Supporting Information

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SI Materials and Methods

DNA Constructs. For the construction of the HaloTag-GFP-MAO expression vector, human monoamine oxidase B (MAO-B) C-terminal transmembrane domain (TMD) (residues 469–520) was cloned into pEGFP-N vector (Clontech). Then, the HaloTag coding region was amplified with PCR from pFC27K HaloTag CMV-neo Flexi Vector (Promega) and cloned into the N-terminal region of GFP-MAO with the HiFi DNA assembly cloning kit (Invitrogen).

Purification of Proteins. All SNARE proteins (syntaxin 13, vti1a, syntaxin 6, and VAMP4) were prepared as described in ref. 23. Briefly, the proteins were expressed as His6-tagged or GST-tagged fusion proteins in *Escherichia coli* and purified by Ni2+ agarose or glutathione-sepharose, respectively. The tags of all proteins were removed by using thrombin cleavage. All proteins were further purified by ion-exchange chromatography. All proteins were 95% pure, as judged by SDS/PAGE and Coomassie blue staining.

Liposome Flotation Assay. The 30% and 80% Nycodenz (Progen) stock solutions are prepared with HP150 buffer [150 mM KCl, 20 mM Hepes (pH7.5)]. A total of 20 μL 80% (wt/wt) Nycodenz was added and thoroughly mixed with 20 μL proteoliposomes. Next, 40 μL of 30% (wt/wt) Nycodenz was overlaid and finally 40 μL HP150 buffer was added as a top layer. Samples were spun at 275,000 × *g* for 60 min at 4 °C. After centrifugation, 20-μL aliquots were taken from the top of the gradient and analyzed with SDS/PAGE and Western blotting.

Cell Culture and Preparation of Mitochondria. HeLa cells and HEK293 cells were grown in Dulbecco's modified Eagle medium (DMEM, Lonza GmbH) with the following additions: 10% FCS (PAA Laboratories GmbH), 4 mM glutamine (Lonza GmbH), and 100 units/mL each of penicillin and streptomycin (Lonza GmbH). For PC12 cells, 5% FCS and 10% horse serum (HS) (PAN-Biotech) were added instead of 10% FCS.

Isolation of fluorescence-labeled mitochondria was performed as described (40). To label mitochondria with GFP, HeLa cells expressing GFP-MAO were used. PNS fraction in mitochondria resuspending buffer [250 mM mannitol, 5 mM Hepes (pH 7.4) and 0.5 mM EGTA] was centrifuged at 600 × *g* for 5 min and the supernatant was centrifuged again at 7,000 × *g* for 10 min. The pellet was resuspended in mitochondria resuspending buffer.

Immunocytochemistry. Injected cells were fixed with 4% PFA in PBS for 10 min and washed with PBS three times. The cells were incubated overnight at 4 °C with primary antibody diluted 1:300–1,000 in PBS containing 0.05% saponin and 1% goat serum. The coverslips were then washed three times with PBS and incubated with Cy2- or Cy3-labeled secondary antibodies, diluted at 1:600 in PBS containing 0.05% saponin and 1% goat serum for 90 min at room temperature. After washing with PBS, the coverslips were mounted in Dako Fluorescent Mounting Medium (DakoCytomation). For immunostaining of (proteo)liposomes in vitro, the vesicles were transferred in PBS on poly-L-lysine–coated coverslips and centrifuged for 45 min at 5,868 × *g* at 4 °C. After fixation with 4% PFA for 10 min, the coverslips were incubated with primary antibody for 60 min at room temperature and developed with labeled secondary antibodies as described above.

Cell Viability Assay. One day after microinjection, the cells were incubated with 0.2 μM calcein-AM (Invitrogen) in culture medium for 15 min and washed with PBS three times. The cells were observed using a confocal microscope.

Image Acquisition. Images of injected cells were acquired using an SP5 confocal microscope (Leica Microsystems) with a 63× oil-immersion objective and Leica LCS software.

The extent of colocalization between injected proteoliposomes and organelles was determined using a custom written Matlab algorithm (The Mathworks, Inc.) (see ref. 19 for details), kindly provided by Silvio Rizzoli (University Medical Center Göttingen, Germany). At least 100 injected vesicles were analyzed for the colocalization with each organelle marker in an experiment of microinjection. Time-lapse images were taken using an LSM 780 confocal microscope (Carl Zeiss) with a 63× water-immersion objective and Zen software.

Drug Treatments. Isolated early endosomes were reacted with trypsin immobilized on magnetic beads (450 units/mL Mag-trypsin; Clontech) overnight at 4 °C. After reaction was completed, trypsin proteins were removed by a magnetic stand. For *N*-ethylmaleimide (NEM) treatment, HeLa cells were preincubated with indicated final concentrations of NEM for 10 min. Microinjection of isolated endosomes or (proteo)liposomes was performed in injection medium containing the same NEM concentration. To disrupt polymerization of microtubules or inhibit cytoplasmic dynein, respectively, 1 μM nocodazole or 20 μM ciliobrevin D was added 30 min before the experiments, respectively.

Protein Recruitment to Liposomes. GFP-tagged protein expressing or nontransfected HeLa cells were homogenized in homogenization buffer with protease inhibitor. Debris and membranes were removed by two consecutive centrifugation steps at 3,000 × *g* for 15 min and at 100,000 × *g* for 60 min, respectively. Liposomes were incubated with the cytosol fraction at 37 °C and unbound proteins were removed by flotation. Proteins bound to liposomes were detected by Western blotting.

In Vitro Membrane Association. PNS fractions, prepared from HeLa cells expressing GFP-MAO or HaloTag-GFP-MAO, were incubated with liposomes for 30 min at 37 °C. To immunoisolate GFP-MAO–expressing mitochondria, the resulting mixture was incubated for 3 h with anti-GFP antibody. Subsequently, protein G-sepharose beads (GE Healthcare) were added to each sample and further incubated for 1 h under constant rotation. The samples were then washed thrice with PBS buffer with protease inhibitor. Bound proteins were detected by Western blotting.

