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Guiding Plant Virus Particles to Integrin-Displaying Cells

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Supporting Information

Experimental Details

Abbreviations. PBS, phosphate buffered saline; FBS, fetal bovine serum; DMEM, Dulbecco's Complete Modified Eagle's Medium; IgG, Immunoglobulin G; HRP, horseradish peroxidase; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate; HCTU, 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate; DIEA, diisopropylethylamine; HOBt, 1-hydroxybenzotriazole; TIPS, triisopropylsilane; TFA, trifluoroacetic acid; NHS, *N*-hydroxysuccinimide ester.

Instrumentation. Optical spectra were collected on an Agilent 8453 diode-array spectrophotometer or a Perkin-Elmer Lambda 25 UV-visible spectrometer. Virus samples were analyzed by size-exclusion chromatography on an Amersham Pharmacia Biotech Äkta Explorer instrument (GE Healthcare), equipped with a Superose-6 column, using 40 μg particles in 500 μL 0.1 M potassium phosphate buffer, pH 7.0 for analytical purposes (flow rate 0.4 mL/min). Sucrose gradients were prepared on a Biocomp Gradient Master instrument. Sucrose gradient ultracentrifugation separation of virus samples was performed on 40 mL 10 – 40% w/v gradients at 28,000 rpm for 3 hr with a Beckman Optima L8-M Ultracentrifuge equipped with an SW28 rotor, and ultrapelleting of virus samples was carried out at 42,000 rpm for 2 h (BC and EF mutants) or 3 h (WT, L189C, and T184C) using a 50.2 Ti rotor. Gels were photographed using a FluorChemSP imaging system. Cells were imaged using a Bio-Rad (Zeiss) Radiance 2100 Rainbow laser scanning confocal microscope, equipped with a 60x oil immersion objective, and image analysis was performed with ImageJ (TreeStar) software. Protein sequencing of the BC and EF mutants by Edman degradation was performed at the TSRI Center for Protein Sciences. Flow cytometry was performed on a FACS Calibur instrument (BD Biosciences).

Peptides were analyzed on a Hewlett Packard Series 1100 reverse phase analytical HPLC (1.0 mL/min, 4.7 x 150 mm Zorbax 300SB-C18 5 μm particle size column), and purification of peptides was performed by preparatory HPLC on a Dynamax instrument model SD-1 (10 mL/min, 22 x 250 mm Vydac 218TP series C18 10 *μ*m particle size column). Routine mass spectra were obtained using an Agilent 1100 (G1946D) ESI MSD with the mobile phase composed of 9:1 MeCN/H_2O containing 0.1% trifluoroacetic acid, or on a Finnigan LCQ instrument interfaced with a Hewlett Packard HP1100 HPLC, eluting with 9:1 MeCN/H_2O containing 0.1% formic acid. MALDI-TOF analyses of peptides were performed on a PerSeptive Biosystems Voyager-DE instrument. NMR spectra were obtained on a Varian Mercury-200 spectrometer in methanol-d_4.

Antibodies. Polyclonal rabbit antibodies against Ad2 penton base (anti-Ad2 penton base) were isolated as previously described.[1] Rabbit anti-CPMV antibodies were purchased from ATCC, HRP-conjugated goat anti-rabbit IgG antibodies were obtained from Sigma, and Alexa Fluor 488-conjugated goat anti-rabbit antibodies was purchased from Invitrogen.

Reagents. All technical-grade solvents were distilled prior to use or purchased as anhydrous solvents. Reagents purchased from commercial suppliers were used without purifications. Trityl-chloride polystyrene (TCP) resin (1.05 mequiv. per gram, 1% divinylbenzene crosslinked) was purchased from

[1] Wickham, T.J.; Mathias, P.; Cheresh, D.A.; Nemerow, G.R. Integrins αvβ3 and αvβ5 promote adenovirus internalization but not virus attachment. *Cell* **1993**, *73*, 309-319.

