~2min. Last section of movie shows a duplicate of only CT traffic to aid visualization of vesicles and tubules.

Movie 3. Caveolin structures mediate CT internalization. BSC1 cells transiently expressing caveolin1-EGFP for 24h were placed inside a chamber for live-cell imaging and stabilized at 37°C with HMEMB medium. Time-lapse recording started immediately before addition of 20nM CT(E112D), and images were captured every 30sec for a total of ~25min.

Movies 4a-b. CT-induced morphological change. Y-1 cells placed inside a chamber for live-cell imaging and stabilized at 37°C with HMEMB medium. Time-lapse recording started immediately before addition of wild type CT (movie 4a in Supplement) or 10 μ M forskolin (movie 4b in Supplement), and phase-contrast images were captured every 30sec for a total of ~70-80min. The morphological change induced by CT had a much longer lag-phase (~35min) compared to forskolin (~2min), consistent with the need for the toxin to traffic from the plasma membrane to the ER, where CT is retro-translocated into the cytosol and then induces toxicity.