An artificial amphiphilic peptide promotes endocytic uptake by inducing membrane curvature

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Materials and Methods

Materials

All reagents including salts and incubation media were obtained from Sigma-Aldrich and Wako unless otherwise specified. Monoclonal anti-CHC (1:2000 for immunoblotting; 610500, BD bioscience), monoclonal anti-Cav1 (1:10000 for immunoblotting; EPR15554, Abcam), monoclonal anti-Flot1 (1:10000 for immunoblotting; EPR6041, Abcam), monoclonal anti-Rac1 (1:1000 for immunoblotting; 23A8, Merck) and monoclonal anti-β-actin (1:10000 for immunoblotting; AC-74, Sigma Aldrich) were purchased. An Amp-YFP expression vector was kindly provided by Dr. Milos Galic (Institute of Medical Physics and Biophysics of the University Hospital and the University of Münster, Germany). CellMask™ Deep Red Plasma membrane Stain was obtained from Thermo Fisher Scientific.

Peptides

Peptides were synthesized using Fmoc-solid-phase synthesis on a Rink amide resin, as reported previously. The peptides were then deprotected and cleaved from the resin by treatment with a trifluoroacetic acid (TFA)–ethanedithiol mixture (95:5) at room temperature (~20°C) for 3 h, followed by lyophilization. Purification of the desired peptides on reverse-phase high-performance liquid chromatography (RP-HPLC) followed by lyophilization of the eluent yielded the pure peptides. Purity and identity of the respective peptides were confirmed by RP-HPLC and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOFMS) (Supporting Information Table S1).

Cell culture

HeLa cells obtained from the European Collection of Authenticated Cell Cultures (ECACC), were cultured in α-minimum essential medium (α-MEM) supplemented with 10% (v/v) heat-inactivated bovine serum (BS) (α-MEM(+)). Cells were maintained at 37°C in a humidified 5% CO_2 incubator.

Transfection and RNAi

Plasmid transfection was carried out using Lipofectamine LTX Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Transfected cells were examined after 24 h. For CHC knockdown experiments, MISSION siRNAs

(Sigma-Aldrich) against human CLTC (siCHC,SASI_Hs02_00338467; siCHC-2,SASI_Hs01_00208103) were used. MISSION siRNA Universal Negative Controls (Sigma-Aldrich) was used as control siRNA. siRNAs (SASI_Hs02_00338467, 10 nM; SASI_Hs01_00208103, 20 nM) were transfected into cells with Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. For Ctr, Cav1, Flot1 and Rac1 knockdown experiments, siRNAs synthesized by Nippon gene (sequences are listed in Table S3, 10 nM) were transfected into cells with Lipofectamine RNAiMax (Thermo Fisher Scientific) according to the manufacturer's instructions. Universal negative control siRNA (Nippon gene) was used as control siRNA. Cells were then cultured for 48 h and used for analysis. We confirmed the knockdown of CHC by RT-PCR and western blotting and knockdown of Cav1, Flot1, Rac1 by western blotting.

Microscopy observation

For dextran cellular uptake observation, HeLa cells were seeded into 35 mm glass-bottom dishes (Iwaki), and the cells were allowed to reach 80–90% confluence in 24 h. These cells were washed with serum-free α-minimum essential medium (α-MEM(–)), and then incubated with 100 μg/mL dextran (10 kDa) labelled with Alexa Fluor 594 (Dex10-AF594) (Thermo Fisher Scientific) in the presence of a peptide (20 μM) in α-MEM(–) for 30 min at 37°C. Cells were washed with α-MEM(–) and the cellular uptake of dextran was analyzed in live cells using an FV1000 Olympus confocal laser scanning microscope.

For observation of Amp-YFP distribution, HeLa cells were plated onto 35 mm glass-bottomed dishes (Iwaki) and transfected with Amp-YFP expression vector using Lipofectamine LTX according to the manufacturer's instructions. One day after transfection, the culture medium was changed to α-MEM(–) (volume, 150 μL). The cells were placed at 37°C in a microchamber (MI-IBC, Olympus) attached on the stage of the inverted microscope equipped with a UPlanSApo 60× oil objective lens, NA 1.35 (Olympus). The peptides dissolved in α-MEM(–) (volume, 50 μL) was added to yield final peptide concentration of 20 μM, and images were acquired every 1 min using a confocal microscope (FV1000, Olympus). The number of YFP-Amp puncta signals in each cell was analyzed and quantified using Fiji/ImageJ (NIH, Bethesda, MD, USA). TIRF microscopy was performed using a TIRF microscope (IX81; Olympus) equipped with an EM-CCD camera (Ixon3; Andor), a CMOS camera (ORCA Flash4.0LT; Hamamatsu), a UAPON 100 × 1.49 NA (Olympus), and MetaMorph software.