Quantification and Statistical Analysis. Images were analyzed with Zen software and ImageJ software. Analysis of motion of vesicles was carried out using ImageJ and plugins (TrackMate, PTA and View5D). To calculate the value of Pearson's correlation coefficient, Coloc2 plugin of ImageJ was used. Calculation of perinuclear index was performed as described (41). In short, fluoresence intensity of Alexa 633-Tfn was measured for the whole cell except of nuclear area (I_{total}), the area within 5 μm of nucleus ($I_{perinuclear}$), and the area >10 μm of nucleus ($I_{peripheral}$), respectively, and calculated $I_{<5} = I_{perinuclear}/I_{total} \times 100$, $I_{>10} = I_{peripheral}/I_{total} \times 100$. The perinuclear index was defined as $I_{index} = I_{<5} - I_{>10}$. KaleidaGraph and Excel software was used to perform statistical analysis. Statistical *P* values were calculated using two-tailed Student's *t* test for unpaired samples.

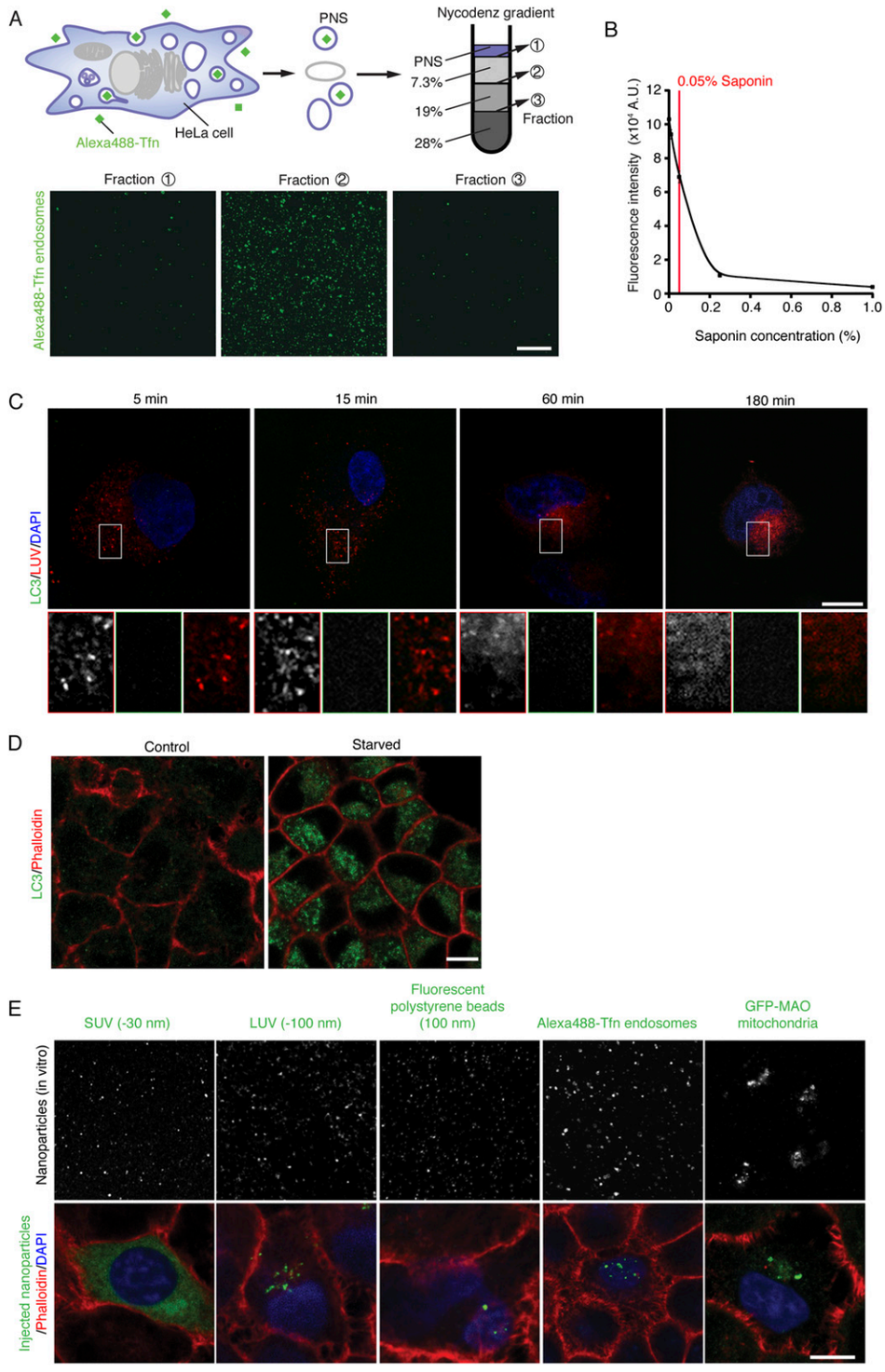


Fig. S1. Efficient introduction system of macromolecular complexes. (*A*) Schematic overview over the procedure for isolating early endosomes from HeLa cells. To label early/recycling endosomes, cells were incubated for 5 min with Alexa-Fluor–labeled transferrin (Tfn) (50 μg/mL), which was internalized by endocytosis. Following homogenization using a ball-bearing cell cracker, a postnuclear supernatant (PNS) was prepared from these cells and loaded onto noncontinuous Nycodenz gradients, followed by centrifugation (see *Methods* for details). Tfn-positive endosomes were highly concentrated in fraction 2 (boundary between 7.3% and 19% Nycodenz) and the fraction was used for microinjection. (*B*) Saponin-dependent extraction of rhodamine-PE from liposomes. LUVs containing 0.3% rhodamine-PE were adsorbed on coverslips and incubated with PBS containing various concentrations of saponin overnight, and the fluorescence intensity of rhodamine-PE was measured by confocal microscopy. The concentration of saponin (0.05%) used for permeabilization is indicated; note that under these conditions only a minor fraction of the lipid label is extracted. (*C* and *D*) Microinjection does not induce autophagocytosis. (*C*) Immunostaining of HeLa cells, injected with 100 nm LUVs (red) for LC3, a marker for autophagosomes. At 5, 15, 60, and 180 min after injection, the cell were fixed and immunostained with anti-LC3 antibody (green). DAPI is included as a marker for injected cells (blue). *Bottom* images show the areas framed by white boxes at high magnification. (Scale bar, 12 μm.) (*D*) Immunostaining of control and starved (to induce autophagocytosis) HeLa cells with anti-LC3 antibody. HeLa cells were incubated in 10 mM HN_4Cl/Earle's balanced salt solution (EBSS) medium overnight and immunostained. Compared with control cells, strong dot-like signals were detected in the starved cells, thus showing induction of autophagocytosis as positive control. Phalloidin staining shows the cell boundaries. (Scale bar, 10 μm.) (*E*, *Top*) Representative images of nanoparticles used for injection after adsorption to coverslips in vitro. (*Bottom*) Images of HeLa cells following injection of the nanoparticles shown in the field above. DAPI is a injection marker and phalloidin staining shows cell boundary. (Scale bar, 10 μm.)

