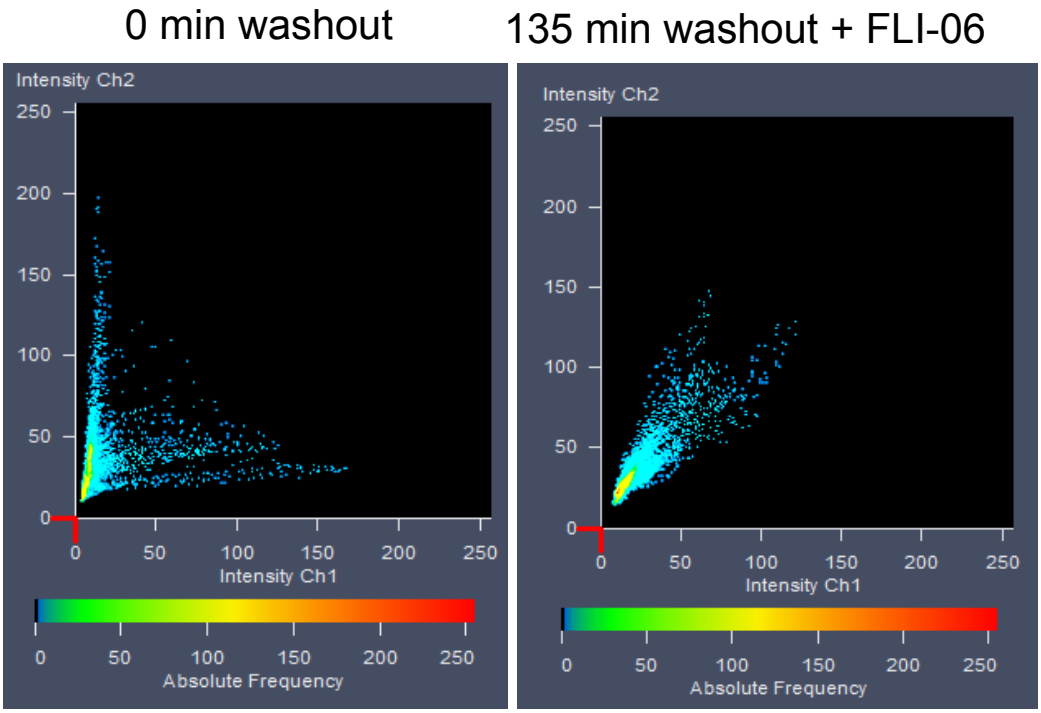


Fig.S1A: Related to Fig. 5. Scatterplots of pixel intensities of ROIs shown in Fig. 5, 0min and 135 min washout with FLI-06. Such data were used to calculate the overlap coefficients in Fig. 5B according to Manders et. al., 1993, J. Microscopy 169. Shown are screenshots from Zen software. Left, low colocalization; right, high colocalization.

