

Description of Additional Supplementary Files

Supplementary Data 1: Data from quantitative proteomic analysis of cell surface proteins in conditions of inhibition of clathrin-mediated endocytosis (CME). HeLa cells were treated for 72h with control or μ 2-adaptin siRNAs, and quantitative mass spectrometry analysis (iTraq) was performed on isolated cell surface proteins. 489 proteins (with Unused score ≥ 2) were identified, 378 (77.3%) of which were containing 'membrane' in their GO-annotations. Among these hits, 262 (53.6%) were containing the following exact GO-annotations: 'cell surface', 'plasma membrane', 'intracellular space/matrix', 'integral component of plasma membrane' (hits highlighted in green in the table). The iTraq analysis was performed on 4-plexes, where reporters 114 and 115 were assigned to two technical replicates of negative control siRNA-treated cells, and reporters 116 and 117 were assigned to two technical replicates of μ 2-adaptin siRNA-treated cells. In the table, fold-change ratios are calculated relatively to the reporter 114 (negative control condition). Ratios highlighted in red indicate hits showing an increased abundance in the μ 2-adaptin-depleted condition compared to the negative control, while in blue, they indicate a decreased abundance. The color intensity depicts the significance of the ratios: no color, not significant; light, $*P < 0.05$; medium, $**P < 0.01$; dark, $***P < 0.001$.

Supplementary Movie 1: 3D movie of anti-CD166 antibody endocytosis imaged by Lattice light-sheet microscopy on live cells expressing CLTA-mRFP, related to Figure 1e-h and Supplementary Figure 2i.

Genome-edited U2OS expressing CLTA-mRFP were incubated for 1 min with $5 \mu\text{g ml}^{-1}$ anti-CD166-ATTO647N antibody and transferred to the Lattice light-sheet microscope for imaging at 30°C . Full 3D volume of 60 planes per U2OS cell was acquired within 3 sec. Spot color indicates the degree of clathrin co-localization, from blue (clathrin negative) to green (clathrin positive). Tracks are depicted in red.

Supplementary Movie 2: 2D movie of anti-CD166 antibody endocytosis imaged by Lattice light-sheet microscopy on live cells expressing CLTA-mRFP, related to Figure 1e-h and Supplementary Figure 2i.

Same conditions as Movie 1: genome-edited U2OS expressing CLTA-mRFP were imaged after a 1 min incubation with $5 \mu\text{g ml}^{-1}$ anti-CD166-ATTO647N antibody and transferred to the Lattice light-sheet microscope for imaging at 30°C . Full 3D volume of 60 planes per U2OS cell was acquired within 3 sec. This 2D movie shows a side view of a cell slice perpendicular to the detection objective. Insets: note CD166 endocytic events (red) that are negative (left) or positive (right) for clathrin (green).

Supplementary Movie 3: Uptake of fluorescently labeled anti-CD166 in an endoA3-GFP-positive structure, related to Supplementary Figure 5d.

HeLa cells expressing endoA3-GFP were incubated continuously at 37°C with $5 \mu\text{g ml}^{-1}$ anti-CD166-A647, and observed by live-cell imaging using a spinning disk microscope at 1.078 sec intervals (exposure time: 200 msec for each channel). Note the tubule that extend inwards from cell periphery. Scale bar, $2 \mu\text{m}$.

Supplementary Movie 4: Uptake of fluorescently labeled anti-CD166 in an endoA3-GFP-positive structure, related to Supplementary Figure 5d.

Same conditions as Movie 3: HeLa cells expressing endoA3-GFP were incubated continuously at 37°C with 5 $\mu\text{g ml}^{-1}$ anti-CD166-A647, and observed by live-cell imaging using a spinning disk microscope at 1.191 sec intervals (exposure time: 200 msec for each channel). E, Extracellular medium. N, Nucleus. Scale bar, 2 μm .

Supplementary Movie 5: Fission of an endoA3-GFP-positive tubule.

Same conditions as Movie 3: HeLa cells expressing endoA3-GFP were incubated continuously at 37°C with 5 $\mu\text{g ml}^{-1}$ anti-CD166-A647, and observed by live-cell imaging using a spinning disk microscope at 1.191 sec intervals (exposure time: 200 msec for each channel). E, Extracellular medium. N, Nucleus. Scale bar, 2 μm .

Supplementary Movie 6: TIRFM analysis of endoA3-GFP cells incubated with fluorescently labeled anti-CD166, related to Supplementary Figure 5e-g.

HeLa cells expressing endoA3-GFP were incubated continuously at 37°C with 5 $\mu\text{g ml}^{-1}$ anti-CD166-A555, and observed by live-cell imaging using a TIRF microscope at 1 sec intervals (exposure time: 50 msec for each channel). Scale bar, 5 μm .

Supplementary Movie 7: TIRFM analysis of cells co-expressing endoA3-GFP and μ 2-mCherry, related to Supplementary Figure 8f.