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PepChem (Tübingen, Germany), and NMP for solid phase synthesis was a kind gift from BASF, Ludwigshafen, Germany. The N-methylated lysine was synthesized using an optimized Mitsunobu procedure.[2] Amino acids were obtained from NovaBiochem. Sulfonated bathophenanthroline was obtained from GFS. The compounds 5(6)-carboxyfluorescein, *N*-methoxycarbonyl maleimide, and bromoacetyl bromide were purchased from Aldrich. The nuclear stain ToPro-3 was obtained from Life Technologies. The complexes $[Cu(MeCN)_4](OTf)$,[3] 3-azidopropylamine,[4] and fluorescein alkyne **5**[5] were synthesized according to published procedures. CAUTION: Molecules such as azidopropylamine containing a high number of azides relative to saturated carbon and oxygen atoms should never be handled away from solvent.

Synthesis of cyclic peptides. *cyclo*(RGDf^{Me}K) and *cyclo*(RβADfK) were prepared on solid support using standard Fmoc chemistry.[6] Peptide couplings were performed on TCP resin with 2 equiv of amino acid, HOBt, and TBTU, and 5 equiv iPr_2NEt in *N*-methylpyrrolidinone (NMP).[7] Peptide coupling immediately following *N*-methylated amino acids required the use of HATU and HOAt (2 equiv each) along with 5 equiv iPr_2NEt in NMP. Fmoc cleavage was performed with 20% piperidine in DMF. After coupling of Fmoc-Lys(NCbz)-OH towards the arginine residue followed by Fmoc deprotection, the amino functionality was activated with *o*-NBS-Cl (3 equiv) and collidine (10 equiv) in NMP (15 min). *N*-methylation was performed under Mitsunobu conditions using PPh_3, DIAD and methanol in THF (10 min). Cleavage of *o*-NBS from the alkylated amine was carried out by the use of β-mercaptoethanol and DBU (2 x 5 min). HATU/HOAt and iPr_2NEt in NMP had to be used for coupling of Fmoc-D-Phe-OH towards ^{Me}Lys(NCBz)-OH. Treatment of the resin with 20% hexafluoroisopropanol (HFIP) in DCM for 15 min (3x) and removal of the solvent yielded the linear peptides.[8] Cyclization was performed in the presence of 1.5 equiv HATU/HOBt and 10 equiv of iPr_2NEt over 24 h with stirring. Following evaporation of the solvent, the crude cyclic peptide was purified by reversed-phase HPLC (C_{18} column). Finally, the side-chain protecting groups of ^{Me}Lys and Lys were removed by hydrogenation (Pd/C, MeOH) to give the cyclic peptides in 20% yield with respect to the resin loading. MS (ESI): *cyclo*(RGDf^{Me}K) (cpd. **D**) calcd. for $C_{45}H_{67}N_9O_{10}S$ 925.5; found 926.4 $[M+H^+]^+$; *cyclo*(RβADfK) (cpd. **E**) calcd. for $C_{45}H_{67}N_9O_{10}S$ 925.5; found 926.5 $[M+H^+]^+$.

Synthesis of propargyl-O-(CH_2CH_2O)$_{11}$-N_3 (Figure S1, compound **A**). The starting hydroxy-PEG-azide [$HO(CH_2CH_2O)_{11}N_3$, 0.9 mmol) (Polypure AS, Oslo, Norway) was added to NaH (50 mg, 20.8 mmol) in dry DMF (5 mL). The mixture was stirred for 5 min, followed by addition of propargyl bromide (0.1 mL, 1.1 mmol). The solution stirred for 12h at room temperature, and the solvent was then removed under reduced pressure. The crude product was purified via C_{18} flash chromatography (water/MeCN 10%-100% MeCN) to give **A** (320 mg, 0.5 mmol, 52%). ^{1}H NMR (200 MHz, CD_3OD) δ 4.19 (d, 2H, J= 2.4

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Hz), 3.64 (broad m, 48H), 2.87 (t, 1H, J= 2.4 Hz). MS (ESI) calcd. for $C_{27}H_{51}N_3O_{12}$ 609.3; found 610.3 $[M+H^+]^+$.