Flow Cytometric Analysis

Cells were seeded in a 24-well microplate (Iwaki) and cultured at 37°C in a humidified 5% CO_2 atmosphere up to 80−90% confluence. Then, the cells were washed with each medium and treated with fresh medium (200 μL) containing Dex10-AF594 (50 μg/mL) or EGF-pHrodo (0.2/μg/mL) and peptides. After washing with PBS(−), the cells were treated with 0.01% trypsin for 5 min at 37 °C. The cells were collected, washed with PBS(−), and subjected to flow cytometry analysis using Attune NxT (Thermo Fisher Scientific).

For ATP depletion experiments, cellular energy was depleted by incubating HeLa cells in PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (PBS(+)) supplemented with 10 mM 2-deoxy-D-glucose and 10 mM NaN_3 for 15-25 min at 37°C. Then, the cells were treated with PBS(+) supplemented with 10 mM 2-deoxy-D-glucose and 10 mM NaN_3 (200 μl) containing Dex10-AF594 (50 μg/mL) and R6W3 (20 μM). Following treatment is same as mentioned above.

For the 4°C experiment, the cells were preincubated at 4°C for 30 min. The cells were then washed with cold α-MEM(−) and incubated with Dex10-AF594 (50 μg/mL) in the presence of peptide in α-MEM(−) (200 μL) at 4°C for 30 min. After incubation, the cells were washed twice with cold PBS(−). Following treatment is same as mentioned above.

Quantitative RT-PCR

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen), 24 hours post-transfection. cDNA was synthesized from total RNA using a PrimeScript RT reagent Kit (Takara), and real-time PCR was performed with 7300 Real-Time PCR System (Thermo Fisher Scientific) with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). The primer sequences are listed in Supporting Information Table S2.

Cell viability assay

Cell viability was determined using Cell Counting Kit-8 (CCK8) (Dojindo), following the manufacturer's protocol. Briefly, HeLa cells were treated with peptides at various concentrations for 30 min in α-MEM(-). CCK8 was then added, and the cells were incubated for another 2 h. No peptide represents the viability of the cells similarly incubated in α-MEM(-) without containing peptides.

Transmission Electron Microscopy (TEM).

Cells were seeded on a coverglass in 24-well microplate (Iwaki) and cultured at 37 °C in a humidified 5% CO_2 atmosphere up to 90−100% confluence. They were washed twice with PBS(−) and then incubated with R6W3 (20 μM) in α-MEM(−) (200 μL) for 5 min at 37 °C. The cells were fixed with 2.5% glutaraldehyde in 0.1 M HEPES at pH 7.4 containing 1mM $CaCl_2$ for overnight. After washed with 0.1 M HEPES at pH 7.4 containing 1mM $CaCl_2$, cells were postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) containing 0.1% potassium ferrocyanide and 1 mM $CaCl_2$ for 1 h, dehydrated in a graded series of ethanol, and embedded in epoxy resin. Ultrathin sections were electrostained with lead citrate and observed under a JEM1011 electron microscope (JEOL, Japan) at 100 kV.

Table S1. Reverse-phase high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses of the peptides.

Peptide	R_t (min)[a)]	m/z (obtained)	MS (calcd)[b)]
R6W3	10.79	1513.061	1512.860
Inverse-R6W3	10.91	1513.032	1512.860
R6DW3	10.08	1512.925	1512.860

a) Retention time (R_t) by HPLC (column: Cosmosil $_5C_{18}$-AR-Ⅱ [4.6 × 150 mm]); gradient: 10–90% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH) over 40 min; flow: 1 mL/min; detection: 220 nm. b) Calculated monoisotopic mass for $(M+H)^+$.

Table S2. Sequences of the primers used for quantitative RT-PCR.

Protein Name	Forward Primer	Reverse Primer
Clathrin Heacy Chain 1	5'-TGAAGTTGGCACACCACCTA-3'	5'-TGGCTTCATGAGGTGCAGTA-3'

Table S3. Sequences of the siRNAs.

siRNA Name	Sense	Antisense
si Cav1	5’-CCUUCACUGUGACGAAAUACUGGtt-3'	5'-AACCAGUAUUUCGUCACAGUGAAGGUG-3'
si Flot1	5'-GGUGAAUCACAAGCCUUUGAGAAca-3'	5'-UGUUCUCAAAGGCUUGUGAUUCACCUG-3'
si Rac1	5'-GGAACUAAACUUGAUCUUAGGGAtg-3'	5'-CAUCCCUAAGAUCAAGUUUAGUUCCCA-3'

siRNAs had a single 2-base 3′-overhang on the antisense strand and a blunt end modified with DNA bases (shown in lower case).

