Supplemental Material

Fluorescence microscopy

Cells were transfected with different DNA constructs 30 h prior to experiment using Fugene 6 (Roche), infected with Ad3-eGFP or Ad5-eGFP at 37° C for 60 min, washed and incubated at 37° C for 15 h. Cells were fixed and mounted with fluorescence mounting medium (DAKO, Carpinteria, CA). Confocal laser scanning microscopy was performed on a Leica-DM SP2 RXA2-TCS-AOBS microscope (Leica Microsystems, Wetzlar, Germany) equipped with an Ar-ArKr laser, a He-Ne 543-594 laser, a He-Ne 633 laser, a diode laser at 405 nm, and a 63 x oil immersion objective (N.A. 1.4 PL APO). The pinhole value was 1.0, airy 1, yielding optical sections of ~0.48 µm with a voxel of 0.233 by 0.233 by 0.48 µm. The zoom factor was 2. Image processing was performed with Leica and Photoshop software (Adobe), and fluorescence intensities determined using Image J (http://rsb.info.nih.gov/ij/) on cell total projections. For CtBP1 colocalization with dextran-positive endosomes cells were cold synchronized with Ad3-TR (2 µg/ml) for 60 min on ice, washed with warm RPMI-BSA and pulsed with 0.5 mg/ml of dextran-FITC at 37° C for 10 min. Cells were washed extensively with RPMI-BSA and PBS, fixed and analyzed by immunofluorescence using a CtBP1 mouse monoclonal antibody (BD Transduction laboratories) and a secondary Alexa647-conjugated goat anti-mouse antibody.

Transmission electron microscopy

Ad3 or Ad2 ts1 (30-50 µg/ml, moi 3000-5000) were cold bound for 60 min, unbound virus washed off and bound particles internalized as indicated. Cells

were fixed in 2% formaldehyde-1.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 (CaCo) overnight, and washed several times in CaCo, followed by postfixation in 1% OsO_4 (Electron Microscopy Sciences) and 1.5% potassium ferricyanide (FeK_3N_6) in double distilled water at 4°C for 60 min. Specimens were rinsed in 0.1 M sodium cacodylate, contrasted with 1% tannic acid in 0.05 M sodium cacodylate at room temperature for 45 min, washed in 1% sodium sulfate, rinsed in H_2O, stained in 2% uranylacetate in H_2O over night, and embedded in Epon as described previously (Meier et al., 2005). Virus particles were quantified at 50000 x magnification in ultrathin sections at the plasma membrane, endosomes and cytosol, and viewed in a transmission electron microscope (Zeiss EM 902A) at an acceleration voltage of 80,000 V. To distinguish endosomal from plasma membrne bound virus, surface staining of HeLa cells was performed with cationized ferritin (2.5 mg/ml, Sigma) on ice for 10 min.

BSA-gold

15 nm collodial gold was prepared by citrate reduction of $HAuCl_4$ (Horisberger and Rosset, 1977). To 20 ml of collodial gold solution (pH adjusted to 5.9) 50 µl of 10 mg/ml BSA (Sigma, fatty acid free) solution was added (De Roe et al., 1987). To stabilize the BSA-gold complex, 1ml of 1% PEG 20000 (Roth, Switzerland) were added, the sample centrifuged at 28'000 g for 60 min, and the pellet dissolved in 2 ml gold-buffer (sterile filtered PBS containing 0.2% PEG-20000) and stored at 4° C. BSA-gold internalization was performed after cold binding of Ad3 or Ad2-ts1 using a 1:1 dilution of BSA-gold with RPMI-BSA (approximately 0.1 mg/ml of BSA) at 37° C for 10 min.

Supplemental figures

Sup. 1: Ad3-TR nuclear targeting and Ad3-eGFP transduction are independent of amphiphysin.

A: Ad3-TR (0.5 µg/ml) was cold bound to HeLa cells transfected with D36R amphiphysin2 SH3 domain (24 h). This mutant protein binds very tightly to dynamin, and blocks transferrin uptake, while D38A or F89S amphiphysin2 SH3 domains which do not bind dynamin did not affect transferrin uptake (Owen et al., 1998). None of the amphiphysin constructs affected Ad3-TR targeting to the nucleus 150 min p.i.. measured by subcellular localization of fluorescent virus particles (Nakano and Greber, 2000). B: D36R potently blocked the uptake of transferrin labeled with texas red, as expected. C: Ad3-TR (0.5 µg/ml) was cold bound to HeLa cells transfected with EHdelta2 Eps15, which blocks transferrin uptake, or D3D2 Eps15 which does not affect transferrin uptake (Benmerah et al., 1999), and internalized for 150 min. D: EHdelta2 Eps15 but not D3D2 Eps15 blocked the uptake of fluorescent transferrin.