Synthesis of propargyl-O-(CH_2CH_2O)$_{11}$-NH_2 (Figure S1, compound **B**)**.** Compound **A** (320 mg, 0.50 mmol) in dry THF (15 mL) was treated with PPh_3 (1.5 g, 5.7 mmol) under a dry argon atmosphere. After stirring for 12h, water (15 mL) was added, the mixture stirred for an additional 15 min, and the solvent was removed under reduced pressure. The residue was taken up in ethyl acetate and an equal volume of water. The water layer was separated and concentrated under reduced pressure to give **(B)** in quantitative yield. ^1H NMR (200 MHz, CD_3OD) δ 4.20 (d, 2H, J= 2.4 Hz), 3.65 (br, complex m, 46H), 3.08 (m, 2H), 2.89 (t, 1H, J= 2.4 Hz). MS (ESI) calcd. for $C_{27}H_{53}NO_{12}$ 583.4; found 584.3 $[M+H^+]^+$.

Synthesis of propargyl-O-(CH_2CH_2O)$_{11}$-amino-4-oxo-butanoic acid (Figure S1, compound **C**). To a solution of 10 mL dry MeCN and 100 mg (0.2 mmol) of **B**, 250 mg (2.5 mmol) succinic anhydride and 0.5 mL (2.9 mmol) *i*Pr_2NEt were added. The solution was allowed to stir for 10 h. The solvent was removed under reduced pressure and the crude product purified via C_{18} flash column chromatography (water/MeCN 0%-60% MeCN) to give 78 mg (0.1 mmol, 57%) of **(3)**. ^1H NMR (200 MHz, CD_3OD) δ 4.19 (d, 2H, J= 2.4 Hz), 3.65 (br, complex m, 46H), 3.08 (m, 2H), 2.89 (t, 1H, J= 2.4 Hz), 2.53 (m, 4H). MS (ESI) calcd. for $C_{31}H_{57}NO_{15}$ 683.4; found 684.3 $[M+H^+]^+$.