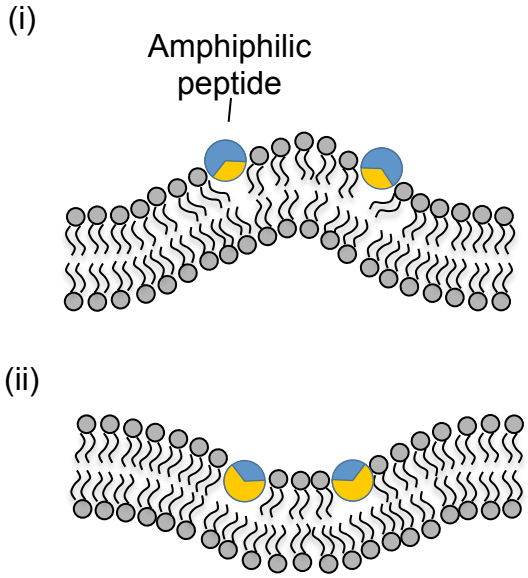

Figure S1. Mechanistic models of amphiphilic peptides-induced membrane curvature. (i) Amphiphilic peptides insertion/wedging induces positive membrane curvature. (ii) Deeper insertion of amphiphilic peptide induces negative curvature.

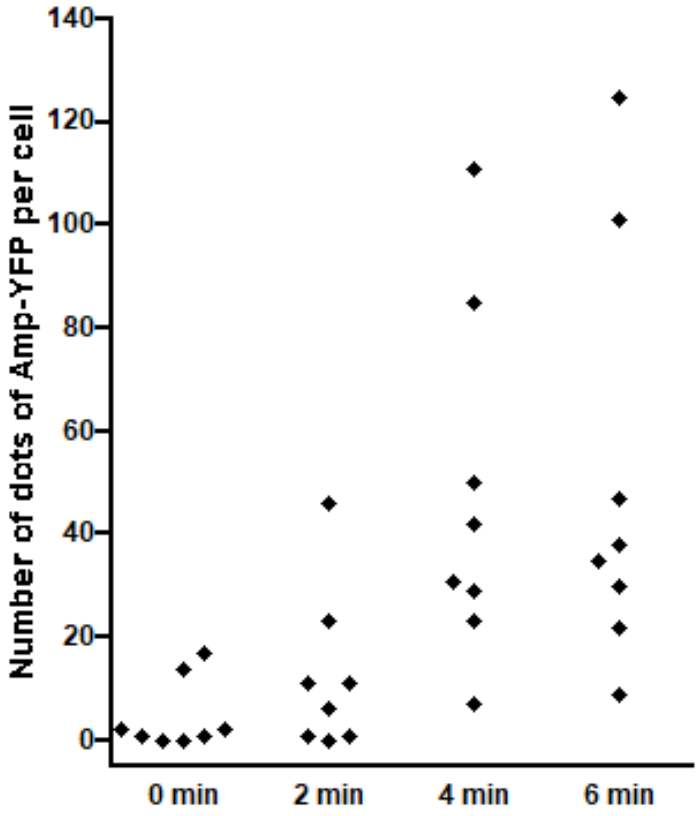

Figure S2. The number of Amp-YFP dot signals per cell in Figure 2A was analyzed. The data were derived from 8 cells.