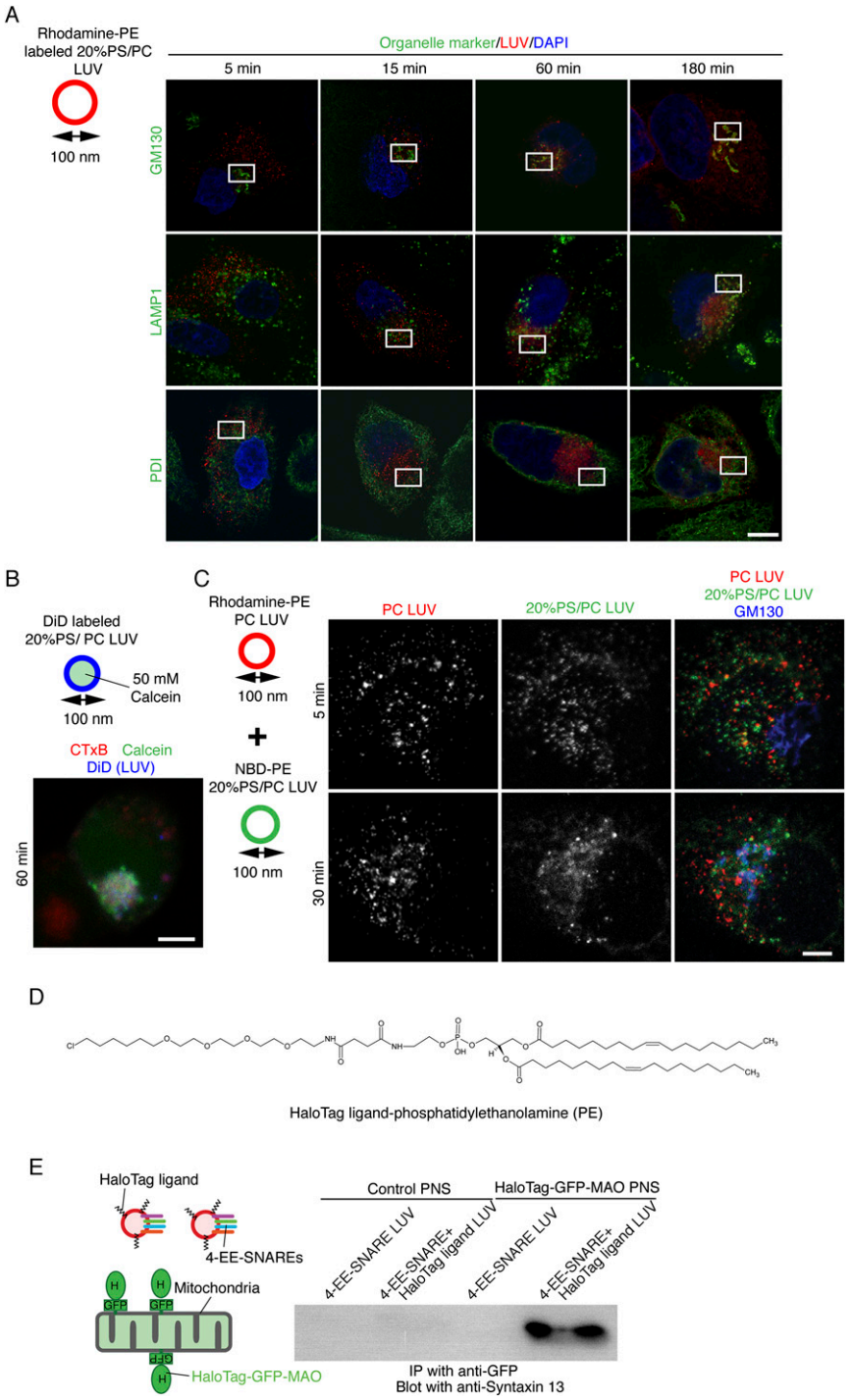


Fig. S2. Fate of nanoparticles injected into HeLa cells. (*A*) Lower magnification overview of the images (boxed areas) shown in Fig. 2*A*. (Scale bar, 12 μm.) (*B*) Fifty millimole calcein-encapsulated LUVs were injected into HEK293 cells preinternalized with Alexa 594-cholera toxin B (CtxB) to label the Golgi. Membranes of LUVs were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine (DiD). Sixty minutes after injection, dequenched calcein signal (green) was highly colocalized with the Golgi marker (red) and LUV marker (blue). (Scale bar, 5 μm.) (*C*) A mixture of nitrobenzoxadiazole (NBD)-labeled 20% PS/PC LUVs and rhodamine-labeled PC LUVs was injected into HeLa cells. Both classes of LUVs showed a punctate distribution across the cytoplasm 5 min after injection. Sixty minutes after injection, however, the NBD signal showed dim and amorphous morphologies around the perinuclear region where it colocalized with GM130, in contrast to the rhodamine signal that remained punctate. (Scale bar, 5 μm.) (*D*) Structure of HaloTag ligand-PE. (*E*) Covalent binding of 4-EE-SNARE + HaloTag ligand LUVs to mitochondria expressing HaloTag-GFP-MAO. A postnuclear supernatant (PNS) obtained from HeLa cells expressing HaloTag-GFP-MAO was incubated with 4-EE-SNARE LUVs (see Fig. 4*D* for details) reconstituted in the presence or absence of HaloTag ligand for 30 min in vitro. HaloTag-GFP-MAO–positive mitochondria were immunoisolated with an anti-GFP antibody, and bound LUVs were detected by Western blotting using an anti-syntaxin 13 antibody.

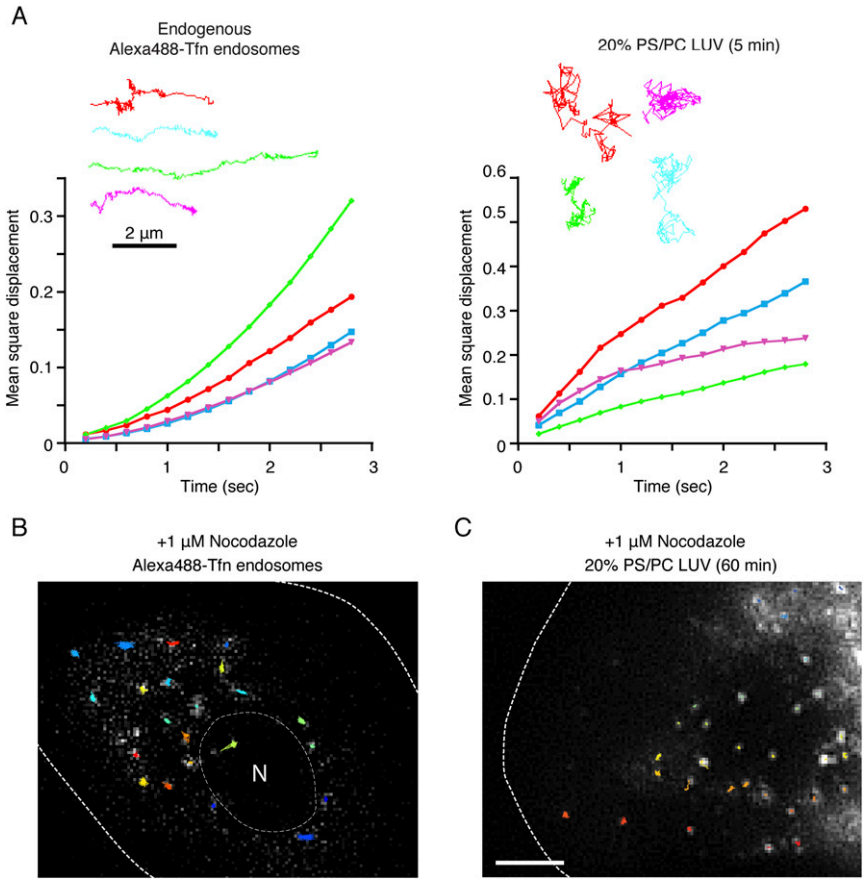


Fig. S3. Movement of injected endosomes and LUVs. (*A*, *Left*) Representative examples of mean squared displacement (MSD) of Alexa 488-Tfn–positive endosome over time. The increasing steepness of the curves indicates that the endosomes are directionally transported. (*A*, *Right*) MSD of representative 20% PS/PC LUVs at 5 min after injection over time. The linear increase of the curves indicates that the vesicles move by Brownian movement, but not by directional movement. The colors of each plot correspond to those of the trajectories shown on *Top*. (*B*) Trajectories of Alexa 488-Tfn–positive endosomes in 1 μM nocodazole-treated cells at 5 min after internalization (see also Movie S5). Trajectories of endosomes continuously tracked over 10 s are shown. N, nucleus. (*C*) Trajectories of injected 20% PS/PC LUVs in HeLa cells treated with 1 μM nocodazole at 60 min after injection (see also Movie S6). Trajectories of vesicles continuously tracked over 10 s are shown. (Scale bar, 3 μm.)