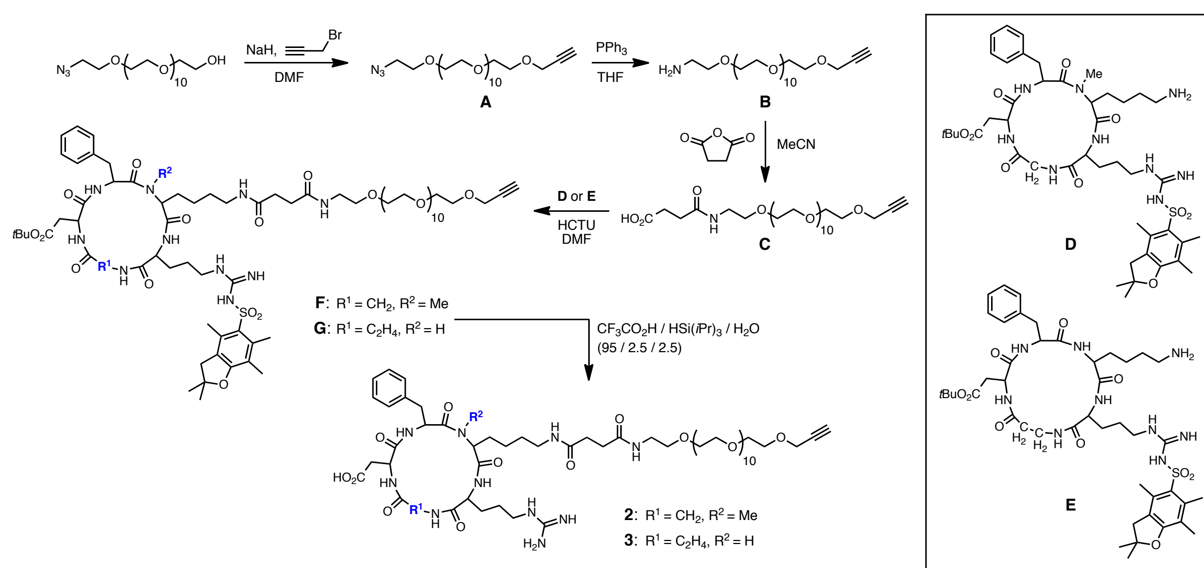

Figure S1. Synthesis scheme of RGD and RβAD peptides with a short PEG_{11} spacer.

Synthesis of *cyclo*(-D-Phe-^{Me}Lys(propargyl-O-(CH_2CH_2O)$_{11}$-N^1,N^4 succinic amide)-Arg(Pbf)-Gly-Asp(OtBu)) (F) and *cyclo*(-D-Phe-Lys(propargyl-O-(CH_2CH_2O)$_{11}$-N^1,N^4 succinic amide)-Arg(Pbf)-βAla-Asp(OtBu)) (G). 5 mg (0.005 mmol) of cyclic peptide (**D** or **E**) and 5.6 mg (0.008 mmol) **C** were dissolved in 5 mL DMF. After addition of 2.5 mg (0.006 mmol) HCTU and 10 μL (0.06 mmol) *i*Pr_2NEt the solution was allowed to stir for 16h. The solvent was removed under reduced pressure and the crude product used without further purification.

Synthesis of *cyclo*(-D-Phe-^{Me}Lys(propargyl-O-(CH_2CH_2O)$_{11}$-carbamoyl-N^1,N^4 succinic amide)-Arg-Gly-Asp) (2) and (-D-Phe-Lys(propargyl-O-(CH_2CH_2O)$_{11}$-carbamoyl-N^1,N^4 succinic amide)-Arg-βAla-Asp) (3). The crude products **F** and **G** were each dissolved in 5 mL TFA/TIPS/water (95/2.5/2.5) and allowed to stir for 1 hour. The solution was concentrated under reduced pressure and purified via

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reversed-phase HPLC (C18 column) to give **2** and **3**, respectively, as colorless oils, each in 70% yield. For **2**, reversed-phase HPLC (water/MeCN 0%-95% MeCN over 20 min, 1 mL/min): retention time = 8.9 min. MS (ESI) calcd. for $C_{59}H_{98}N_{10}O_{21}$ 1282.7; found 642.4 = ½[M+2H$^+$]. For **3**, reversed-phase HPLC retention time = 10.2 min. MS (ESI) calcd. for $C_{59}H_{98}N_{10}O_{21}$ 1282.7; found 1283.7 [M+H$^+$]$^+$.

Synthesis of Alexa Fluor 568 cadaverine pent-4-ynamide (8). 1 mg AlexaFluor 568 cadaverine diammonium salt (1.23 mmol) was dissvoled in 0.5 mL DMSO. After addition of 25 eq. (30.8 mmol) of 2,5-dioxopyyrolidin-1-yl pent-4-ynoate (6 mg) and *i*Pr_2NEt (4 mg, 5.26 µL) the solution was stirred in the dark at room temperature for 16 h. Afterwards, the solution was diluted by addition of 1 mL water and purified by reversed-phase HPLC on a standard C_{18} column.

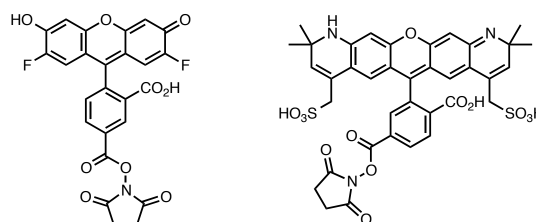

Figure S2. Structures of Oregon Green 488 (left) and AlexaFluor 568 (right) NHS esters. The structure of AlexaFluor 555 has not been released by Invitrogen.

