**Legends for Figure Supplements. Bitsikas *et al*.**

**Figure 1-figure supplement 1. Removal of extracellular fluorophore from BG-SS-fluorophore labelled SNAP-tag by reduction with MESNa is highly efficient. A**. Cells expressing SNAP-GPI were simultaneously labeled at 4˚C with BG-SS-488 and SNAPsurface549. The latter is not reducible. They were then treated with PI-PLC *or* PI-PLC plus MESNA. Both treatments reduced the amount of label significantly (top row of images). However when remaining cell surface label was inspected by adjusting the intensity post-acquisition (lower panels), differential effects in the removal of the two fluorophores were observed. PI-PLC reduced the signal of both fluorophores evenly. However, when cells were treated with MESNa followed by PI-PLC the levels of reducible BG-SS-488 become practically undetectable. **B.** Quantification of images from the same experiment shown in A. Images used for quantification were confocal cross sections of cells. Line profiles across the plasma membrane of the cell were combined to produce average traces of fluorescence intensity. The removal of BG-SS-488 in the MESNa + PI-PLC treated cells is clearly more efficient than removal of SNAP-surface546.

**Figure 2-figure supplement 1. Macropinosomes are readily identified by labelling with sulfo-NHS-SS-biotin.**  Cells were labeled with sulfo-NHS-SS-biotin and transferrin-546, allowed to endocytose at 37˚C for the times indicated, MESNA-treated, fixed, and stained with streptavidin-488. Occasionally, cells contained larger endocytic structures (>0.5µm) that were intensely labeled with fluorescent streptavidin but did not contain transferrin, despite the presence of transferrin in the medium during incubation at 37˚C. These structures correspond to macropinosomes. No clear correlation between their abundance and incubation time at 37°C was observed. Bar is 15m.

**Figure 2-figure supplement 2. Surface-bound transferrin is highly concentrated within clathrin-coated pits.**  Cells were transfected with clathrin light chain-GFP, cooled to 4˚C, labelled with transferrin-546, fixed, and imaged. Bar is 20m. The boxed region is shown in the lower panels.

**Figure 2-figure supplement 3. Quantification of percent co-localisation.** All processing was carried out in Image J. 2 channel raw images were acquired by confocal microscopy. The channels were separated, subjected to Gaussian blur with =0.7, and then contrast adjusted using the histogram of pixel intensities as shown. In the streptavidin / biotin channel, the base of the histogram was used to set pixel intensity=0, maximal pixel intensity was not altered. In the transferrin channel, which is used to generate a binary mask, pixel intensity=0 and maximal pixel intensity were both set to the base of the histogram of pixel intensities as shown. Following dilation of positive pixels in the binary mask a logical “AND” operation was carried out to isolate those pixels in the streptavidin channel that also are positive in the transferrin binary mask. This image was combined with the original biotin image in a 2 colour overlay, and manually drawn regions of interest were used to calculate total pixel intensity in the biotin channel, and total pixel intensity in the same channel from transferrin-positive pixels.

**Figure 2-figure supplement 4. Co-localisation between internalised sulfo-NHS-SS-biotin and transferrin after labelling at 4˚C and 90s of internalisation at 37˚C.** Confocal images of co-internalisation of total membrane protein, labeled at 4˚C with sulfo-NHS-SS-biotin, and transferrin-546. Internalisation was for 90s at 37˚C. Biotin was detected with streptavidin-488 after MESNA treatment. Note that labelling with biotin and transferrin at 4˚C was carried out consecutively, so transferrin was not biotinylated. Zoomed in area of the lower panel is indicated with a box. Bar is 20m.

**Figure 2-figure supplement 5. Co-localisation between internalised sulfo-NHS-SS-biotin and clathrin after 20s and 60s of internalisation at 37˚C**. HeLa cells labeled at 4˚C with sulfo-NHS-SS-biotin, were moved to 37**°**C for the indicated time-points. Surface biotin was removed by MESNA treatment, the cells were fixed and permeabilised, and then stained with streptavidin-488, and antibodies against clathrin heavy chain by indirect immunofluorescence. Box indicates zoomed region, bars are 20m. 20s image also shown in Figure 2.

**Figure 2-figure supplement 6. Total endocytosed protein and transferrin co-localise after 90s uptake in Cos7 and RPE cells**. Confocal images of co-internalisation of total membrane protein, labeled at 4˚C with sulfo-NHS-SS-biotin, and transferrin-546. Internalisation was for 20s at 37˚C. Biotin was detected with streptavidin-488. Note that labelling with biotin and transferrin at 4˚C was carried out consecutively, so transferrin was not biotinylated. Zoomed in area of the lower panel is indicated with a box. Bar is 20m.

**Figure 2-figure supplement 7. Absence of membrane-positive, transferrin-negative vesicles.** **A**. Cells were labelled with the membrane dye FM1-43FX and transferrin at 4˚C, warmed to 37˚C for 90 seconds, plasma membrane dye was removed by washes with ice-cold PBS, and were imaged at 4˚C without fixation. Transferrin-positive puncta that do not contain FM1-43FX are likely to represent clathrin-coated pits that have not budded from the plasma membrane. Contrast levels have been set so that residual background plasma membrane staining with FM1-46FX is excluded. Zoomed in area of the lower panel is indicated with a box. Bar is 20μm. **B**. Quantification of the proportion of FM1-43FX signal detected in intracellular puncta that is present in transferrin-positive pixels. Note that cells where FM1-43FX clearly stained many intracellular membranes due to cell disruption were excluded from the analysis. Bars are mean, SD. Each point is one cell region.