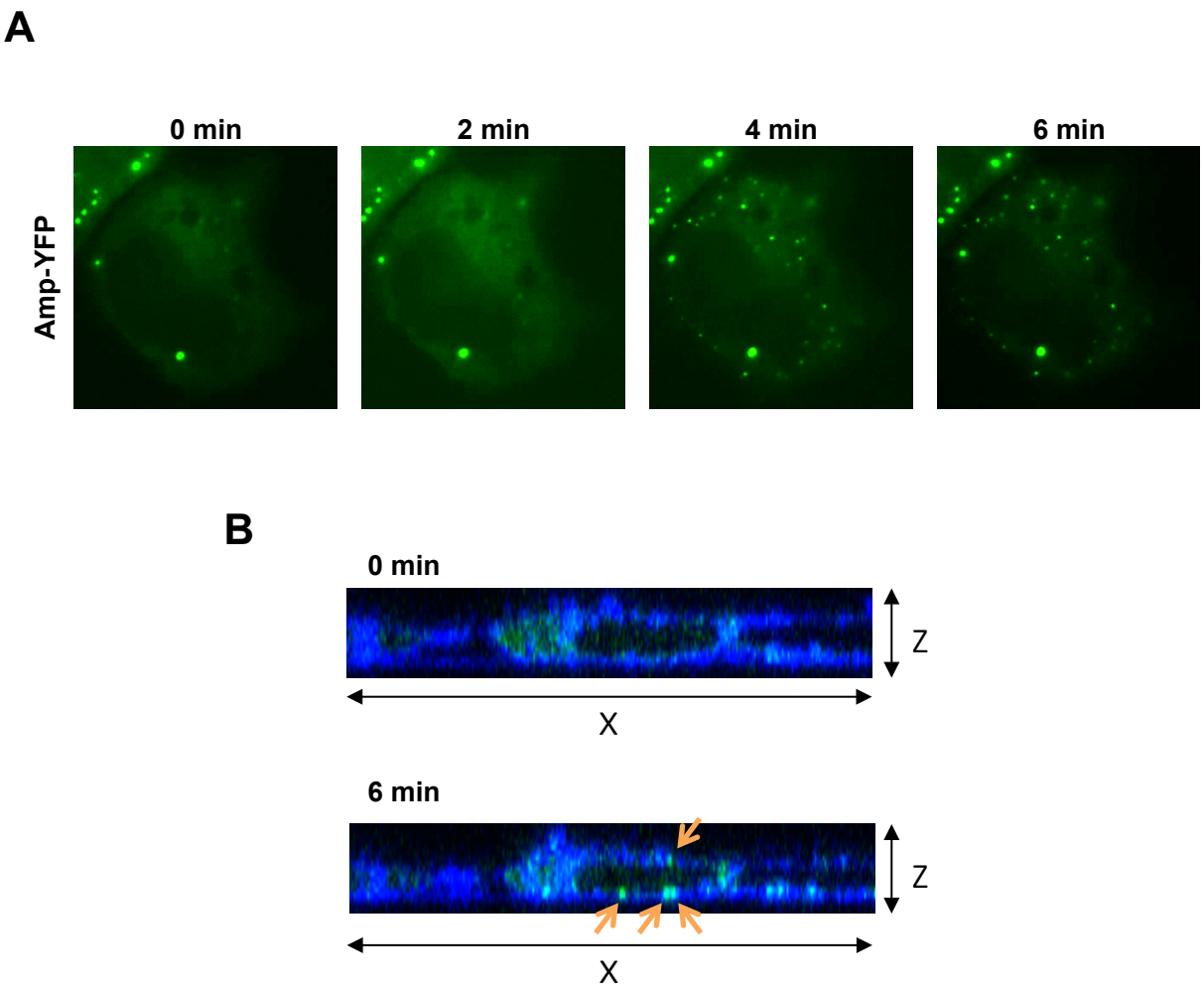

Figure S3. (A) Time-lapse TIRF images of HeLa cells expressing Amp-YFP after the addition of R6W3 (20 μM). Time 0 (zero) denotes immediately after peptide addition. (B) Orthogonal view of z-stack images of Amp-YFP (green) and CellMask (blue) after R6W3 (20 μM) addition. Time 0 (zero) denotes immediately after peptide addition. Arrows indicate Amp-YFP signals.

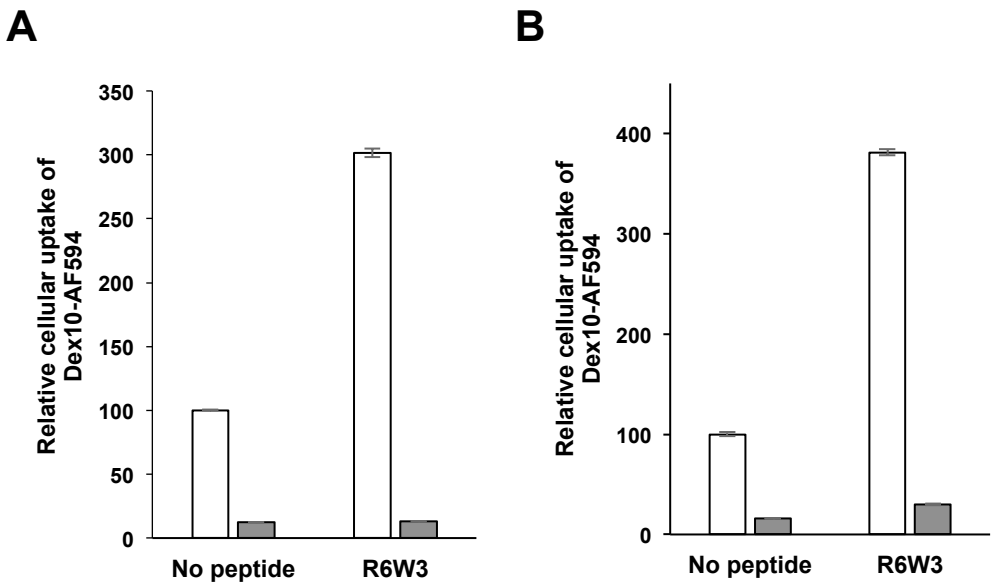

Figure S4. Uptake of Dex10-AF594 in HeLa cells treated with R6W3 for 30 min under ATP-depleted conditions (A) and at 4 °C (B), analyzed by flow cytometry. Open and filled columns in (A) represent without and with ATP depletion, and those in (B) 37°C and 4°C, respectively. The results are the means ± SEM (n = 3).