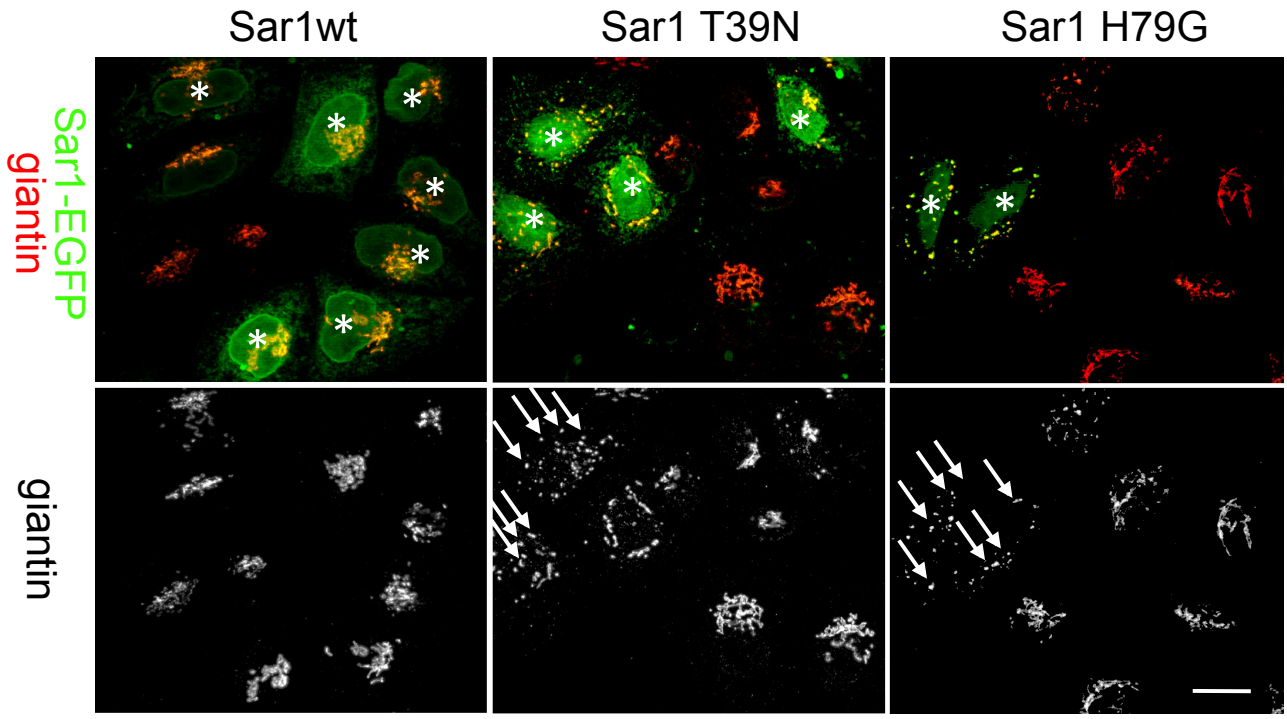


Fig. S1B: Related to Fig. 6D. Sar1-EGFP mutants disrupt the Golgi. HeLa cells were transfected with the indicated constructs, incubated for 24h, fixed and stained with giantin antibodies. Asterisk indicates transfected cells, arrows indicate fragmented Golgi. Overexpression of T39N and H79G mutants disrupt the Golgi, indicating they act in a dominant-negative manner as expected. Only highly overexpressing cells show a Golgi phenotype, lower expressing cells have intact Golgi (not shown). Scalebar 10 µm.