Sup. 2: Thin section EM analyses of incoming Ad3 in drug-treated cells.

HeLa-ATCC cells were treated with drugs in growth medium at 37°C for 30 min as indicated, incubated with Ad3 at high moi (30 µg/ml) in the cold for 60 min, washed and internalized in the presence of drugs for 30 min, fixed, stained and prepared for EM analyses. Virus particles in macropinosomes are indicated by arrow heads, viruses in small vesicles by small arrows, and viruses at the plasma membrane by large arrows. Bar = 1 µm, except for Jas (bar = 0.5 µm).

Sup. 3: Thin section EM analyses of incoming Ad3.

HeLa-ATCC cells were infected with Ad3 at high moi (30 µg/ml) for 30 min, fixed, stained and prepared for EM analyses. Virus particles in macropinosomes are indicated by arrow heads, and 2 particles in clathrin-coated vesicles are indicated by arrows. Bar = 1 µm.

Sup. 4: Ad3-eGFP infection depends on Rac1 but not Cdc42.

HeLa-ATCC cells were transfected with plasmids encoding CFP-Rac1, CFP-Rac1 T17N (panel A), or CFP-Cdc42 and CFP-Cdc42 T17N (panel B) for 30 h, infected with Ad3-eGFP for 15 h, fixed, and analyzed by confocal laser scanning microscopy. Transfected cells are pointed out by arrows. Bars = 10 µM.

Supplemental references

Benmerah, A., Bayrou, M., Cerf-Bensussan, N. and Dautry-Varsat, A. (1999) Inhibition of clathrin-coated pit assembly by an Eps15 mutant. *J Cell Sci*, 112, 1303-1311.