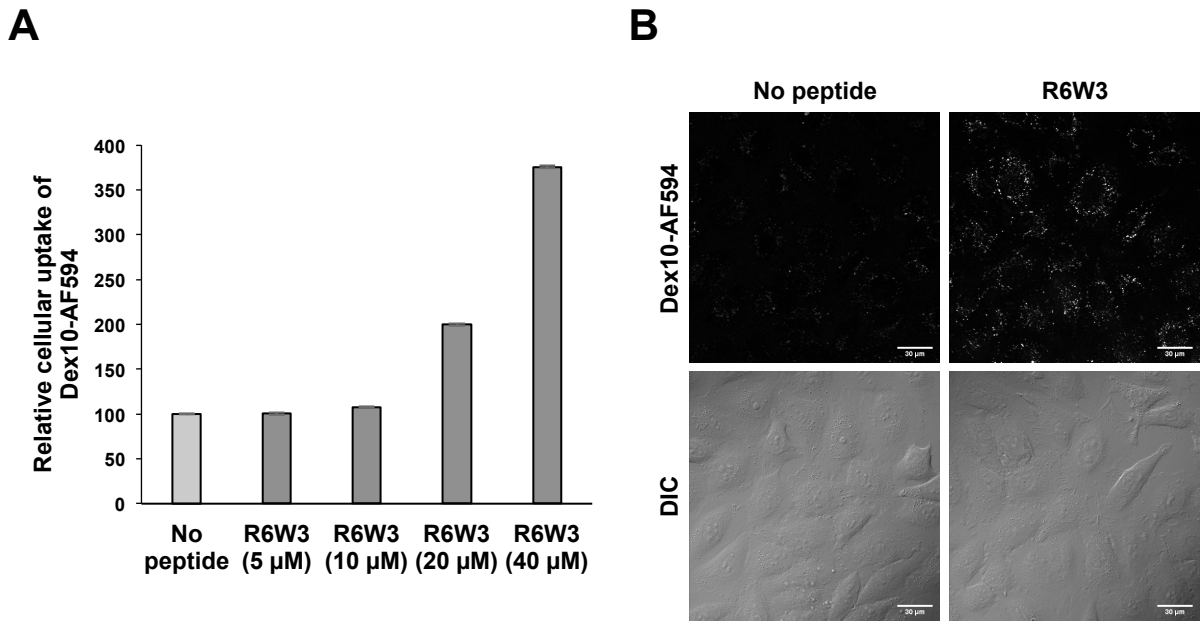

Figure S5. R6W3 increased dextran uptake in the presence of serum. (A) Uptake of Dex10-AF594 in HeLa cells treated with R6W3 for 30 min in the presence of serum, analyzed by flow cytometry. The results are the means ± SEM (n = 3). (B) Confocal laser scanning micrographs of Dex10-AF594 in HeLa cells treated with R6W3 (40 μM) for 30 min in the presence of serum. Scale bars, 30 μm.

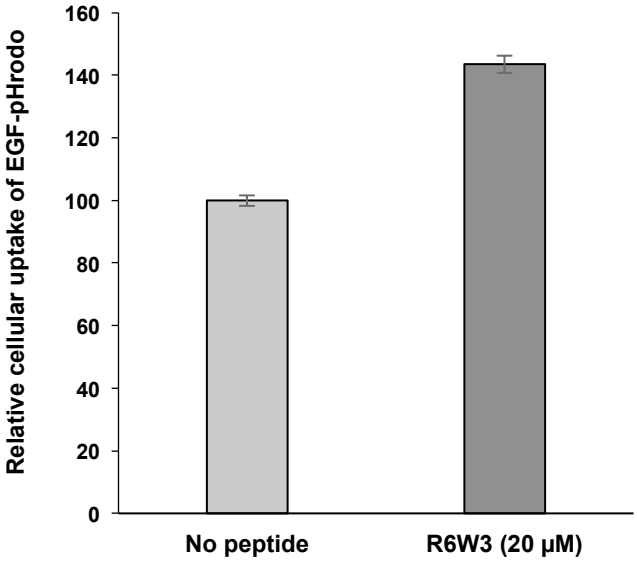

Figure S6. Uptake of EGF-pHrodo in HeLa cells treated with R6W3 in α-MEM(–) for 30 min, analyzed by flow cytometry. The results are the means ± SEM (n = 3).

