Supplementary Text

The DISplay Mechanism

Assuming that the dimerization of Golgi- and PM-localizing proteins changes their intracellular trafficking patterns, we tested different pairs of FKBP and FRB fusion proteins (fig. S1). For anchoring to the Golgi, we used giantin, glucosylceramide synthase, and TGN38 (*9*), whereas for anchoring to the plasma membrane (PM), we used Lyn (*10*) and the CAAX tail of K-Ras (*33*). Among all of the combinations that we tested, the pair of giantin and the CAAX gave the most distinct contrast in localization before and after the addition of rapamycin. We assume that this difference can be explained by the idea that heterodimerization on the Golgi initiates the secretion process. According to this idea, K-Ras, which cycles between the PM and Golgi (*9*), can be rapidly trapped on the Golgi by dimerization with the giantin construct, which results in heterodimers likely beginning to translocate to the PM. This idea is consistent with the observation that FRB-CAAX was transiently accumulated at the Golgi immediately after the addition of rapamycin addition (<10 min), which was followed by a gradual increase in the abundance of FYG-Myc at the PM (15 to 60 min after addition) (fig. S2). FYG-Myc may also traffic between the PM and Golgi and get trapped by FRB-CAAX at the PM in the presence of rapamycin. However, this contribution to cell-surface display seems marginal given that FYG-Myc exhibited only a minor increase in abundance at the PM, when Lyn, which does not cycle between the Golgi and PM, is used to anchor to the PM. Therefore, the cell-surface display of FYG-Myc is primarily achieved by two sequential steps: (i) dimerizing to FRB-CAAX at the Golgi, and (ii) vesicular trafficking to the PM as a dimerized complex. Note that the transient accumulation of RCh-CAAX at the Golgi was observed immediately after the addition of rapamycin (fig. S3C), which requires dimerization with its counterpart at the Golgi, such as FYG-Myc (fig. S2B). These results suggest that FYG-Myc briefly traps RCh-CAAX at the Golgi before the heterodimerized complex can enter the conventional secretion pathway to be delivered to the PM. To further investigate the vesicular trafficking mechanism of DISplay, we next repeated the DISplay procedure in the presence of BFA and monensin, both of which inhibit the conventional secretory pathway. Whereas BFA blocks GTP-GDP exchange in the ADP-ribosylation factor and promotes retrograde transport from the Golgi to the endoplasmic reticulum (ER), monensin acts as an ionophore and inhibits the transport of cargo proteins at Golgi. Consistent with this, BFA (30 μg/ml) localized FYG-Myc mainly to the ER. Subsequent treatment with rapamycin for 60 min led to Myc staining at the PM in permeabilized, but not non-permeabilized, cells (fig. S9), suggesting that the FYG-Myc–containing retrograde vesicles traffic to the periplasma membrane region, but not the outer leaflet of the PM. Treatment with 10 μM monensin, which severely affected Golgi morphology as expected, resulted in marked inhibition of the cell-surface display of FYG-Myc (fig. S10). Furthermore, we confirmed that the addition of rapamycin at 4°C to block membrane transport also impaired the cell-surface display (fig. S11). These observations led us to think that the DISplay at least partially uses the conventional secretory pathway.

A

		Plasma Membrane			Golgi
		–	Lyn-FRB	FRB-CAAX	FRB-Giantin
Golgi	FKBP-Giantin	G	G+PM	PM	G
	FKBP-Glucosylceramide synthase	G+PM	G+PM	G+PM	
	FKBP-TGN38	G	G+PM	G+PM	