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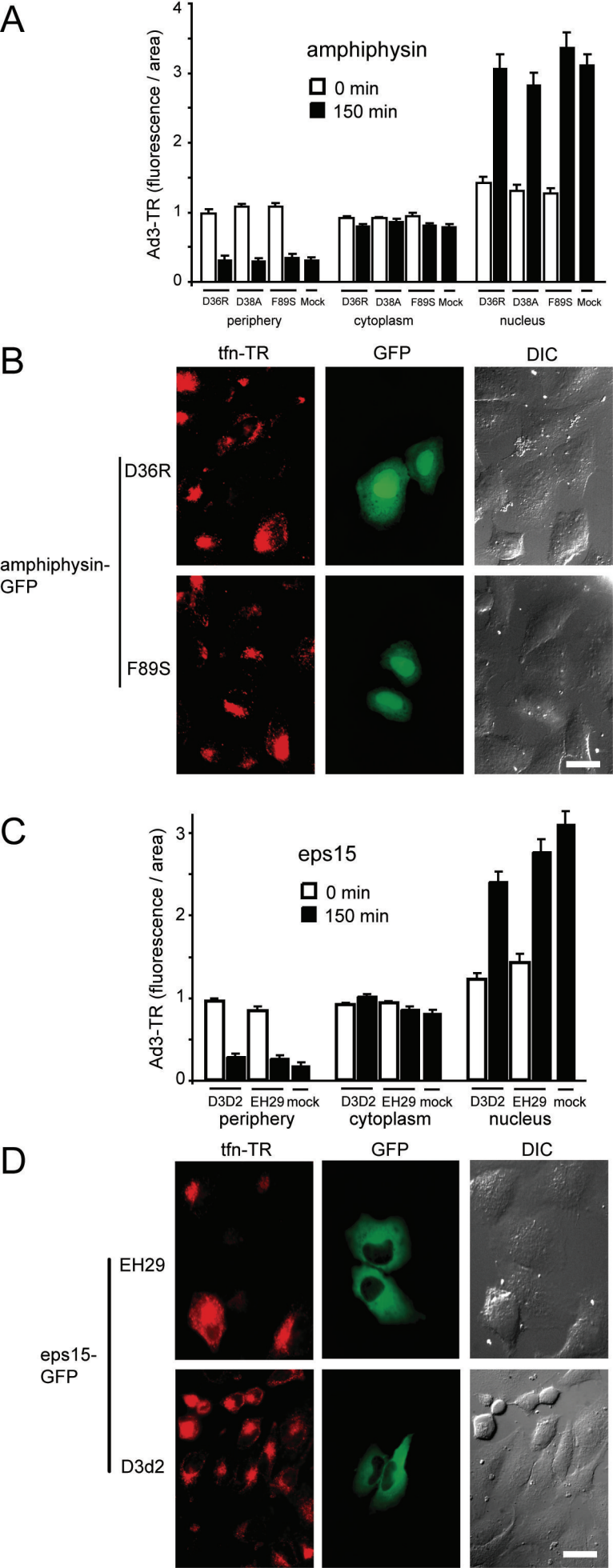
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0 min
150 min
Ad3-TR (fluorescence / area)
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1
2
3
4
D36R D38A F89S Mock
periphery
D36R D38A F89S Mock
cytoplasm
D36R D38A F89S Mock
nucleus
B
tfn-TR
GFP
DIC
amphiphysin-GFP
D36R
