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Supporting Information

SI Text

Cell Culture. BS-C-1 cells, an African green monkey kidney epithelial cell line, from the American Type Cell Collection (ATCC), were maintained in a 5% CO2 environment at 37°C in EMEM medium (ATCC) and supplemented with 10% (vol/vol) FBS (HyClone), 100 international units/ml penicillin, and 100 μg/ml streptomycin (ATCC). For fluorescence imaging, BS-C-1 cells were cultured in the same medium in 50-mm glass-bottom culture dishes (MatTek). Before experiments, cells were washed with F-MEM (serum-free, phenol red-free MEM (Invitrogen), fortified with 1% glucose (wt/vol) and 100 mM Hepes, pH 8.0), and then kept in this medium for experiments.

Influenza Virus Labeling. Influenza virus X-31 was purchased from Charles River Laboratories and labeled with lipophilic dye DiD (Invitrogen). For labeling influenza virus, 100 μl of the original virus stock was incubated with 2 μl of 25 mM DiD dissolved in DMSO at room temperature for 2 h with gentle vortexing. Unincorporated dye was removed by buffer exchange into the Hepes 145 buffer (50 mM Hepes, pH 7.4, 145 mM NaCl) by using NAP-5 gel filtration columns (GE Healthcare). The labeled virus was aliquoted, snap-frozen with liquid nitrogen, and stored at −70°C. Immediately before experiments, the labeled virus was thawed and filtered through a 0.2 μm pore size syringe filter (Supor membrane, Pall) to remove viral aggregates.

Antibodies and Immunofluorescence. Primary antibodies against the clathrin light chain (C1985, Sigma-Aldrich), clathrin heavy chain (Ab21679, Abcam), α-adaptn (MA1-064, Affinity BioReagents), µ2-adaptin (611350, BD Transduction Laboratories), epsin 1 (sc-8673, Santa Cruz Biotechnology), epsin 2 (provided by P. De Camilli), Eps15 (sc-534, Santa Cruz Biotechnology), Eps15R (1857–1, Epitomics), influenza nucleoprotein (NP) (Ab20343, Abcam), and α-tubulin (Ab7750, Abcam), and secondary antibodies (Alexa Fluor 488 goat anti-mouse, Alexa Fluor 555 donkey anti-mouse, tetramethylrhodamine (TMR) goat anti-mouse, Alexa Fluor 633 donkey anti-goat, Alexa Fluor 633 donkey anti-mouse, and TMR goat anti-rabbit, all from Invitrogen) were used in immunofluorescence and Western blot analyses.

For immunofluorescence imaging, cells were fixed in 2% (vol/vol) formaldehyde in PBS at room temperature for 20 min, after which cells were washed with PBS and permeabilized in blocking buffer (PBS containing 10% (vol/vol) FBS, 3% (wt/vol) BSA, and 0.5% (vol/vol) Triton X-100) at room temperature for 15 min. Cells were then incubated with primary antibodies against clathrin light chain, clathrin heavy chain, epsin 1, α-adaptin, or influenza NP in blocking buffer either at room temperature for 2 h or at 4°C overnight. Cells were washed with wash buffer (PBS containing 0.2% BSA (wt/vol) and 0.1% (vol/vol) Triton X-100) and incubated with secondary antibodies diluted in blocking buffer at room temperature for 30 min. Unbound antibodies were removed by washing with wash buffer before imaging. In experiments to determine the colocalization between transferrin and clathrin, the cells were permeabilized in blocking buffer at room temperature for 5 min and incubated with antibodies diluted in PBS containing only 0.2% BSA (wt/vol), which was also used to remove unbound antibodies.

Plasmids and Transfection. The original GFP-clathrin-LCa construct was a gift from J. H. Keen. The clathrin-LCa-EYFP and clathrin-LCa-ECFP plasmids were generated as described by using pEYFP-N1 and pECFP-N1 vectors (Clontech), respectively (1). The plasmid containing Venus cDNA was a gift from A. Miyawaki. The plasmid containing rat epsin 1 full cDNA was a gift from P. De Camilli. To generate epsin 1-Venus, the Venus cDNA was PCR-amplified and used to replace the EYFP gene in the pEYFP-N1 vector by using AgeI (5’') and NotI (3’) sites. The epsin 1 cDNA was PCR-amplified and ligated into the Venus vector by using HindIII (5'') and AgeI (3'') sites. Epsin1ΔUIM and epsin1U1M were similarly cloned into the Venus or ECFP vector.