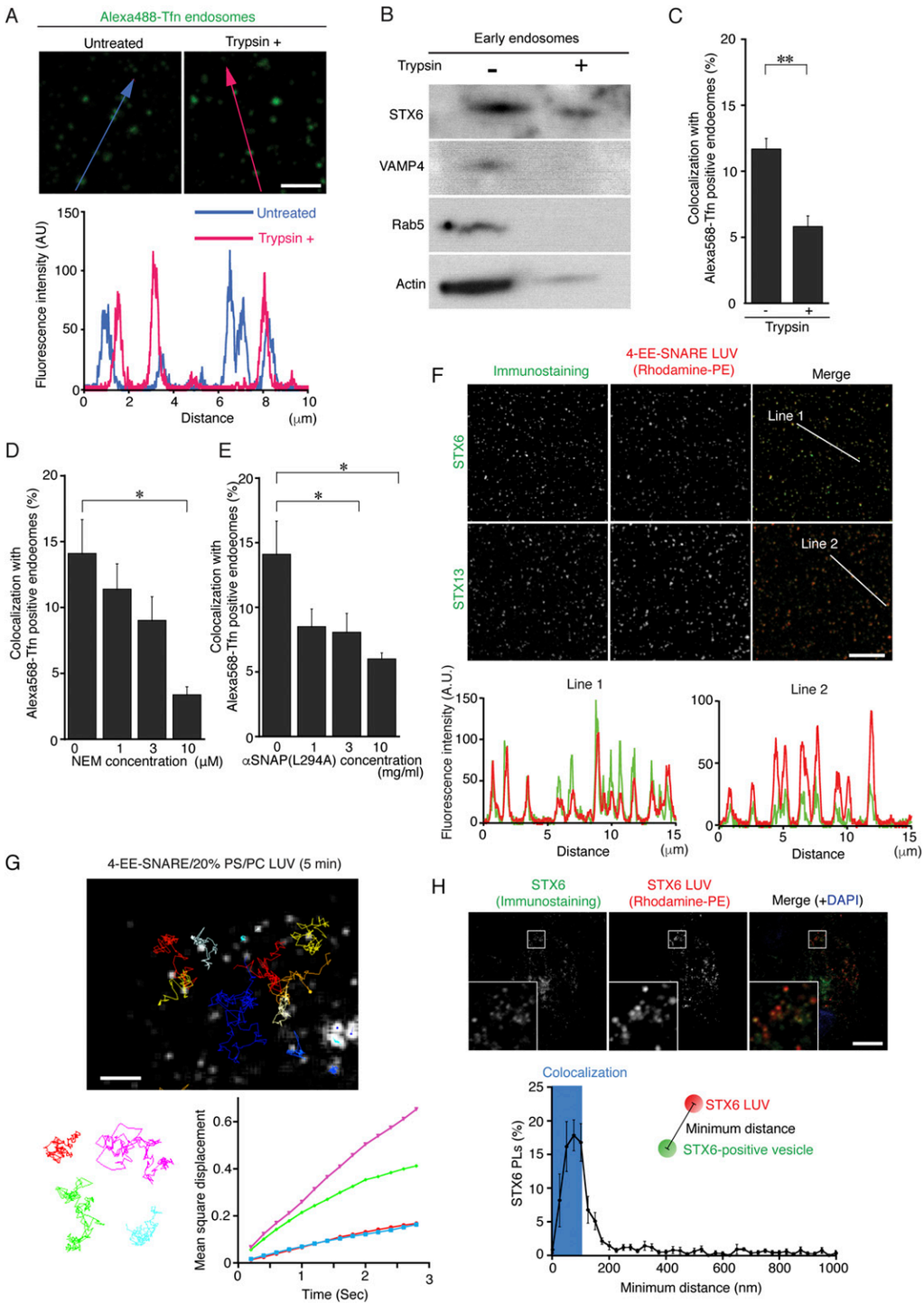


Fig. S4. SNARE proteins determine targeting specificity. (*A*, *Top*) Fluorescent images of trypsin-treated or -untreated Alexa 488-Tfn–labeled endosomes in vitro. (*Bottom*) Fluorescence intensity profiles (arbitrary units) of the line scans indicated in the micrographs above. Trypsin treatment did not change the vesicle size and the fluorescent intensity of Alexa 488-Tfn. (Scale bar, 3 μm.) (*B*) Western blotting of trypsin-treated or -untreated endosomes. The level of early endosomal markers exposed on the endosome surface was decreased by trypsin treatment. (*C*) Trypsin-pretreated Alexa 488-Tfn–positive endosomes were injected cells and examined for colocalization with endogenous endosomes. (*D*) Colocalization between injected Alexa 488-Tfn–positive endosomes and endogenous Alexa 568-Tfn–positive endosomes in cells treated with increasing concentrations of NEM. (*E*) Colocalization between injected Alexa 488-Tfn–positive endosomes and endogenous Alexa 568-Tfn–positive endosomes following coinjection of increasing amounts of a dominant negative mutant of αSNAP [αSNAP(L294A)]. For all plots, error bars indicate SEM, $*P < 0.05$, $**P < 0.01$, determined by unpaired *t* test. (*F*) Immunostaining of 20% PS/PC LUVs containing 4-EE-SNARE for syntaxin 6 (STX6) and syntaxin 13 (STX13) in vitro. Liposomes were deposited on coverslips, fixed, and stained. Intensity plots of the line scans in the pictures on the *Top*, demonstrating that the two channels are highly correlated. The correlation coefficients for STX6 and lipids and for STX13 and lipids were 0.80 ± 0.03 ($n = 6$) and 0. 71 ± 0.05, ($n = 6$), respectively, showing that almost all liposomes contain detectable quantities of the SNAREs. (Scale bar, 5 μm.) (*G*, *Top*) Representative trajectories of 4-EE-SNARE LUVs at 5 min after microinjection. Time-lapse imaging was performed with one acquisition every 200 ms in HeLa cells. The first frame is shown as a background, with the trajectories plotted in different colors to better distinguish individual trajectories. Trajectories represent vesicles that could be tracked over 10 s during 40-s acquisition. (Scale bar, 3 μm.) (*Bottom*) MSD of representative 4-EE-SNARE LUVs at 5 min after injection over time. (*H*) Calibration of minimum distance analysis using double-labeled particles. Immunostaining of HeLa cells preinjected with syntaxin 6 LUVs (labeled with rhodamine-PE) using anti-syntaxin 6 antibodies. (Scale bar, 5 μm.) *Bottom* histogram shows the minimum distance between rhodamine-PE (as marker for the injected liposomes) and particles immunolabeled for syntaxin 6. Since the vesicles contain both signals, the peak observable defines colocalization (i.e., fusion in our experiments), with our cutoff area (0–100 nm) highlighted in blue. This pattern corresponds to what is expected for identity of particles where the error of the position determination (limited by the point spread function) is normally distributed (Rayleigh distribution, see ref. 19 for details).