F89S
C
eps15
0 min
150 min
Ad3-TR (fluorescence / area)
0
1
2
3
D3D2 EH29 mock
periphery
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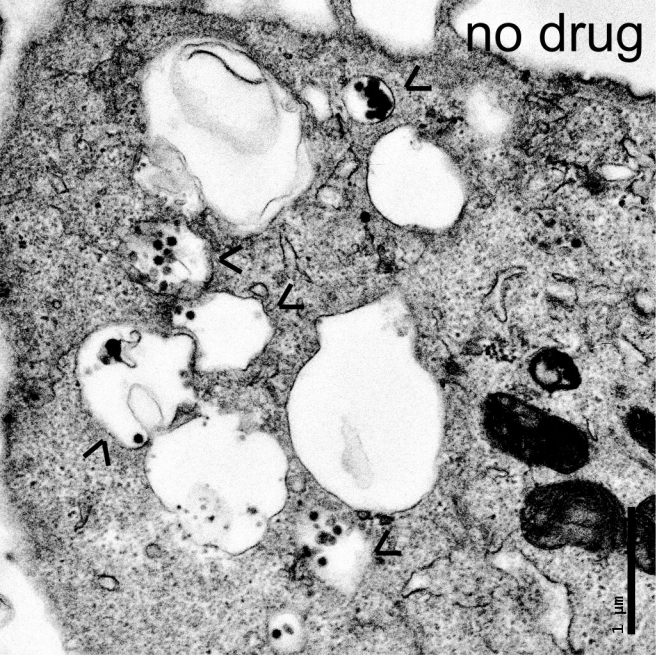
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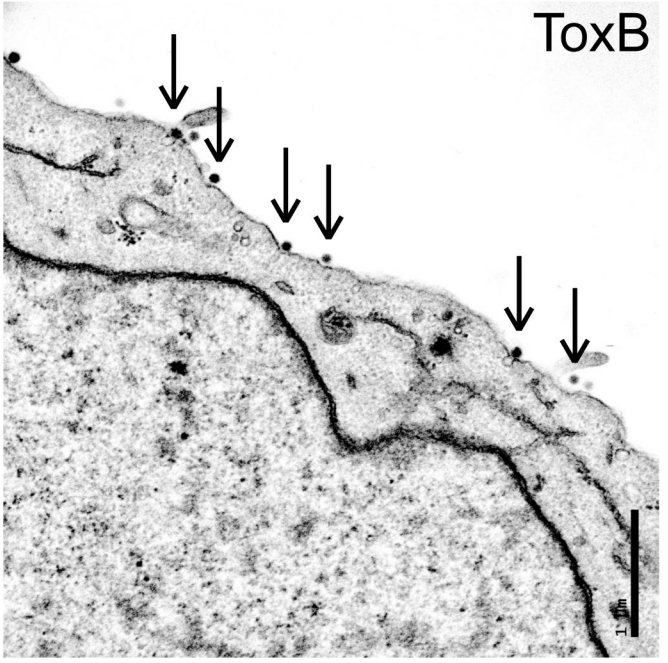
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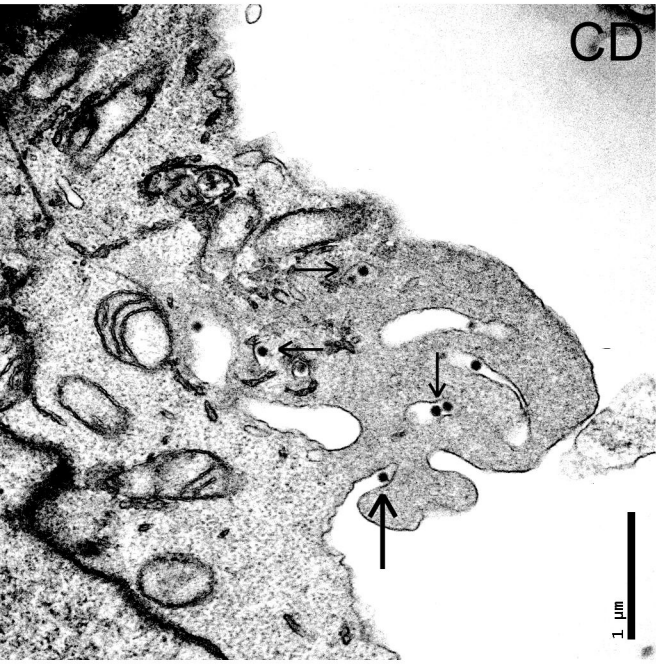