We transiently transfected BS-C-1 cells with fluorescent protein constructs 24 h after cells were plated. A solution containing 1–2 μg of plasmids and 3–6 μl of FuGENE 6 (Roche) in 100 μl of EMEM was used for transfection. Experiments were conducted either 24 or 48 h after transfection.

Knockdown by siRNA. The siRNAs for epsin 1 (GGACCUUGCU-GAGCUUCU), epsin 2 (CCUUGAGCUUCUCAUGUA), and Eps15R (GAAGUUAUCCUGAGCAAU) were obtained from Dharmacon’s predesigned siGENOME collection. The siRNAs for the μ2 subunit of AP-2 (GUGGAUCCCU-UCCGGAUCAU, Dharmacon) and for Eps15 (GAGUUUGGAGUGAGUGA) were described (2, 3). The nontargeting siRNA was obtained from Dharmacon (siCONTROL Non-targeting siRNA #1).

The siRNAs were transfected by Oligofectamine (Invitrogen) into approximately 70% confluent cells. For each 10-cm culture dish, 50 μl Oligofectamine was added to 100 μl of Opti-MEM I medium (Invitrogen), and 25 μl of the 40 μM siRNA (in the cases of single knockdown) was added to 807 μl of Opti-MEM I medium; the two solutions were mixed at room temperature for 15–20 min and added to 4035 μl of Opti-MEM I to make a final volume of 5 ml of transfection mixture. Cells were rinsed with Opti-MEM I and then incubated with the transfection mixture in a CO2 incubator for 4 h, after which 5 ml of EMEM containing 20% FBS without antibiotics was added to the cells. After 24 h, the cells were trypsinized, split into two 10-cm dishes and grown for 24 h before the second round of siRNA transfection. Approximately 24 h after the second siRNA transfection, the cells were split into 50-mm glass-bottom culture dishes for experiments. For epsin 1 and epsin 2 double knockdown, 8 μl of epsin 1 and 20 μl of epsin 2 siRNAs were mixed and 56 μl of Oligofectamine was used. For Eps15 and Eps15R double knockdown, 20 μl of Eps15 and 20 μl of Eps15R siRNAs were mixed and 80 μl of Oligofectamine was used. The efficiency of siRNA knockdown was assessed by immunofluorescence and/or Western blot analyses.

Flow Cytometry. BS-C-1 cells were infected with low MOI (<1) of influenza viruses for 4 h at 37°C. Infected cells were trypsinized and fixed in 2% formaldehyde in PBS at room temperature for 20 min, after which the cells were permeabilized in buffer P (PBS containing 10% FBS and 0.075% Saponin) at room temperature for 5 min. Cells were incubated with mouse anti-influenza NP (1:1000 dilution in buffer P) at room temperature for 1 h and washed with buffer P before incubation with Alexa Fluor 633 donkey anti-mouse (1:1000 dilution in buffer P) at room temperature for 30 min. Cells were then washed with buffer P and PBS before being analyzed by flow cytometry.

Uptake of Transferrin, EGF, LDL, and Influenza Virus. The influenza virus was added to cells in situ at 37°C during image acquisition.
Alexa 633-labeled transferrin (Invitrogen), Alexa Fluor 647-labeled EGF (Invitrogen), and LDL (MP Biomedicals) labeled with DiD were prebound to cells at 0°C before cells were warmed up to 37°C for uptake or added to cells in situ at 37°C during image acquisition, as specified. Transferrin, EGF, and LDL were added to cells at a concentration of 50 µg/ml, 0.2 µg/ml, and 33 µg/ml, respectively.

The influenza virus entered cells through both epsin 1-dependent and epsin 1-independent pathways. 

(A) Snapshots of a virus particle (red in white circle) colocalized with epsin 1 (green) before undergoing the directed, microtubule-dependent movement and viral fusion. 

(B) Snapshots of a virus particle (red, in white circle) that entered a cell and exhibited microtubule-dependent movement and viral fusion without colocalization with epsin 1 before its entry. (Scale bars, 2.5 μm.)

(C) Fluorescence time traces of the DiD (red) and epsin 1-Venus (green) signals associated with the virus particle shown in A. 