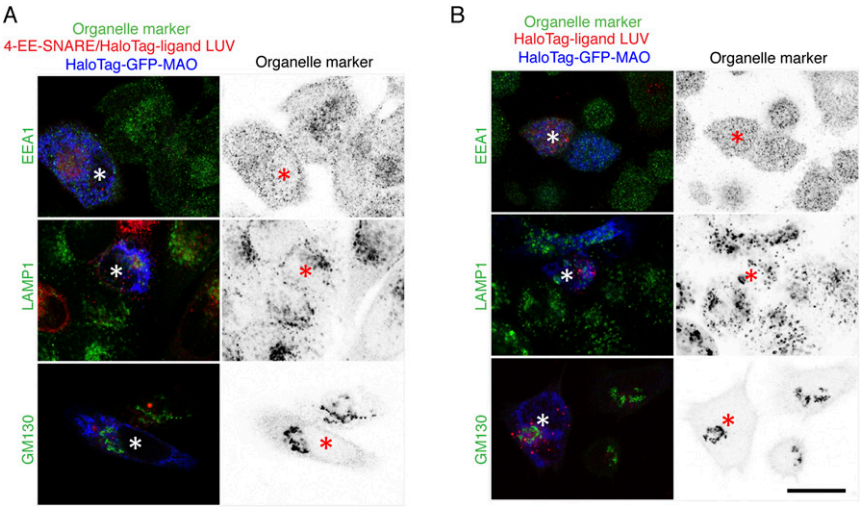
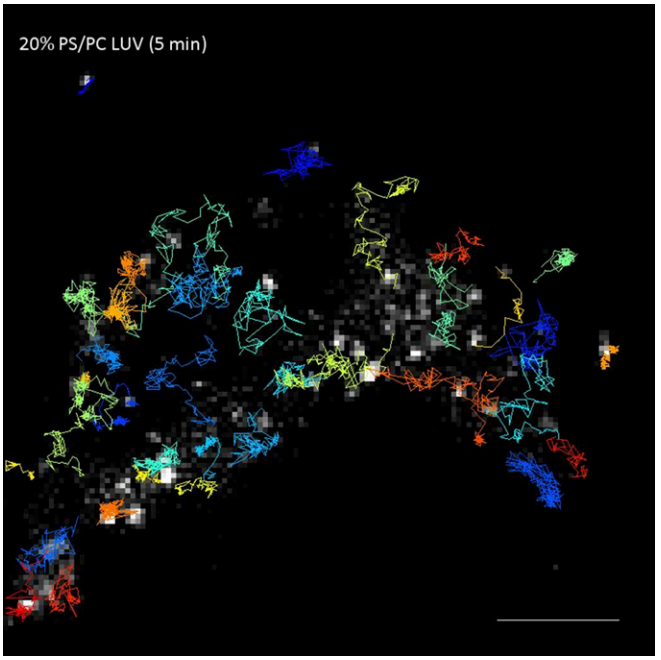
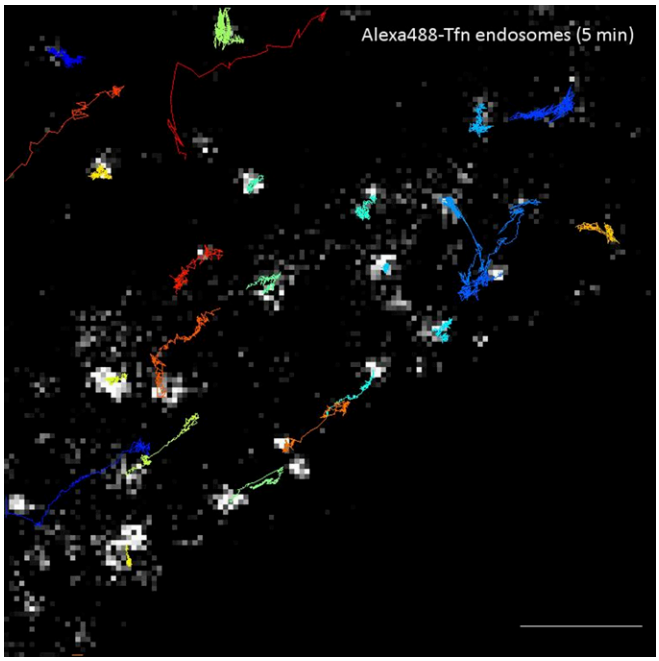


Fig. S5. Interference with endosomal trafficking by diverting endosomes to mitochondria via fusion with targeted liposomes. (*A*) HeLa cells expressing HaloTag-GFP-MAO (blue) were injected with 4-EE-SNARE + HaloTag ligand LUVs (red) and immunostained for the indicated organelle markers (green) 60 min after injection. Asterisks mark injected cells. (*B*) As in *A*, but the injected LUVs lacked the 4-EE-SNAREs. (Scale bar, 20 mm.)