Gel Electrophoresis. 10 µg of CPMV conjugates in PBS (in loading dye, MBI Fermentas) were analyzed on 1.2 % (w/v) agarose gels in 1x TBE buffer, with 0.5x TBE running buffer. After completion of the electrophoretic separation, the particles were visualized under UV light or stained using Coomassie Blue.

Cell Adhesion Assay. Protein samples in PBS (100 µL, 0.1 µg/well) were immobilized overnight on 96-well poly-sorb plates (Nunc) at room temperature, in triplicate. The coating solution was removed and wells were blocked with BSA in PBS (1%, 200 µL/well) for 1 h. The wells were then washed for 5 min with PBS. Meanwhile, cells were detached from growth flasks using 20 mM EDTA at 37 °C and resuspended in serum-free media. Cells were added to a set of wells as 100 µL suspensions containing 200,000 cells, and serial dilutions with serum-free media were used to dilute the number of cells for each immobilized protein sample. The multi-well plates were incubated at 37 °C for 1 h, followed by careful removal of the cell suspension. The wells were washed with serum-free media to remove nonadherent cells. Attached cells were stained with crystal violet (0.1% in saline, 20% MeOH, 100 µL/well) for 5 min, and then rinsed twice with H_2O (200 µL/well). Cell-bound stain was resolubilized in EtOH (200 µL/well), and 100 µL from each well was transferred to a fresh 96-well plate. Absorbance measurements at 595 nm were recorded using a microplate reader.

Confocal microscopy – HeLa cells. Approximately 1 x 10^5 HeLa cells were plated in glass bottom dishes (MatTek) and allowed to adhere overnight at 37°C in a humidified 5% CO_2/95% air atmosphere. CPMV particle solutions were prepared in complete growth media directly before addition to the cells. Cells were rinsed once with PBS before CPMV solutions were added to a final concentration of 9 or 18 nM (3.4 and 6.8 x 10^7 particles/cell). After incubation for 1, 3, or 6 h at 37°C, the cells were washed with PBS at room temperature. The cells were then fixed with 2% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 10 min. Staining of endolysosomes and CPMV particles was carried out sequentially, and all antibody dilutions were made in 1% of the serum used for blocking. The first blocking step was performed with 10% goat serum (Omega Scientific) for 1 h, before incubation with anti-human CD107a (Lamp-1) primary antibody (BioLegend) overnight at 4°C. The following morning, staining was performed with goat anti-mouse AlexaFluor 488 conjugated secondary antibody (Cell Signaling Technologies). The second blocking step was done in 10% donkey serum (Jackson ImmunoResearch), and CPMV particles were stained with rabbit polyclonal anti-CPMV antibody, followed by DyLight 549 conjugated donkey anti-rabbit secondary antibody (Biolegend). Nuclei were stained with DAPI (Biotium, Inc.), cell membranes

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were stained with AlexaFluor 555 conjugated wheat-germ agglutinin (Invitrogen) and glass cover slips were mounted over cells with ImmunO-Fluore mounting media (MP Biomedicals).

Confocal microscopy – SW480 cells. Approximately 50,000 SW480 cells/well were plated on circular glass cover slips in a 24-well tissue culture plate. Following overnight incubation, solutions of CPMV (3.8×10^{12} particles, 38 μg) were added directly to the media and kept at 37 °C for 2 h. The following manipulations were performed at r.t. The cells were first washed 3 x 5 min with PBS, and then fixed with 4% paraformaldehyde for 20 min. The cells were again washed 3 x 5 min with PBS, permeabilized with 0.2% Triton-X for 5 min, followed by washing 3 x 5 min with PBS. Next, the cells were treated with blocking buffer (10% FBS in PBS) for 30 min. The cells were exposed to anti-CPMV antibodies (1:2000) diluted in blocking buffer for 1 h, and then washed 3 x 5 min with PBS. Cells were subsequently treated with Alexa Fluor 488-conjugated goat anti-rabbit antibodies (1:2000) in blocking buffer for 1 h, then with ToPro-3 (1:10,000) for 5 min, and finally washed 3 x 5 min with PBS. The cover slips were mounted on glass slides and examined with a Bio-Rad (Zeiss) Radiance 2100 Rainbow laser scanning confocal microscope in the core microscopy facility at TSRI.