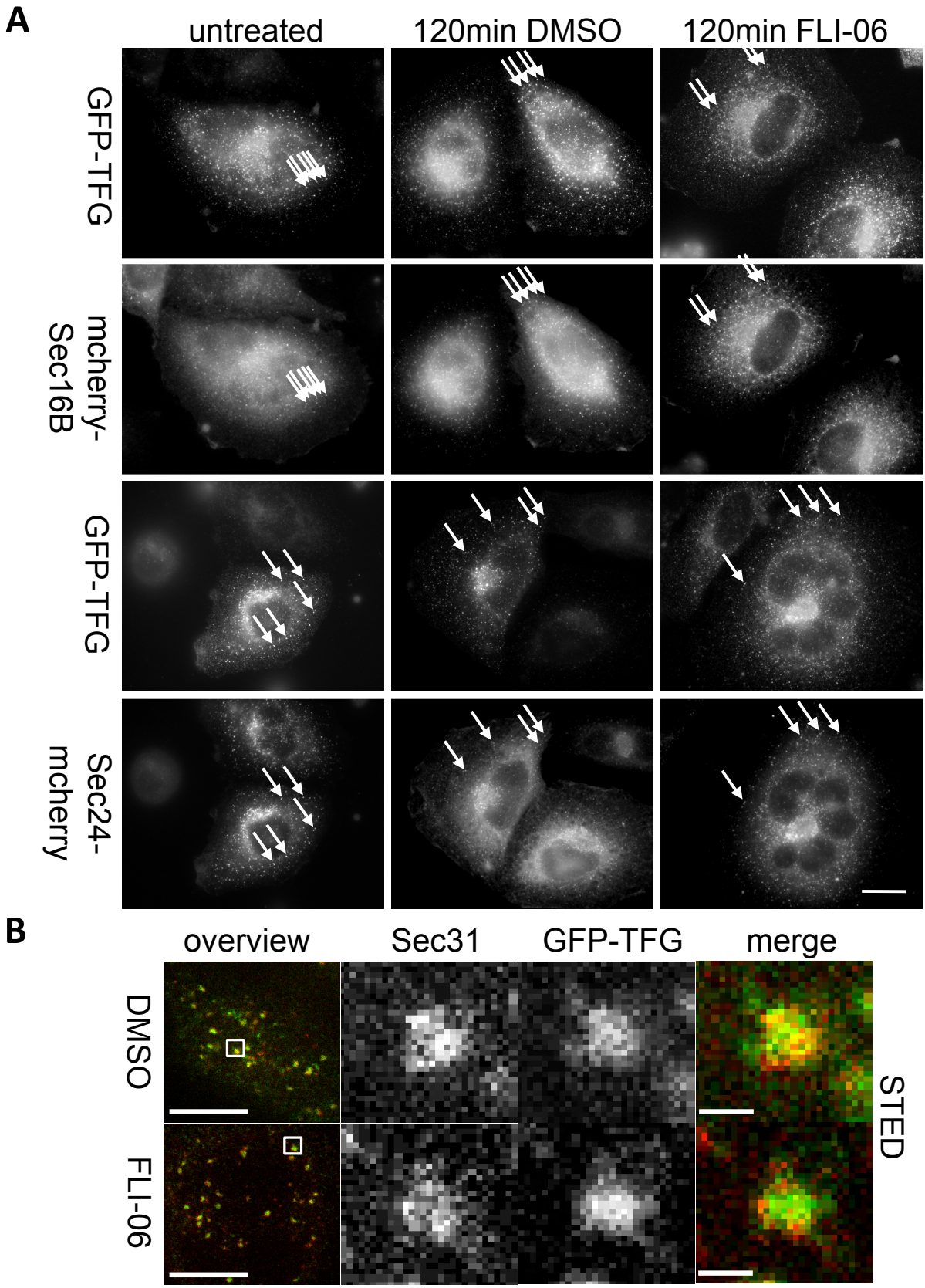


Fig. S2: COPII components persist at ERES in the absence of cargo. HeLa cells transiently co-transfected with GFP-TFG and mcherry-Sec16B (A, top) or with GFP-TFG and Sec24-mcherry (A, bottom) or GFP-TFG (B) were incubated with DMSO or FLI-06 for 120 min (A) or 60min (B), fixed and mounted for conventional immunofluorescence microscopy (A) or STED superresolution microscopy (B). Arrows indicate colocalization. White boxes in (B) in the overviews are magnified on the right. Scalebar in (A): 10 µm, in (B) overview: 5 µm, and in (B) magnified boxes: 100 nm. In all conditions, no differences after FLI-06 treatment are observed.