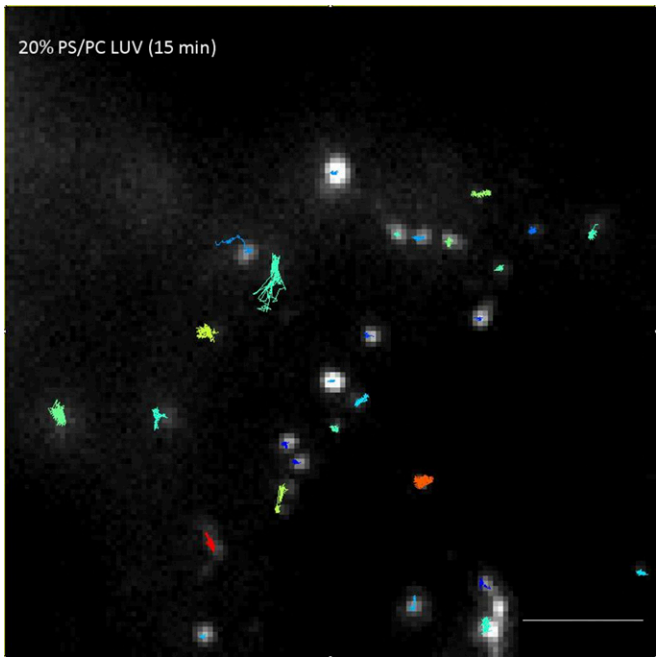
Movie S1. The movement of injected 20% PS/PC LUVs in a HeLa cell 5 min after injection. HeLa cells were injected with 20% PS/PC LUVs labeled with rhodamine-PE, followed by time-lapse confocal imaging 5 min after microinjection. Time interval between acquired frames was 0.2 s and the acquisition length was 40 s. (Scale bar, 3 μm.)

Movie S1



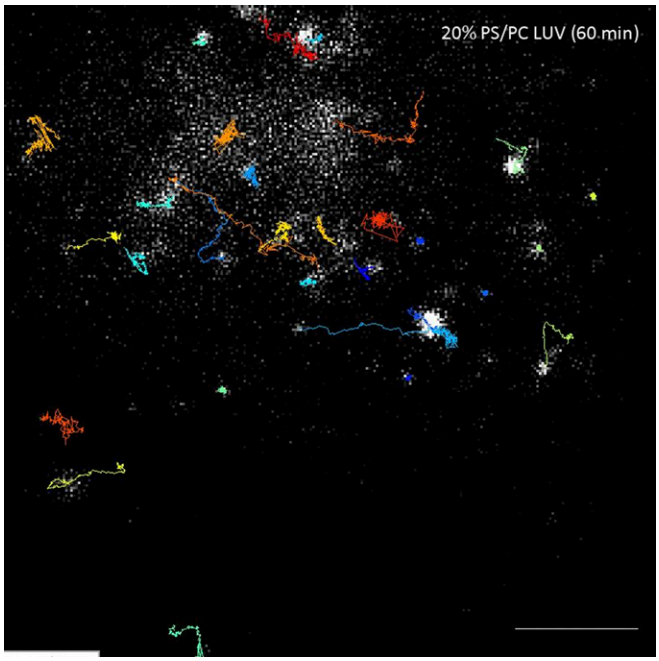
Movie S2. The movement of endogenous endosomes in a HeLa cell. HeLa cells were incubated with Alexa 488-Tfn for 5 min to allow for internalization. Thereafter the cells were washed, followed by time-lapse confocal imaging. Time interval between acquired frames was 0.2 s and the acquisition length was 40 s. (Scale bar, 3 μm.)

Movie S2



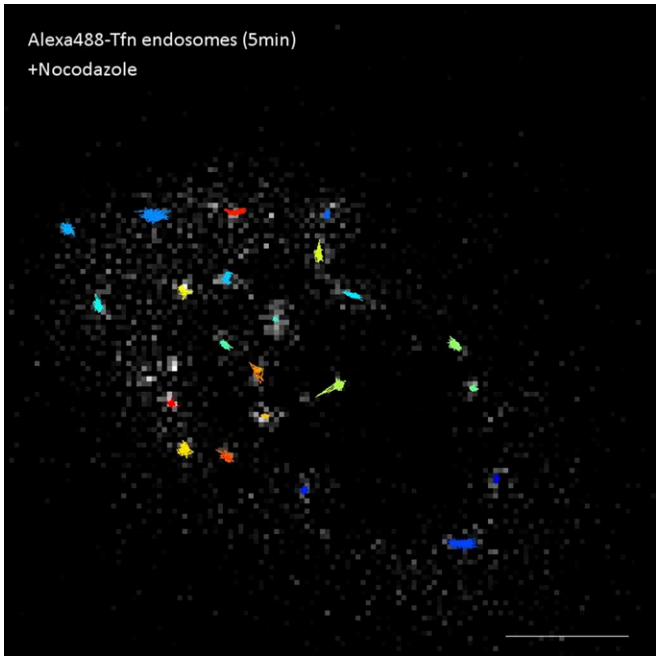
Movie S3. The movement of injected 20% PS/PC LUVs in a HeLa cell 15 min after injection. HeLa cells were injected with 20% PS/PC LUVs, followed by time-lapse confocal imaging 15 min after microinjection. Time interval between acquired frames was 0.2 s and the acquisition length was 40 s. (Scale bar, 3 μm.)

Movie S3



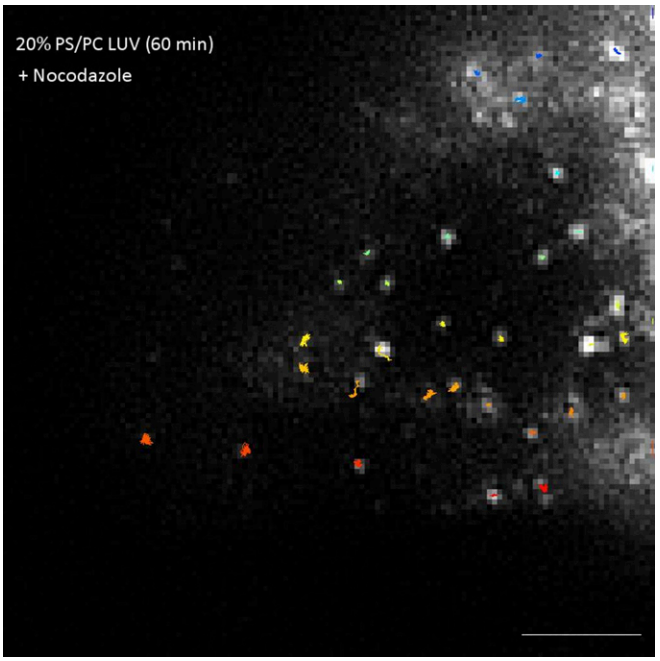
Movie S4. The movement of injected 20% PS/PC LUVs in a HeLa cell 60 min after injection. HeLa cells were injected with 20% PS/PC LUVs, followed by time-lapse confocal imaging 60 min after microinjection. Time interval between acquired frames was 0.2 s and the acquisition length was 40 s. (Scale bar, 3 μm.)