(D) Fluorescence time traces of the DiD and epsin 1-Venus signals associated with the virus particle shown in B.
Fig. S2. Colocalization of epsin 1 and epsin1ΔUIM with clathrin. (A) Epsin 1-Venus (red) and clathrin-ECFP (green) showed extensive colocalization in a cell coexpressing the two fluorescent fusion proteins: 77 ± 7% of the epsin 1-Venus-labeled structures showed clathrin-ECFP signal and 78 ± 8% of the clathrin-ECFP-labeled structures displayed epsin 1-Venus signal. (B) Epsin1ΔUIM-Venus (green) and immunofluorescence of endogenous clathrin (red) showed extensive colocalization in a cell expressing epsin1ΔUIM-Venus at a low level: 92 ± 3% of the epsin1ΔUIM-Venus-labeled structures showed clathrin signal and 74 ± 4% of the clathrin-stained structures displayed epsin1ΔUIM-Venus signal. (Scale bars, 10 μm.)
Fig. S3. Western blot analysis of epsin 1 and epsin 2 in control and knockdown cells. (A) Epsin 1 was detected in control cells (left lane), cells treated with epsin 1 siRNA (center lane), and cells treated with epsin 2 siRNA (right lane). (B) Epsin 2 was detected in control cells (left lane), cells treated with epsin 2 siRNA (center lane), and cells treated with epsin 1 siRNA (right lane). (C) Epsin 1 (Top) and epsin 2 (Middle) were detected in control cells (left lane) and cells treated with both epsin 1 and epsin 2 siRNAs (right lane). In all cases, α-tubulin was used as a loading control (Bottom).
Fig. S4. Epsin 1 knockdown did not affect influenza virus infection. (A) Flow-cytometry measurement of the expression of influenza NP in noninfected cells (Top), cells treated with nontargeting siRNA and infected with the influenza virus (Middle), and epsin 1 knockdown cells infected with the influenza virus (Bottom). Numbers in percentage indicate the fraction of cells infected, i.e., cells with NP expression level exceeding the threshold level indicated by the line marked P2. (B) The percentage of influenza virus particles fused in control and epsin 1 knockdown cells. DiD-labeled influenza virus particles were added to BS-C-1 cells and visualized in live cells at 37°C for 15 min. The number of virus particles that fused with endosomes, as indicated by dequenching of the DiD signal, was counted and divided by the total number of virus particles visualized. In both cases, more than 20 cells were analyzed and hundreds of virus particles were counted.
Fig. S5. Overexpression of epsin1ΔUIM did not perturb clathrin distribution or transferrin uptake. (A) Distribution of endogenous clathrin (Right, green) in cells overexpressing epsin1ΔUIM-ECFP (Left, cyan). Here the cells were transfected only with epsin1ΔUIM-ECFP. The strong and diffuse ECFP signal was used to identify epsin1ΔUIM-ECFP overexpression. Endogenous clathrin was detected by immunofluorescence. (B) Overlay of the clathrin-EYFP (green) and transferrin (red) images shows extensive colocalization between the two in cells overexpressing epsin1ΔUIM-ECFP. Alexa Fluor 633-labeled transferrin (50 μg/ml) was incubated with cells coexpressing clathrin-EYFP and epsin1ΔUIM-ECFP at 4°C for 10 min and imaged at room temperature immediately afterward. Under these conditions, we have found that transferrin largely accumulates in CCPs (1). (C) Uptake of transferrin in cells overexpressing epsin1ΔUIM-ECFP. The cells were incubated with 50 μg/ml Alexa Fluor 633-labeled transferrin at 37°C for 15 min to allow internalization and then subject to acid buffer wash to remove uninternalized transferrin. (Left) The image of epsin1ΔUIM-ECFP (cyan). (Right) The fluorescence image of internalized transferrin (red) in the same cells. (D) A similar transferrin uptake assay was conducted in control cells not expressing epsin1ΔUIM-ECFP. (Scale bars, 10 μm.)
Fig. S6. Double knockdown of Eps15 and Eps15R did not significantly affect the clathrin-mediated entry of the influenza virus. (A) Western blot analysis of Eps15 and Eps15R double knockdown cells. Eps15 (Top) and Eps15R (Middle) were analyzed in control cells (right lane) and in cells treated with both Eps15 and Eps15R siRNAs (left lane). α-Tubulin was used as a loading control (Bottom). (B) The fraction of internalized influenza virus particles that entered through the clathrin pathway in the Eps15 and Eps15R double knockdown cells is shown in comparison with the fractions observed in nontreated cells, cells treated with nontargeting siRNA, and cells treated with epsin 1 siRNA.
Fig. S7. Transferrin entered epsin 1 knockdown cells through clathrin-mediated endocytosis. (A) Transferrin (Tfn, red) colocalization with clathrin (green) in both control (Top) and epsin 1 knockdown cells (Bottom). Transferrin was bound to cells on ice for 1 h. Unbound transferrin was removed by washing cells with cold medium before the cells were fixed and permeabilized for immunofluorescence against clathrin. (Scale bar, 10 μm.) (B) Quantitative analysis of the fraction of transferrin spots that colocalized with clathrin in control and epsin 1 knockdown cells.
**Fig. S8.** Knockdown of epsin 1 did not inhibit the uptake of EGF and LDL through the clathrin pathway. (A) Nontreated (Top) or epsin 1 knockdown (Bottom) cells were incubated with Alexa Fluor 647-labeled EGF (0.2 μg/ml) at 37°C for 20 min. Surface-bound but uninternalized EGF was removed by acid buffer wash. The cells were then fixed and permeabilized for immunofluorescence of epsin 1. (Left) DIC images of the cells. (Center) Corresponding immunofluorescence images of epsin 1. (Right) Fluorescence images of EGF. Quantitative analyses of the amount of EGF internalized are shown in Fig. 5B. (B) A similar uptake assay was used for DiD-labeled LDL (33 μg/ml) in control (Top) and epsin 1 knockdown cells (Bottom). Quantitative analyses of the amount of LDL internalized are shown in Fig. 5B. (Scale bars, 10 μm.)
Fig. S9. EGF and LDL entered epsin 1 knockdown cells through clathrin-mediated endocytosis. (A) Epsin 1 knockdown cells expressing clathrin-EYFP were first incubated with Alexa Fluor 647-labeled EGF (0.2 μg/ml) on ice for 5–10 min to allow EGF binding, and unbound EGF was removed by washing with ice-cold, serum-free and phenol red-free MEM. The cells were then quickly moved to the 37°C temperature-controlled microscope stage for imaging. EGF rapidly formed clusters (red) on the cell surface. The snapshots show one such cluster (indicated by the white circle) colocalizing with clathrin (green) for a period before it entered into the cell and exhibited the rapid, directed, and microtubule-dependent movement. (B) A similar tracking experiment was performed for the clathrin-mediated endocytosis of LDL. Shown in the snapshots is an LDL particle (red, circled in white) colocalized with clathrin (green) before its rapid, directed, and microtubule-dependent motion. (Scale bars, 2.5 μm) (C) The fraction of EGF (Left) and LDL (Right) internalized through the clathrin-dependent pathway as determined from the above tracking experiments.
Fig. S10. The entry kinetics of transferrin (Tfn), EGF, and LDL in control and epsin 1 knockdown cells. The ligands were bound to cells on ice for 30 min (transferrin) or 1 h (EGF and LDL), and unbound ligands were removed by washing the cells with cold medium. The cells were then incubated at 37°C for the indicated amount of time before being washed extensively with cold acid buffer, after which the cells were fixed on ice and imaged. At each time point, 10-15 cells were randomly chosen for analysis. The average total fluorescence intensities were plotted.
Movie S1. A typical trajectory of an influenza virus particle. A DiD-labeled virus particle (indicated by the white circle) initially bound to the cell surface. After some time, the virus began rapid, directed, and microtubule-dependent movement. Eventually, the DiD signal increased dramatically, because of the fusion of the viral membrane with the endosomal membrane. The fluorescence intensity is shown in a thermal scale with blue indicating low intensity, red higher, and yellow the highest. All of the movies are played at 15 times the actual frame rate, which is 2 Hz.