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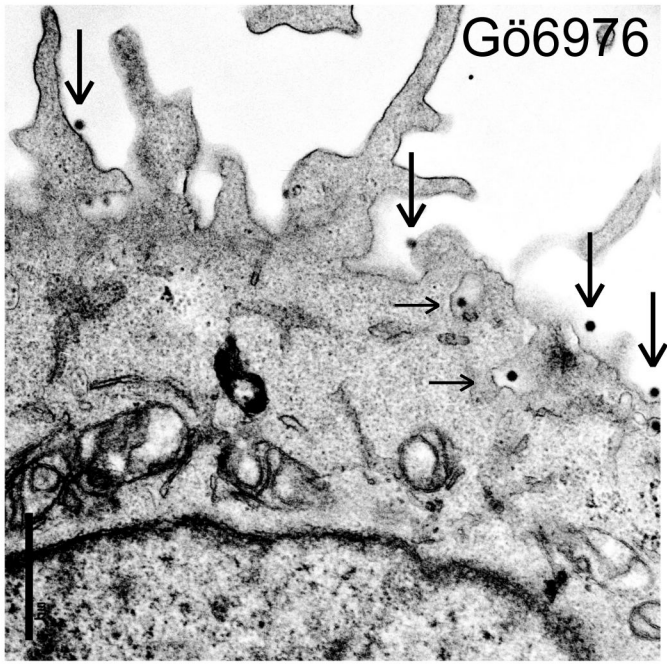
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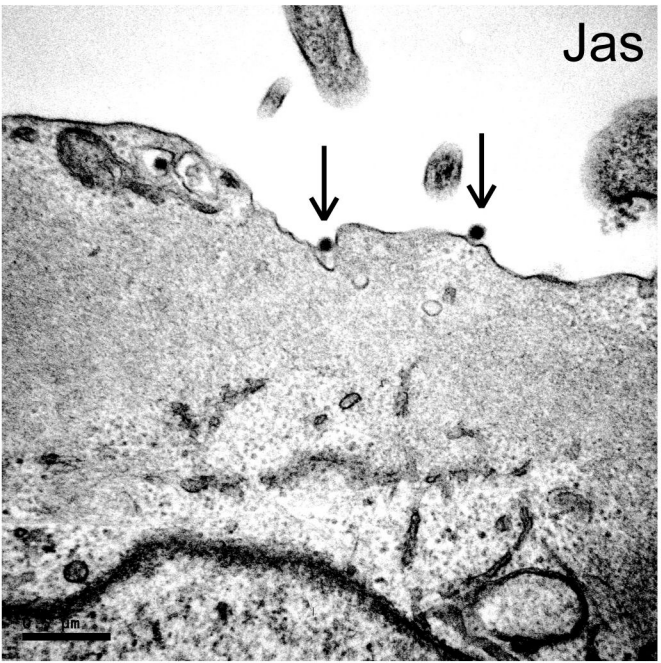
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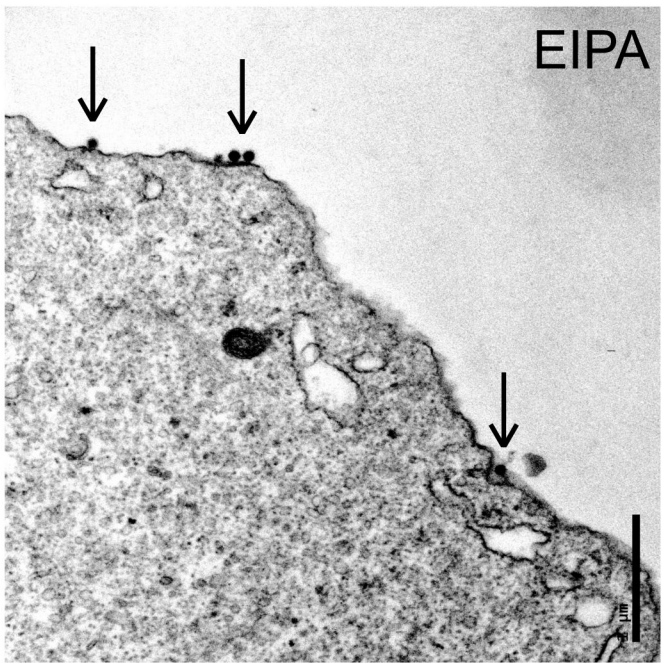
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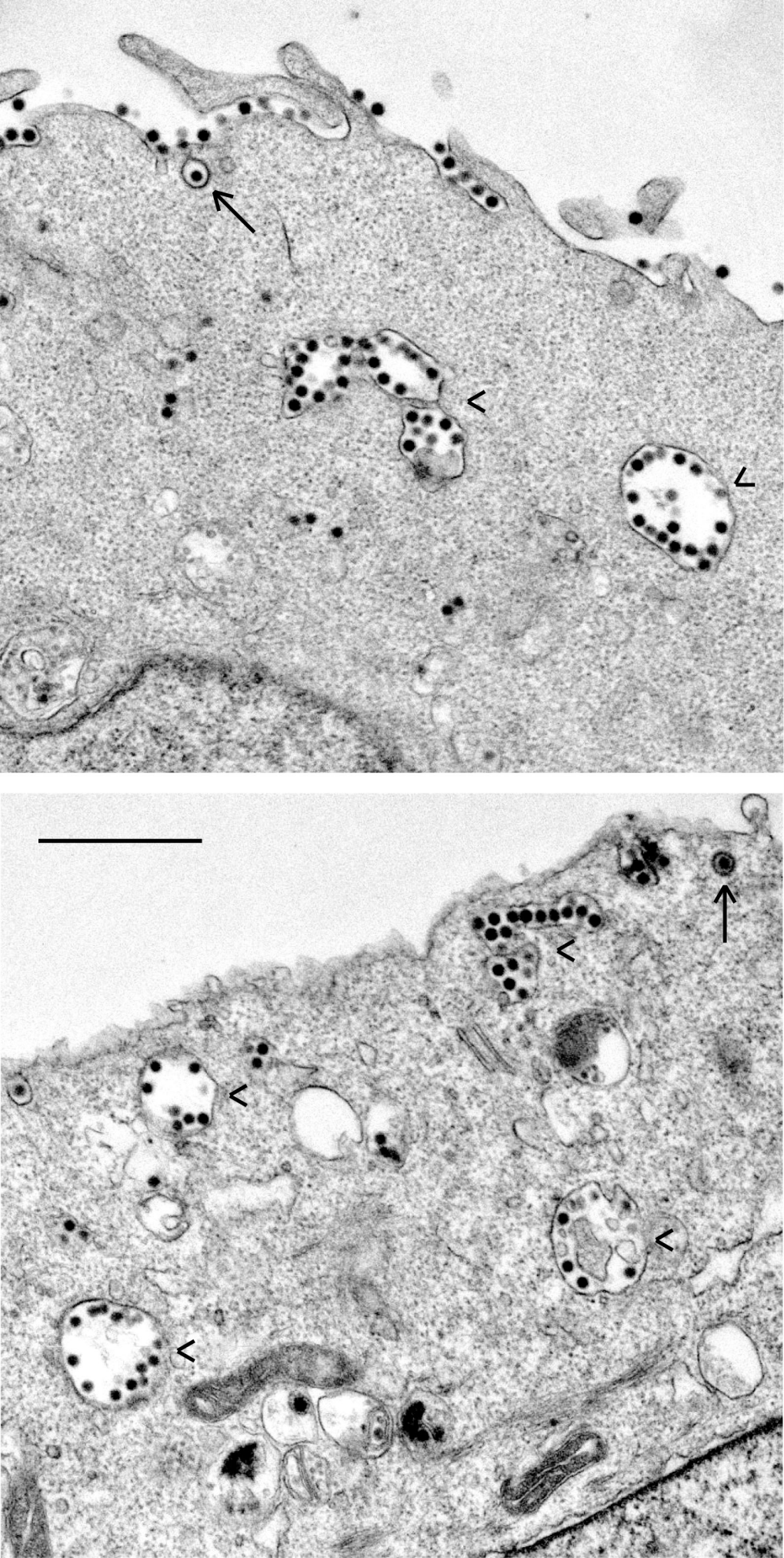