Movie S4



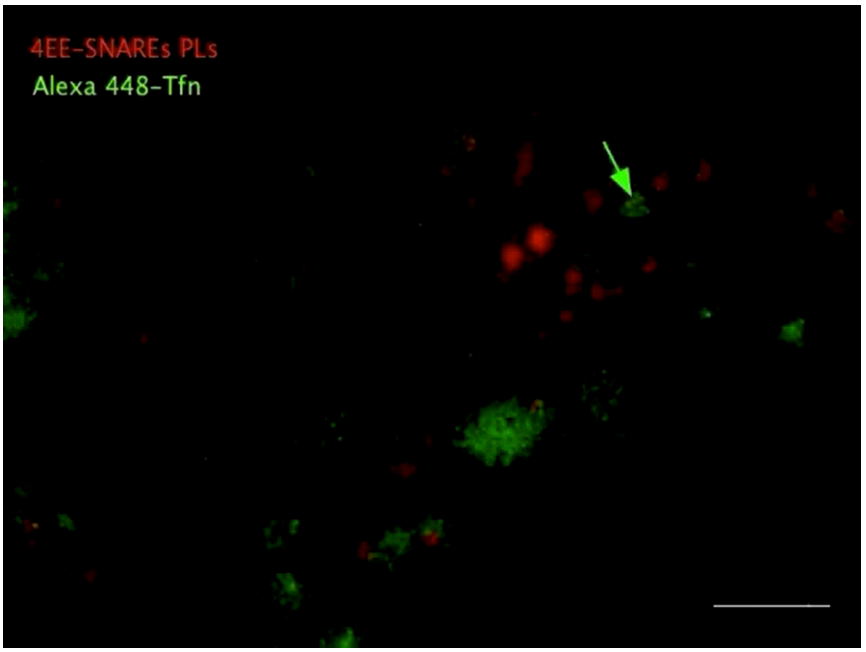
Movie S5. The movement of endogenous endosomes in a HeLa cell after nocodazole treatment. HeLa cells were treated with 1 μM nocodazole for 30 min and then incubated with Alexa 488-Tfn for 5 min to label early endosomes. After internalization, the cells were washed with PBS, followed by time-lapse confocal imaging. Time interval between acquired frames was 0.2 s and the acquisition length was 40 s. (Scale bar, 3 μm.)

Movie S5



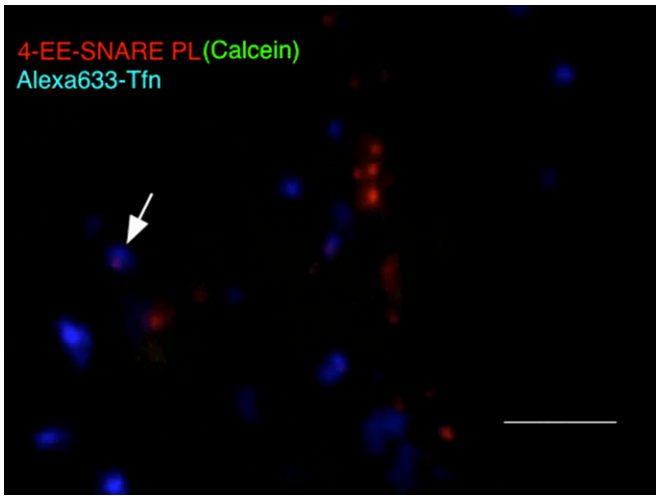
Movie S6. The movement of injected 20% PS/PC LUVs in a nocodazole-treated HeLa cell 60 min after injection. HeLa cells were treated with 1 μM nocodazole for 30 min and then injected with 20% PS/PC LUVs. After further incubation for 60 min with 1 μM nocodazole, time-lapse imaging was collected. Time interval between acquired frames was 0.2 s and the acquisition length was 40 s. (Scale bar, 3 μm.)

Movie S6



Movie S7. Approach between an endogenous endosome and an injected 4 EE-SNARE proteoliposome. Time-lapse confocal imaging of a HeLa cell shortly after injecting 4-EE-SNARE LUVs labeled with rhodamine-PE (red). To label endogenous early/recycling endosomes, Alexa 488-Tfn (green) was internalized during microinjection for 5 min. Images were collected a few minutes after the microinjection. Time interval between acquired frames was 0.4 s and the acquisition length was 9 s. (Scale bar, 3 μm.)

Movie S7



Movie S8. Fusion between an endogenous endosome and an injected 4 EE-SNARE proteoliposome. Time-lapse confocal imaging of a HeLa cell shortly after injecting 4-EE-SNARE LUVs containing 50 mM calcein (red). To label endogenous early/recycling endosomes, Alexa 633-Tfn (blue) was internalized during microinjection for 5 min. Green indicates dequenched calcein signal generated by dilution during fusion. Images were collected for a few minutes after the microinjection. The time interval between the acquired frames was 1 s and the acquisition length was 30 s. (Scale bar, 3 μm.)

Movie S8