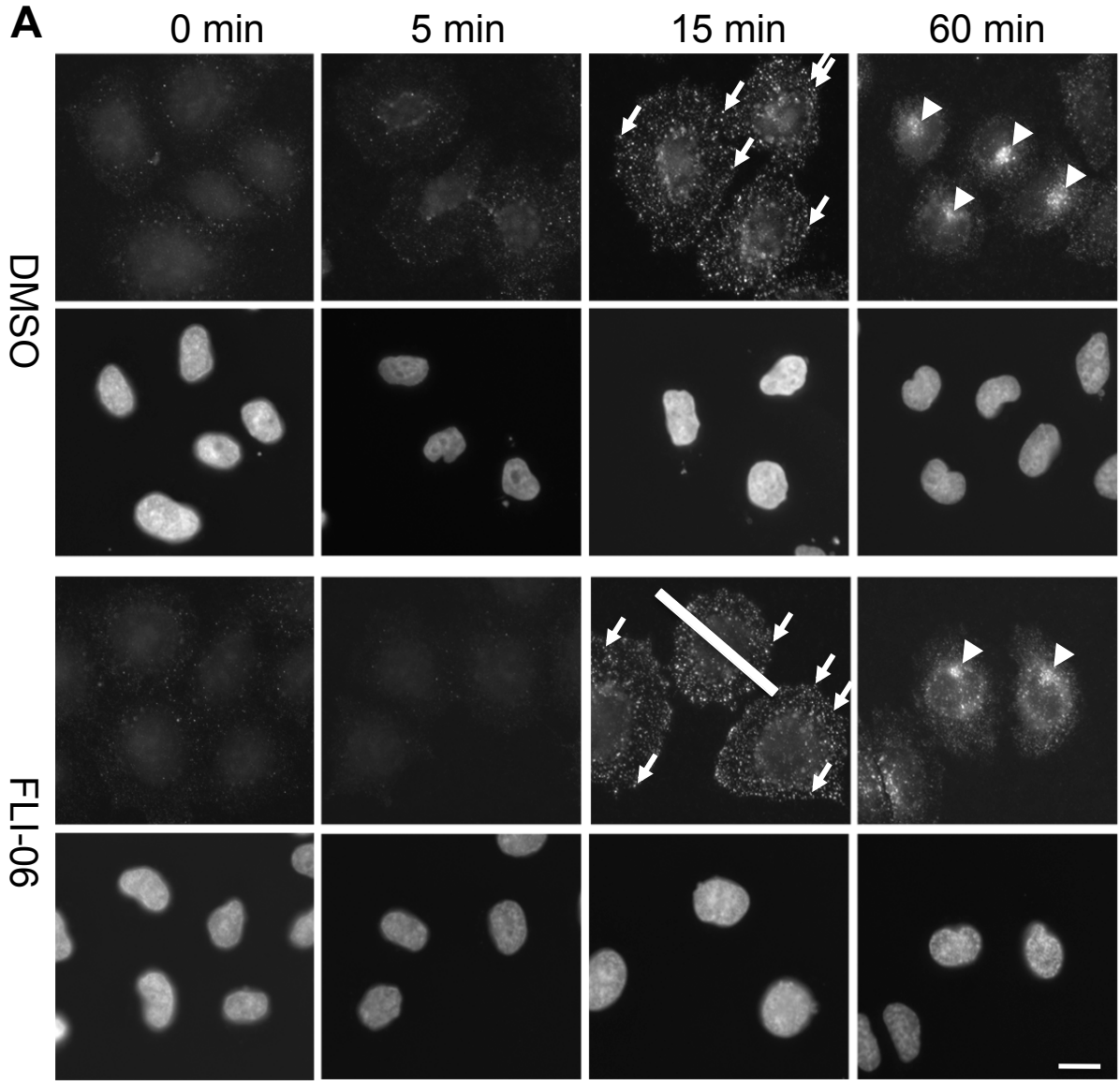


Fig. S3A: FLI-06 does not inhibit endocytosis nor transport from early to late endosomes. Hela cells grown on coverslips were starved in serum-free medium for 30min and incubated on ice for 30min in 25 µg/ml transferrin (Tf)-alexa555 and 10 µM FLI-06 or DMSO. After change to serum-containing pre-warmed medium with 10 µM FLI-06 or DMSO, cells were chased at 37°C for indicated times. Thereafter cells were stripped of PM-resident transferrin, fixed, the nuclei stained with DAPI and imaged by fluorescence microscopy. Representative cells from at least n=3 independent experiments are shown. Arrows, peripheral early endosomes; arrowheads, central late endosomes. Scalebar 10 µm.