Flow Cytometry. Figure S3 shows the flow cytometry data represented in Figure 6, in the form of histograms. The data show selective binding of the RGD coated particles to cells in comparison to uncoated particles and RβAD coated particles.

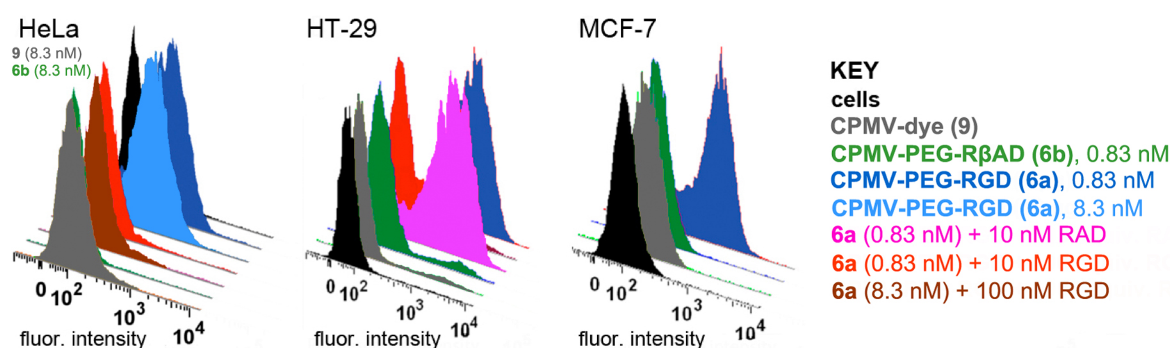
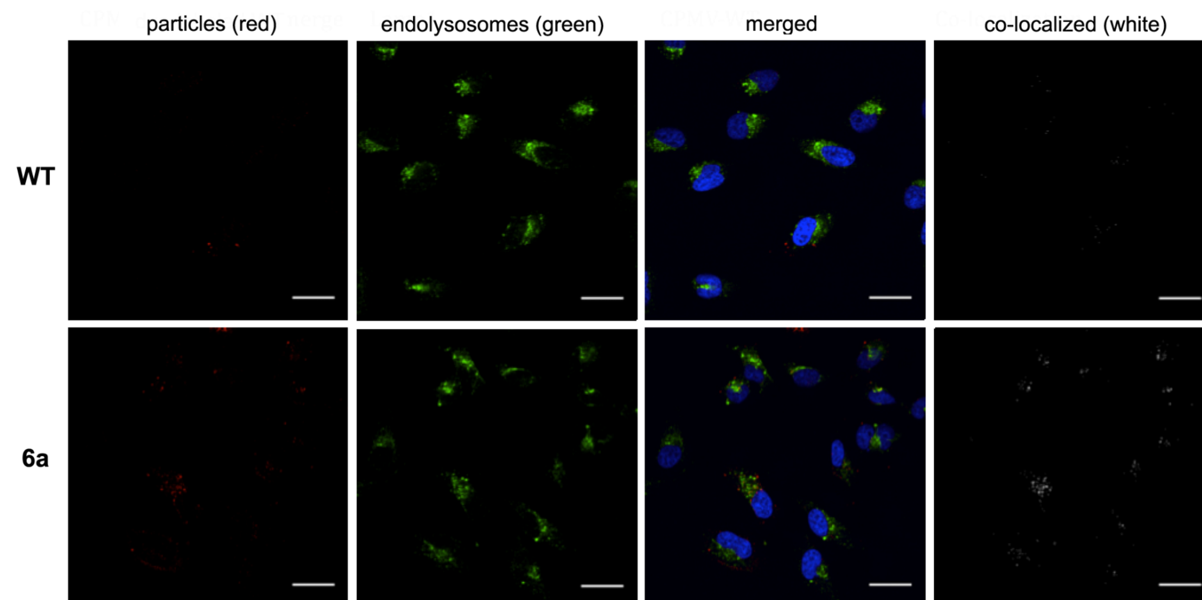


