Supplementary Data

Supplementary Equations

The following equations were used in the normalization of fluorescent data. Signals used in the equations below are labeled as $Signal\ (raw\ fluorescence)_{experimental\ conditions}$:

$$Toxicity = 1 - \left[\frac{GFP_{+Inhibitor,\ -siRNA,\ +LF2K}}{GFP_{-Inhibitor,\ -siRNA,\ +LF2K}}\right] \quad \text{(Supplementary Table S2)}$$

$$Relative\ silencing\ (EGFP) = \left[\frac{1 - \left[\frac{GFP_{+Inhibitor,\ +siRNA,\ +LF2K}}{GFP_{+Inhibitor,\ -siRNA,\ +LF2K}}\right]}{1 - \left[\frac{GFP_{-Inhibitor,\ +siRNA,\ +LF2K}}{GFP_{-Inhibitor,\ -siRNA,\ +LF2K}}\right]}\right] - 1 \quad \text{(Figs. 1 and 2 and Supplementary Fig. S1)}$$

$$Relative\ accumulation\ (siRNA) = \left[\frac{\left[\frac{siRNA_{+Inhibitor,\ +siRNA,\ +LF2K}}{siRNA_{+Inhibitor,\ +siRNA,\ -LF2K}}\right]}{\left[\frac{siRNA_{-Inhibitor,\ +siRNA,\ +LF2K}}{siRNA_{-Inhibitor,\ +siRNA,\ -LF2K}}\right]}\right] - 1 \text{(Figs. 1 and 2)}$$

$$Relative\ accumulation\ (siRNA) = \left[\frac{siRNA_{+p(endocytitc\ protein),\ +siRNA}}{siRNA_{+pd2EGFP-N1,\ +siRNA,\ +siRNA}}\right] - 1 \quad \text{(Fig. 3)}$$

Endocytic Inhibitor Toxicity and Dose Response

Each endocytic inhibitor was evaluated over a range of concentrations for each cell line to assess both toxicity and dose response. EGFP-expressing cells were seeded in 96-well plates at 20,000 cells/well (40,000 cells/well for HepG2 cells) in 100 μL of antibiotic-free Dulbecco's modified Eagle's medium (DMEM)/fetal bovine serum (FBS). After 23 h, cells were washed with DMEM and incubated for 1 h in DMEM containing inhibitors. For toxicity assessment, cells were then treated with 50 μL Opti-MEM (Supplementary Table S3). For dose response, cells were transfected with 50 μL of solution containing Opti-MEM, small interfering RNA (siRNA), and Lipofectamine 2000 (LF2K), yielding final concentrations of 100 nM siRNA and 2.3 μg/mL LF2K (Supplementary Fig. S1). Cells were washed 4 h post-transfection with antibiotic-free DMEM/FBS and incubated in heparin sulfate solution for 5 min to remove extracellular siRNAs. The heparin sulfate solution was subsequently removed and replaced with antibiotic-free DMEM/FBS. At 24 h post-transfection, cells were washed with Dulbecco's phosphate-buffered saline (DPBS; +Mg/Ca) and analyzed using a BioTek Synergy H1 plate reader. All incubations were conducted at 37°C, 5% CO_2, and 100% humidity.

Confocal Microscopy

For the cellular images of the inhibitor experiments, cells were fixed 24 h post-transfection, using a 2% paraformaldehyde solution, and stored in DPBS (+Mg/Ca) at 4°C. Confocal images were taken using a Nikon A1 laser scanning confocal microscope. Nikon Plan Apo 20×/.75NA and Apo 60×/1.4NA objectives were used to acquire all images. EGFP (488/530) fluorescence was measured using an excitation of 488 nm with a multiline Argon laser and displayed as green. Dy547-tagged siRNA (557/574) fluorescence was excited at 560 nm by a HeNe laser and displayed as red. The focal plane for each image was chosen to include the highest intensity EGFP fluorescence and maintained using the Nikon Perfect Focus System. All images were collected sequentially as single *XY* images and used two count Line Kalman averaging. For overexpression images (Supplementary Fig. S2), cells were fixed 1 h after siRNA transfection using a 2% paraformaldehyde solution and stored in DPBS (+Mg/Ca) at 4°C. Confocal images were taken as above.

Supplementary References

S1. Rappoport JZ, BW Taha, S Lemeer, A Benmerah and SM Simon. (2003). The AP-2 complex is excluded from the dynamic population of plasma membrane-associated clathrin. J Biol Chem 278:47357–47360.

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S3. Fork C, J Hitzel, BJ Nichols, R Tikkanen and RP Brandes. (2014). Flotillin-1 facilitates toll-like receptor 3 signaling in human endothelial cells. Basic Res Cardiol 109:439.

S4. Lundmark R, GJ Doherty, MT Howes, K Cortese, Y Vallis, RG Parton and HT McMahon. (2008). The GTPase-activating protein GRAF1 regulates the CLIC/GEEC endocytic pathway. Curr Biol 18:1802–1808.