HeLa cells co-expressing endoA3-GFP and μ 2-mCherry were observed by live-cell imaging using a TIRF microscope at 1 sec intervals (exposure time: 50 msec for each channel). Scale bar, 5 μm .

Supplementary Movie 8: TIRFM analysis of cells co-expressing endoA3-GFP and DNM2-mCherry, related to Supplementary Figure 8e.

HeLa cells co-expressing endoA3-GFP and DNM2-mCherry were observed by live-cell imaging using a TIRF microscope at 1 sec intervals (exposure time: 50 msec for each channel). Scale bar, 5 μm .

Supplementary Movie 9: Fluid-FM – confocal experiment with Gal8-coated gold nanoparticles on endoA3-GFP expressing cells, related to Figure 3e.

HeLa cells expressing endoA3-GFP (green channel) were exposed locally to single Gal8-functionalized nanoparticles (red channel) using a Fluid-FM probe. The recruitment of endoA3-GFP to the site of Gal8 exposure was observed by fast scanning confocal microscopy, recording consecutive images for a total duration of 60 s. Scale bar, 5 μm .

Supplementary Movie 10: Fluid-FM – confocal experiment with Gal1-coated gold nanoparticles on endoA3-GFP expressing cells, related to Supplementary Figure 9i.

HeLa cells expressing endoA3-GFP (green channel) were exposed locally to single Gal1-functionalized nanoparticles (red channel) using a Fluid-FM probe. The movements of endoA3-GFP around the site of Gal1 exposure were monitored by fast scanning confocal microscopy, recording consecutive images for a total duration of 60 s. No significant recruitment of endoA3-GFP was observed. Scale bar, 5 μm .

Supplementary Movie 11: Fluid-FM – confocal experiment with bare probe on endoA3-GFP expressing cells, related to Supplementary Figure 9k.

HeLa cells expressing endoA3-GFP (green channel) were exposed locally to bare Fluid-FM probes, as negative control. The transmitted light channel was used to show the exact positioning of the Fluid-FM probe on the cell. The movements of endoA3-GFP around the Fluid-FM probe were monitored by fast scanning confocal microscopy, recording consecutive images for a total duration of 60 s. No significant recruitment of endoA3-GFP was observed. Scale bar, 5 μ m.

Supplementary Movie 12: Fluid-FM – confocal experiment with control fluorescent gold nanoparticles on endoA3-GFP expressing cells, related to Supplementary Figure 9j.

HeLa cells expressing endoA3-GFP (green channel) were exposed locally to single fluorescently labeled gold nanoparticles with no protein attached to it (red channel) using a Fluid-FM probe, as a negative control. The movements of endoA3-GFP around the nanoparticles were monitored by fast scanning confocal microscopy, recording consecutive images for a total duration of 60 s. No significant recruitment of endoA3-GFP was observed. Scale bar, 5 μ m.

Supplementary Movie 13: Fluid-FM – confocal experiment with Gal8-coated gold nanoparticles on endoA2-GFP expressing cells, related to Supplementary Figure 9l.

HeLa cells expressing endoA2-GFP (green channel) were exposed locally to single Gal8-functionalized nanoparticles (red channel) using a Fluid-FM probe. The movements of endoA2-GFP around the nanoparticles were monitored by fast scanning confocal microscopy, recording consecutive images for a total duration of 60 s. No significant recruitment of endoA2-GFP was observed. Scale bar, 5 μ m.

Supplementary Movie 14: Wound healing assay on U2OS cells, related to Figure 4a-e and Supplementary Figure 10a.

U2OS cells transfected with control siRNAs or siRNAs targeting endoA3 and/or CD166 were followed during 46h for wound healing in bright field microscopy. Images were acquired every 10 min. Quantifications are shown in Figure 4a-e and Supplementary Figure 10a. Scale bar, 100 μ m.

Supplementary Movie 15: Wound healing assay on SUM159 wild-type cells, related to Supplementary Figure 10b.

SUM159 cells transfected with control siRNAs or siRNAs targeting endoA3 and/or CD166 were followed during 46h for wound healing in bright field microscopy. Images were acquired every 10 min. Quantifications are shown in Supplementary Figure 10b. Scale bar, 100 μ m.

Supplementary Movie 16: Wound healing assay on genome-edited SUM159 AP2-GFP cells, related to Supplementary Figure 10c.

SUM159 AP2-GFP cells transfected with control siRNAs or siRNAs targeting endoA3 and/or CD166 were followed during 46h for wound healing in bright field microscopy. Images were acquired every 10 min. Quantifications are shown in Supplementary Figure 10c. Scale bar, 100 μ m.

Supplementary Movie 17: Wound healing assay on LB33-MEL cells, related to Supplementary Figure 10d,e.

LB33-MEL cells, wild-type or stably expressing endoA3-GFP, were followed during 46h for wound healing in bright field microscopy. Images were acquired every 10 min. Quantifications are shown in Supplementary Figure 10d,e. Scale bar, 100 μm .