Figure S3. Histogram representation of flow cytometry data shown in Figure 6. Added peptides: "RGD" = *cyclo*(RGDf^{Me}K); "RAD" = *cyclo*(RβADfK).

Confocal Microscopy. Figure S3 shows additional data of the type shown in Figure 8, after shorter periods of incubation.

1 hour incubation



3 hour incubation

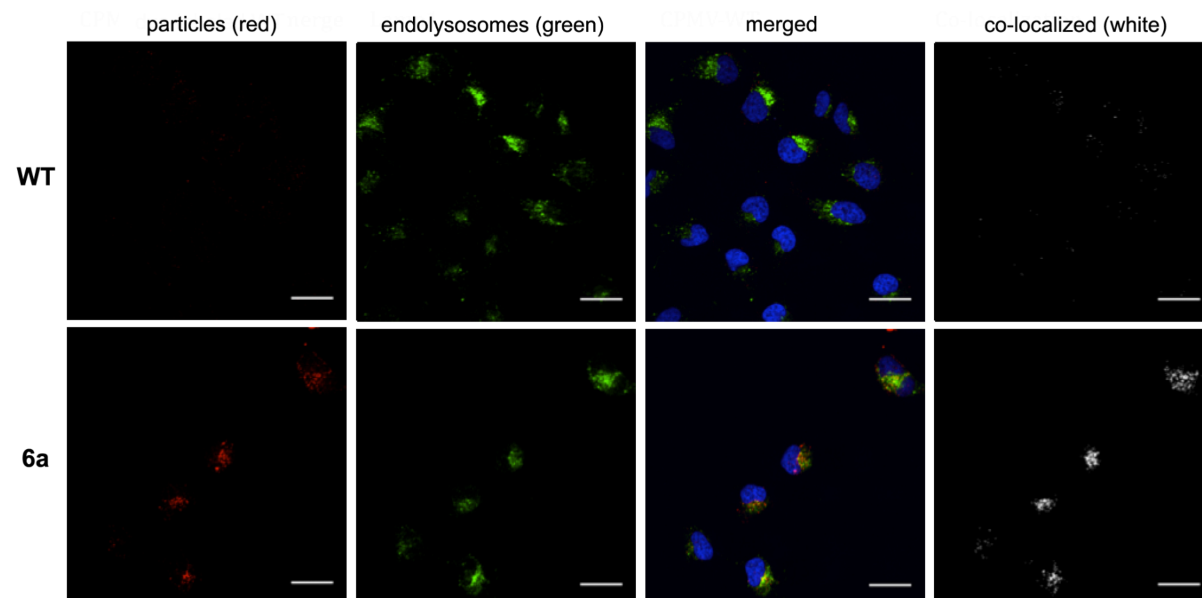


Figure S3. Confocal microscopy of HeLa cells after 1 h (*top*) and 3 h (*bottom*) incubation with the indicated particles (50 µg/mL or 9 nM) at 37°C, followed by washing, cell fixation, and staining with the following reagents: red = dye-labeled anti-Qβ antibody, blue = cell nuclei stain (DAPI), green = endolysosome stain (anti-LAMP1 antibody). Co-localization of particles and endolysosomes was determined using ImageJ software (white). Scale bars = 30 µm.