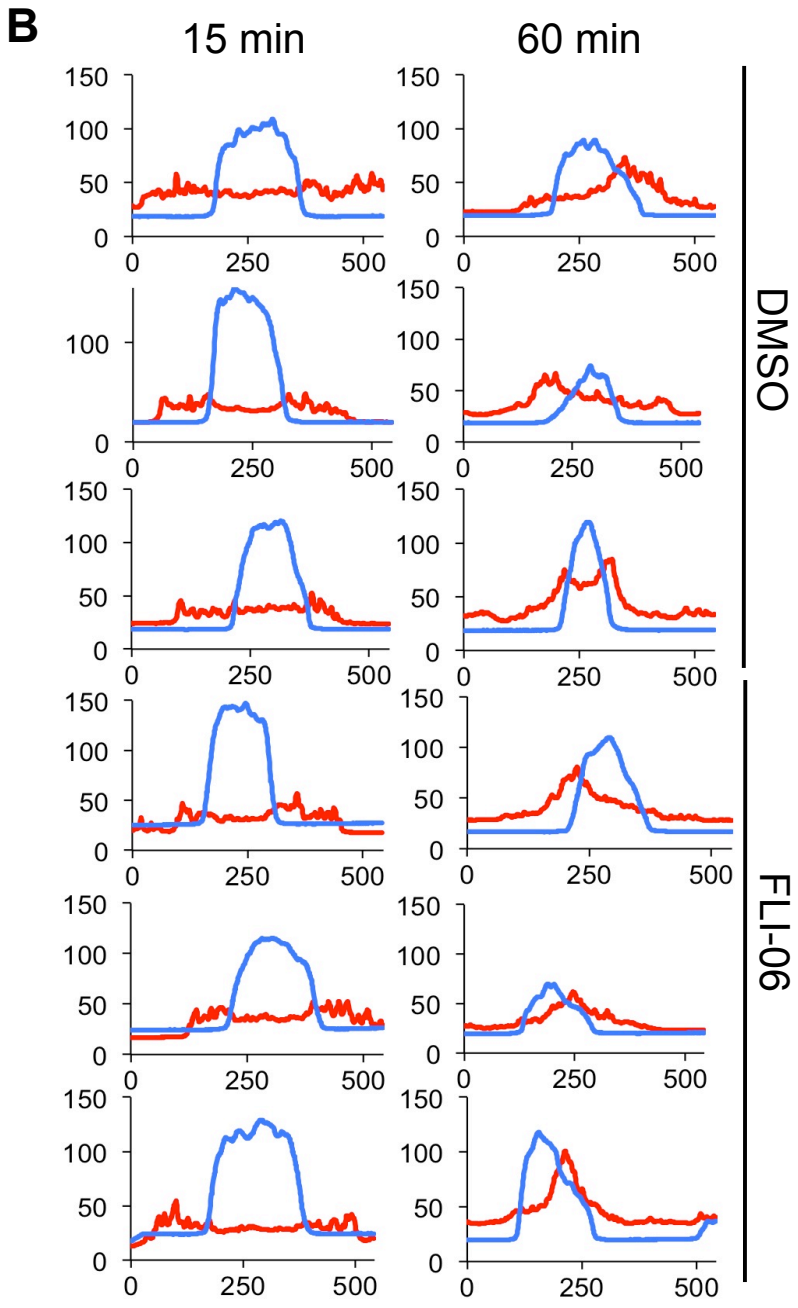
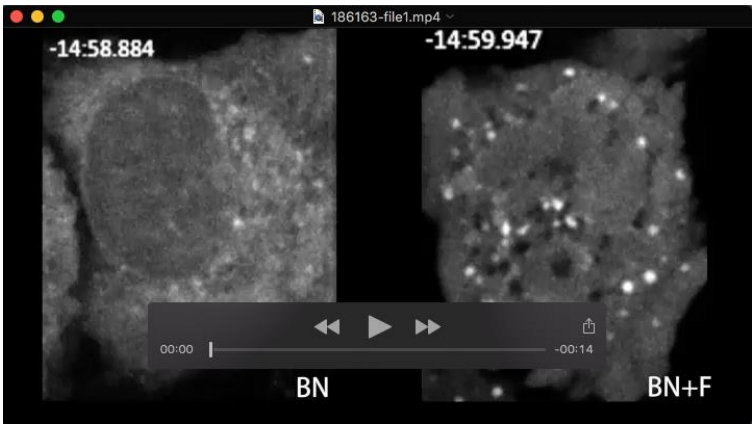


Fig. S3B: FLI-06 does not inhibit endocytosis nor transport from early to late endosomes. Typical intensity profiles of bars as exemplified in S3A (white bar in A) after 15 and 60min Tf-uptake. At 15min uptake there is an even distribution of Tf-alexa555 fluorescence (red) throughout the cell, indicative of early endosomes, whereas at 60 min a peak near the nucleus (blue) is observed, indicating transport to late endosomes. No differences in intensity profiles between DMSO- and FLI-06 are observed, indicating FLI-06 does not change uptake into endosomes nor transport from early to late endosomes. Blue, DAPI; red, Tf-Al555.



Movie 1. Corresponding to Fig. 3. FLI-06 does not disrupt existing VSVG-EYFP-containing ERES. Hela cells transfected with VSVG-EYFP were incubated at 40°C over night, then treated with BFA (B) and nocodazole (N) for 20-30 min on ice. Cells were shifted to 32°C and incubated for 45 min to induce gERES. After 45 min incubation at 32°C, cells were incubated with BN or BN with 10 mM FLI-06 (BN+F). Images were taken every 5 sec on a Zeiss spinning disk confocal microscope. The arrow in the BN treated cell indicates an ERES with the typical increase-collapse-increase intensity profile, the arrowhead in the BN+F condition a collapsing ERES with no new cargo recruitment.

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Movie 2. Corresponding to Fig. 5. FLI-06 does not inhibit export of pre-accumulated cargo. Hela cells transfected with VSVG-EYFP and Sec24-mCherry were incubated at 40°C over night, then treated with GCA and nocodazole for 20-30 min on ice. Cells were shifted to 32°C and incubated for 45 min to induce gERES. After 45 min incubation at 32°C, GCA and nocodazole were washed out and incubated with 10 mM FLI-06 at 32°C. Images were taken every 5 sec on a Zeiss spinning disk confocal microscope. Arrows indicate VSVG-EYFP leaving Sec24-mCherry labelled ERES in the presence of FLI-06.

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