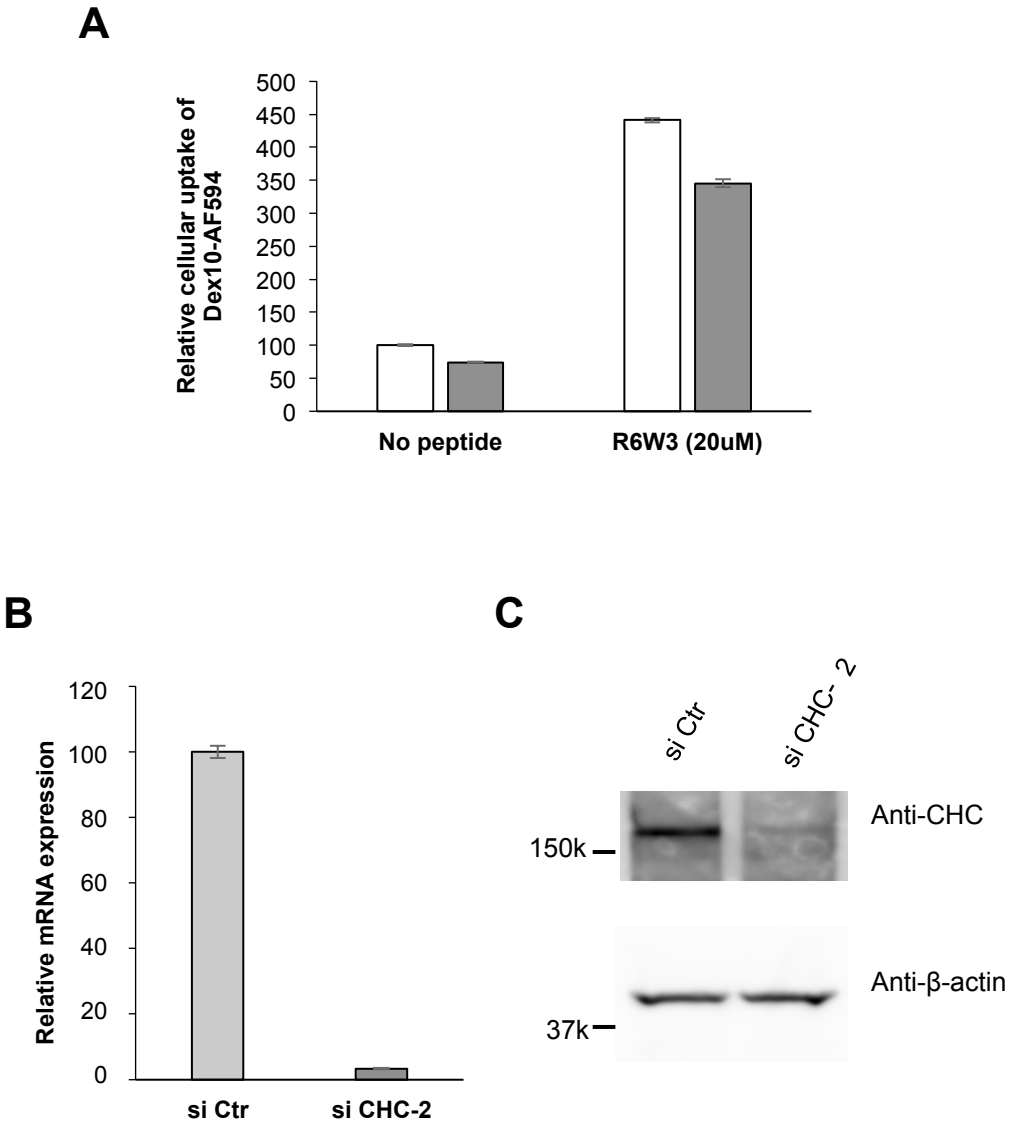

Figure S7. Possible involvement of clathrin heavy chain (CHC) in R6W3-stimulated promotion of Dex10-AF594 uptke. (A) Uptake of Dex10-AF594 in siRNA-treated HeLa cells with R6W3 treatment for 30 min, analyzed by flow cytometry. Open and filled bars represent relative cellular uptake of Dex10-AF594 by siCtr (control) and siCHC-2 treated cells, respectively. The results are the means ± SEM (n = 3). The CHC mRNA (B) and protein (C) levels in HeLa cells transfected with CHC siRNA (siCHC-2, 20 nM) were analyzed by RT-PCR and Western blotting using a specific antibody against CHC, respectively.

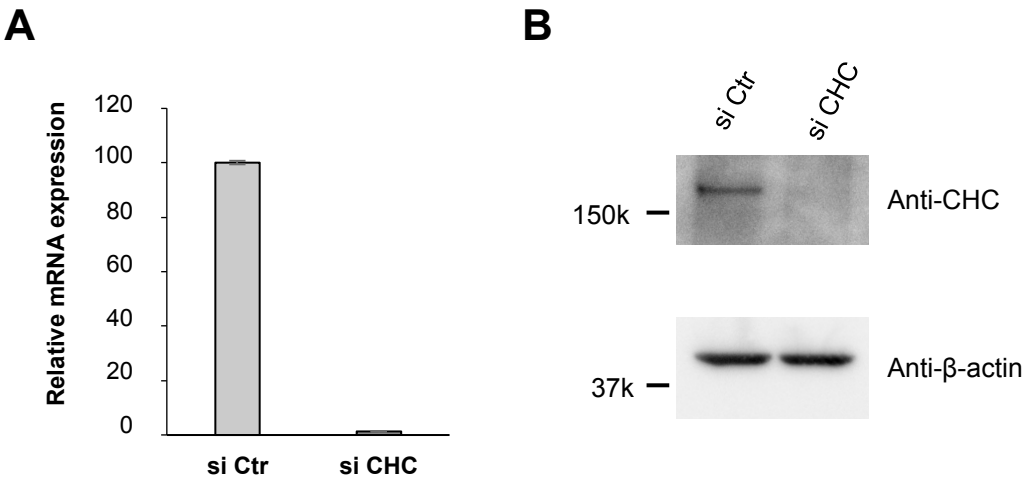

Figure S8. Confirmation of the effect of the siRNA on CHC expression. The CHC mRNA (A) and protein (B) levels in HeLa cells transfected with CHC siRNA (10 nM) were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blotting using a specific antibody against CHC, respectively.