**Figure 3-figure supplement 1. Correlation between streptavidin (total endocytosed protein) and transferrin intensities in endocytic vesicles. A.** Projections of confocal z-stacks showing raw fluorescence images of internalised biotin, labelled with streptavidin, and transferrin, after 90s internalisation. The streptavidin image was used to identify endocytic vesicles using Imaris software as displayed in the lower left panel. The lower right panel displays the software-recognised vesicle objects superimposed on streptavidin and transferrin fluorescence. Bar 15m. **B.** Correlation between the mean fluorescence intensity of streptavidin and transferrin present in individual endosomes. Individual endosomes have has been ranked from high to low streptavidin intensity. Red dots correspond to the transferrin intensity in the particular endosome. Dotted line represents the cut-off for transferrin-positive endosomes, based on 95th percentile of background intensities as described in the main text. Transferrin positive endosomes are within in the greyed area.

**Figure 3-figure supplement 2. Effect of CTB-binding on transferrin intensities in endocytic vesicles.**  Analysis of the transferrin cargo load of endocytic vesicle objects identified as in Figure 3A after 90s of uptake in control and CTB-labeled cells as shown. Frequency distribution of transferrin intensities for the same vesicles after offsetting the transferrin channel by 500nm provides a set of background intensities, shown as a black line and not plotted to the same y-axis scale. Cut-offs are shown as dotted lines and correspond to the 95 percentile for the offset values. The proportion of vesicles that are potentially transferrin-negative (the fraction below the dotted line) is not altered by CTB-binding.

**Figure 4-figure supplement 1.** **Verification of changes in plasma membrane protein levels detected by SILAC.** Flow cytometry was used to analyse plasma membrane abundance of transferrin receptor and CD59. Cells were transfected with control siRNA or siRNA to knock down expression of the alpha adaptin subunit of the AP2 complex (‘AP2 siRNA’) and then labeled at 4˚C with anti-CD59-AlexaFluor647 and Transferrin-546.

**Figure 5-figure supplement 1. Effect of CTB-binding on transferrin intensities in endocytic vesicles defined by uptake of GPI-linked protein.**  Analysis of the transferrin cargo load of endocytic vesicle objects identified as in Figure 3A after 90s of uptake in control and CTB-labeled cells as shown. Cells were expressing SNAP-CD59, endocytic vesicles were defined by BG-SS-488 labelling and MESNa treatment. Frequency distribution of transferrin intensities for the same vesicles after offsetting the transferrin channel by 500nm provides a set of background intensities, shown as a black line and not plotted to the same y-axis scale. Cut-offs are shown as dotted lines and correspond to the 95 percentile for the offset values. The proportion of vesicles that are potentially transferrin-negative (the fraction below the dotted line) is not altered by CTB-binding.

**Figure 6-figure supplement 1. Endocytic structures induced by high dynamin-2-K44A expression. A**. Hela cells stably expressing SNAP-CD59 and transiently transfected with dynamin-2-K44A-dsRed were labeled with BG-SS-488 and transferrin-647 for 15 min 37˚C. Note that the cell shown has a very high level of dynamin-2-K44A expression, and this has induced abundant macropinosomes that are clearly larger than the normal endosomes in neighbouring cells. This is an extreme illustrative example. **B.** Flow cytometry measuring internalisation of SNAP-CD59 in the population of cells transfected with dynamin-2-K44A-dsRed as in A. Note that as the expression of dynamin-2-K44A increases, SNAP-CD59 actually also goes up (Figure 6E).

**Figure 7-figure supplement 1. Labelling of the total population of endocytosed proteins does not provide evidence for significant protein flux through clathrin-independent endocytic pathways. A, B, C.** Confocal images showing distribution of the marker indicated (caveolin 1, flotillin 1, GRAF1, ARF6), together with internalised biotinylated protein after 15min of endocytosis. Labelling was done as in Figure 1A. This is the same experiment as shown in Figure 7, but a longer time-point.

**Figure 8-figure supplement 1.** **Reduction of AP-2 (alpha adaptin) levels affects the amount of uptake of both transferrin and SNAP-CD59, 50 hours after siRNA transfection.** Cells stably expressing SNAP-CD59 were incubated at 37°C for 15 minutes with BG-SS-546 and transferrin-647. Surface label was removed with MESNa and acid wash and then the cells were analysed by flow cytometry.

**Figure 8-figure supplement 2. Incorporation of mutant** **2 subunits into AP2 complexes.** Cells were transfected with plasmids expressing the 2 YXXΦ-binding mutant-myc as shown. Cell lysates were subjected to immunoprecipitation with anti-alpha adaptin, or as a negative control anti-GFP antibodies. Immunoprecipitates were analysed by Western blotting with anti-alpha adaptin and anti-myc antibodies.

**Movie 1**

Object recognition for quantification of cargo load in individual endocytic vesicles. Cells were labelled for 90s at 37˚C with sulfo-NHS-SS-biotin and transferrin-546, after MESNA treatment internalised proteins were labelled with streptavidin-488. 3D reconstructions were obtained from confocal z-stacks of whole cell volumes. Streptavidin channel (green), transferrin channel (red), an overlay of both channels, and then the same overlay with objects recognised as vesicles superimposed in blue, are shown consecutively.