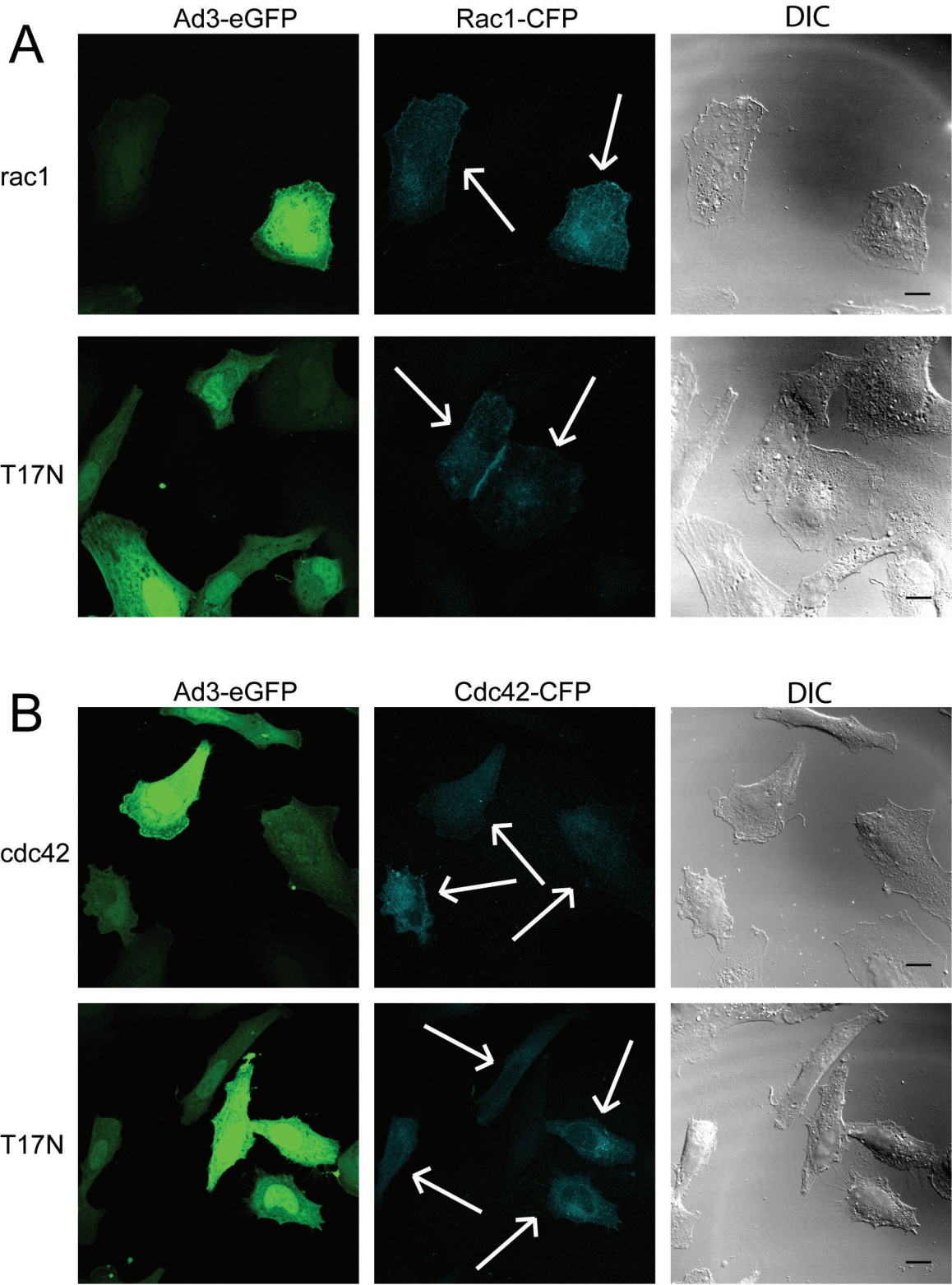
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Ad3-eGFP
Rac1-CFP
DIC
rac1
T17N
B
Ad3-eGFP
Cdc42-CFP
DIC
cdc42
T17N