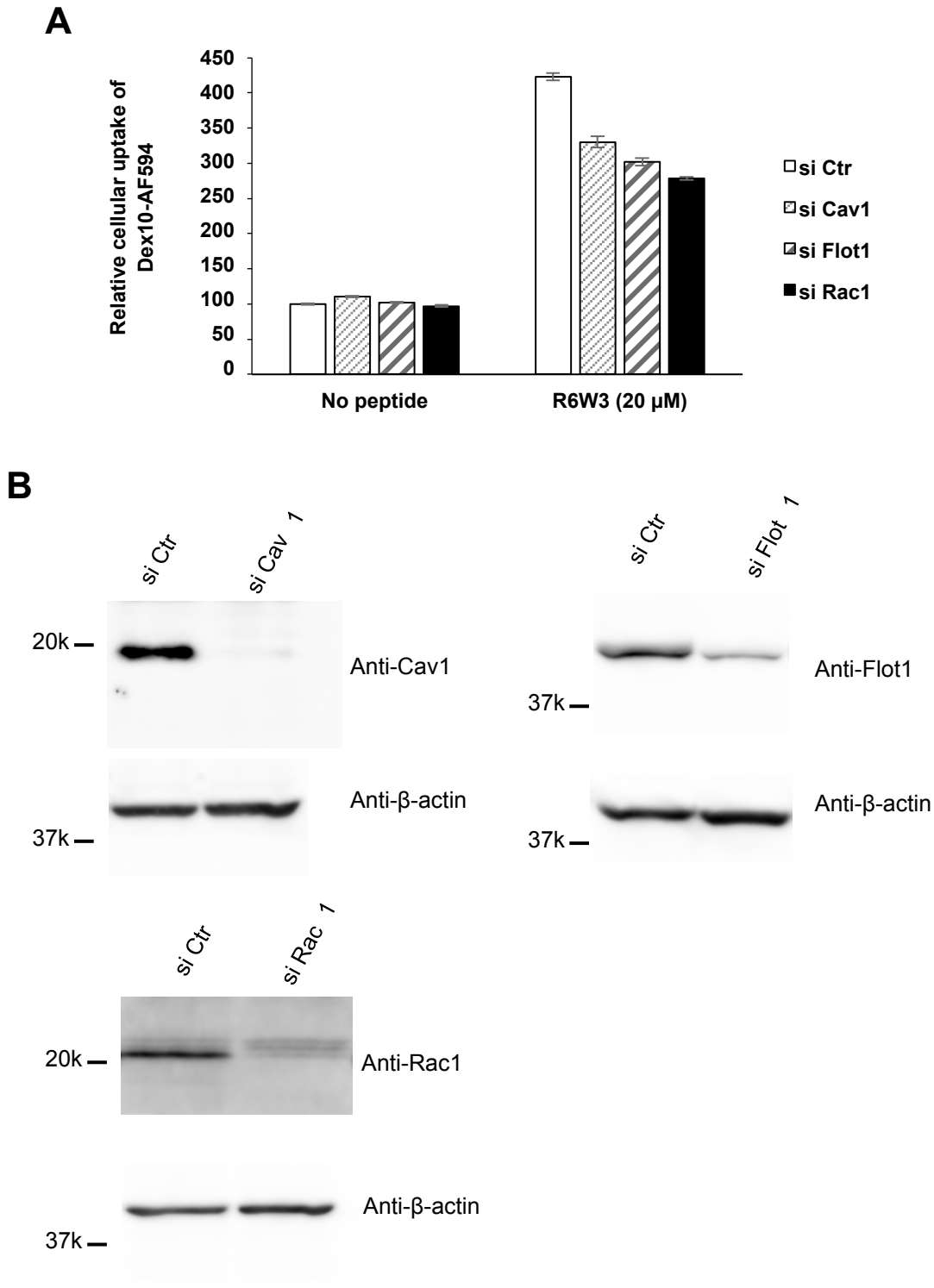

Figure S9. (A) Uptake of Dex10-AF594 in siRNA-treated HeLa cells with R6W3 treatment for 30 min, analyzed by flow cytometry. The results are the means ± SEM (n = 3). (B) The Cav1, Flot1, and Rac1 protein levels in HeLa cells transfected with corresponding siRNA or control (Ctr) siRNA (10 nM) were analyzed by Western blotting using a specific antibodies, respectively.