Movie S1 (MOV)
**Movie S2.** An example of the virus particles that colocalized with clathrin and epsin 1 before entry. Shown are the clathrin (cyan) and virus (red) channels (Left) and the epsin 1 (green) and virus (red) channels (Right) of the three-color movie. The DiD-labeled virus particle (red, circled in white) initially bound to the cell and recruited clathrin and epsin 1. After a period of colocalization, the clathrin and epsin 1 signals disappeared, indicating uncoating of the clathrin-coated vesicle. The virus then began its microtubule-dependent movement and eventually fused with an endosome, indicated by the increase of the DiD signal. The movies are played at 15 times the actual frame rate, which is 2 Hz.

[Movie S2 (MOV)](data:image/jpeg;base64,imagedata)
Movie S3. An example of the virus particles that did not colocalize with clathrin or epsin 1 before entry. Shown are the clathrin (cyan) and virus (red) channels (Left) and the epsin 1 (green) and virus (red) channels (Right) of the three-color movie. The DiD-labeled virus particle (red, circled in white) initially bound to the cell. After a period without showing any colocalization with clathrin or epsin 1, the virus began its microtubule-dependent movement and eventually fused with an endosome, indicated by the increase of the DiD signal. The movies are played at 15 times the actual frame rate, which is 2 Hz.