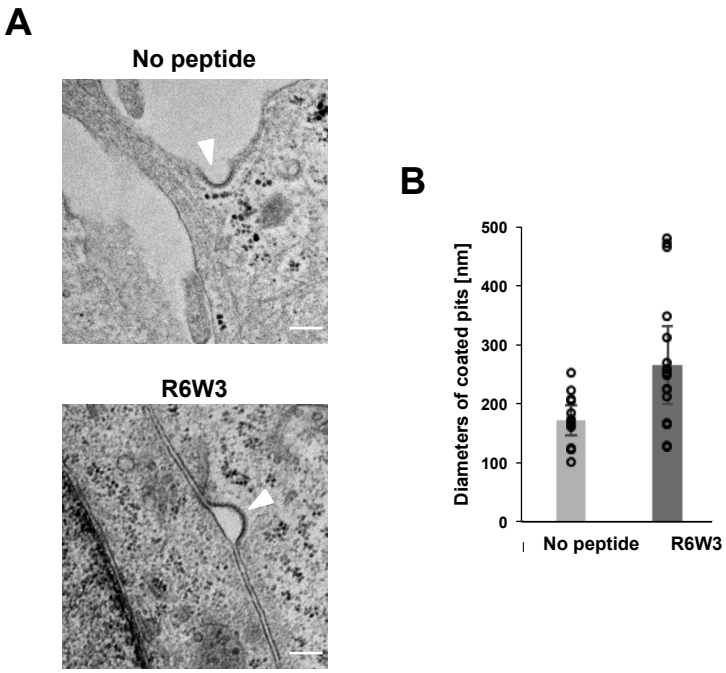

Figure S10. (A) TEM images of representative coated pits (white arrowheads) in HeLa cells treated with R6W3 (20 μM) in α-MEM(–) for 5 min. Scale bars, 200 nm. (B) Quantitative data of (C). The diameter of n = 14 (no peptide) and n = 17 (R6W3) coated pits was analyzed.

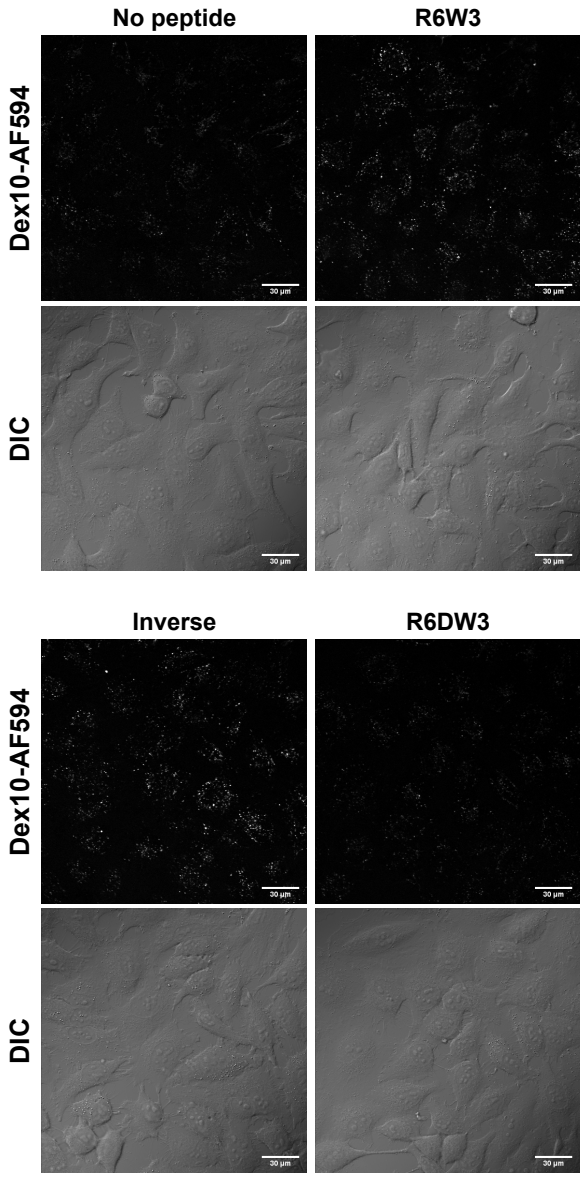

Figure S11. Confocal laser scanning micrographs of Dex10-AF594 in HeLa cells treated with R6W3-derivatives (20 μM) in serum-free medium for 30 min. Scale bars, 30 μm.

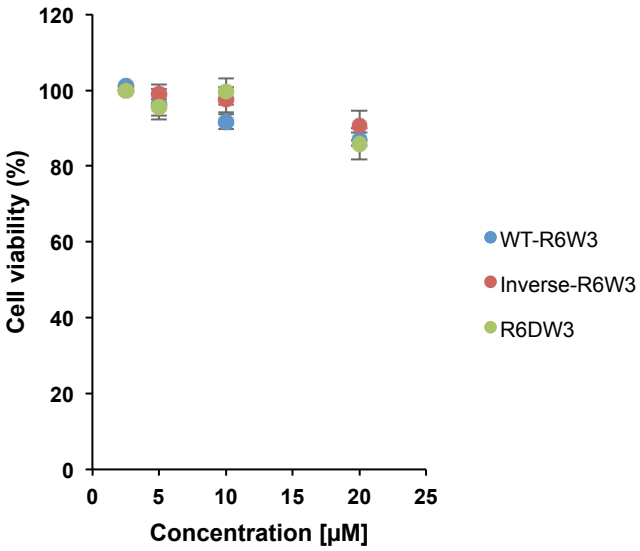

Figure S12. Evaluation of the cytotoxic effects of the R6W3 and R6W3 derivatives. HeLa cells were treated with the peptides for 30 min and then subjected to the cell viability assay. Results are presented as means ± SEM (n = 3).