B

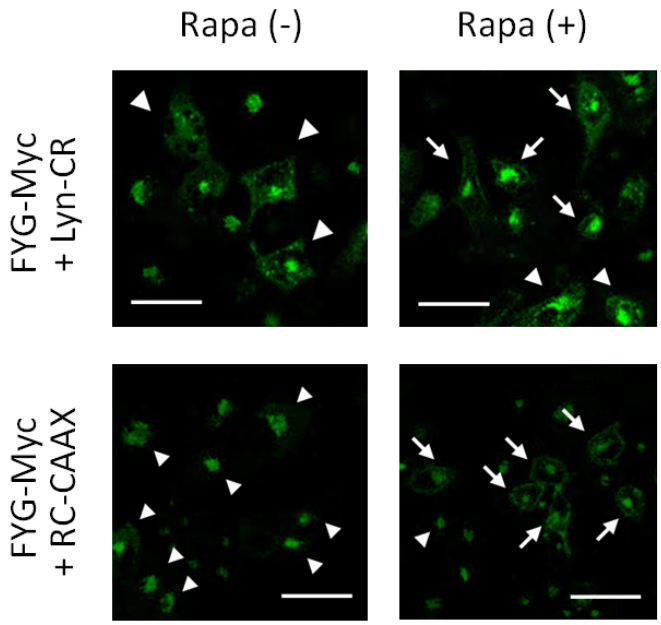

Fig. S1. Analysis of various constructs for rapamycin-dependent dimerization. Based on our idea that Golgi-anchored protein should be transported to the plasma membrane (PM) by binding to a PM-localizing protein, we tested various heterodimerization construct pairs. Only the pair consisting of giantin and FRB-CAAX localized exclusively on the PM after the addition of rapamycin. (**A**) Localization of Golgi-anchored proteins that formed hererodimers with PM-targeting proteins after the addition of rapamycin. G, PM, and G+PM indicate that protein localization was mostly at the Golgi, mostly at the PM, or distributed between the Golgi and PM, respectively. (**B**) HeLa cells were transfected with plasmids encoding FYG-Myc (green) and either Lyn-CFP-FRB (Lyn-CR) or FRB-CFP-CAAX (RC-CAAX) and then were left untreated or were treated with rapamycin before being analyzed by confocal microscopy. Scale bar: 50 μm. White arrowheads indicate cells that expressed both constructs, but did not exhibit the PM localization of FYG-Myc, whereas white arrows indicate those cells that exhibited PM localization of FYG-Myc. Data are representative of three independent experiments.

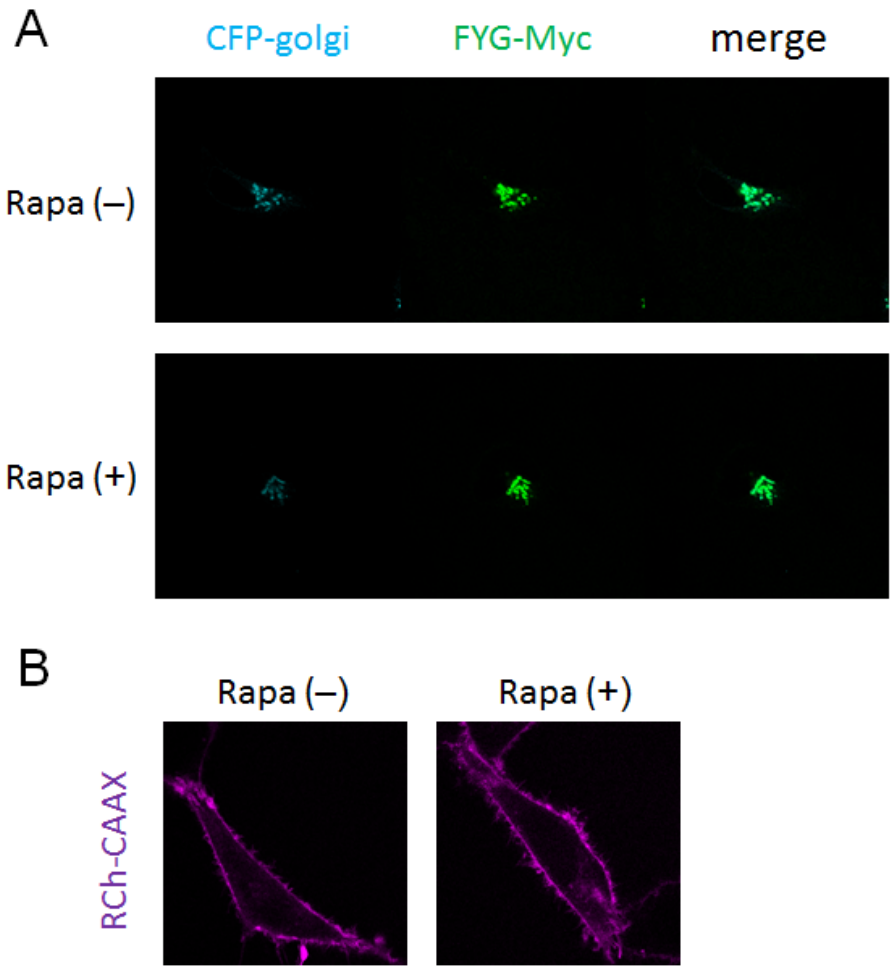

Fig. S2. Rapamycin has no side effects on protein localization. (**A**) HeLa cells transfected with plasmids encoding a Golgi marker fused to CFP (CFP-golgi, cyan) (*9*) and FYG-Myc (green) were left untreated or were treated with rapamycin before being analyzed by fluorescence microscopy. (**B**) HeLa cells transfected with plasmid encoding RCh-CAAX were left untreated or were treated with rapamycin before being analyzed by fluorescence microscopy. Data are representative of three independent experiments.

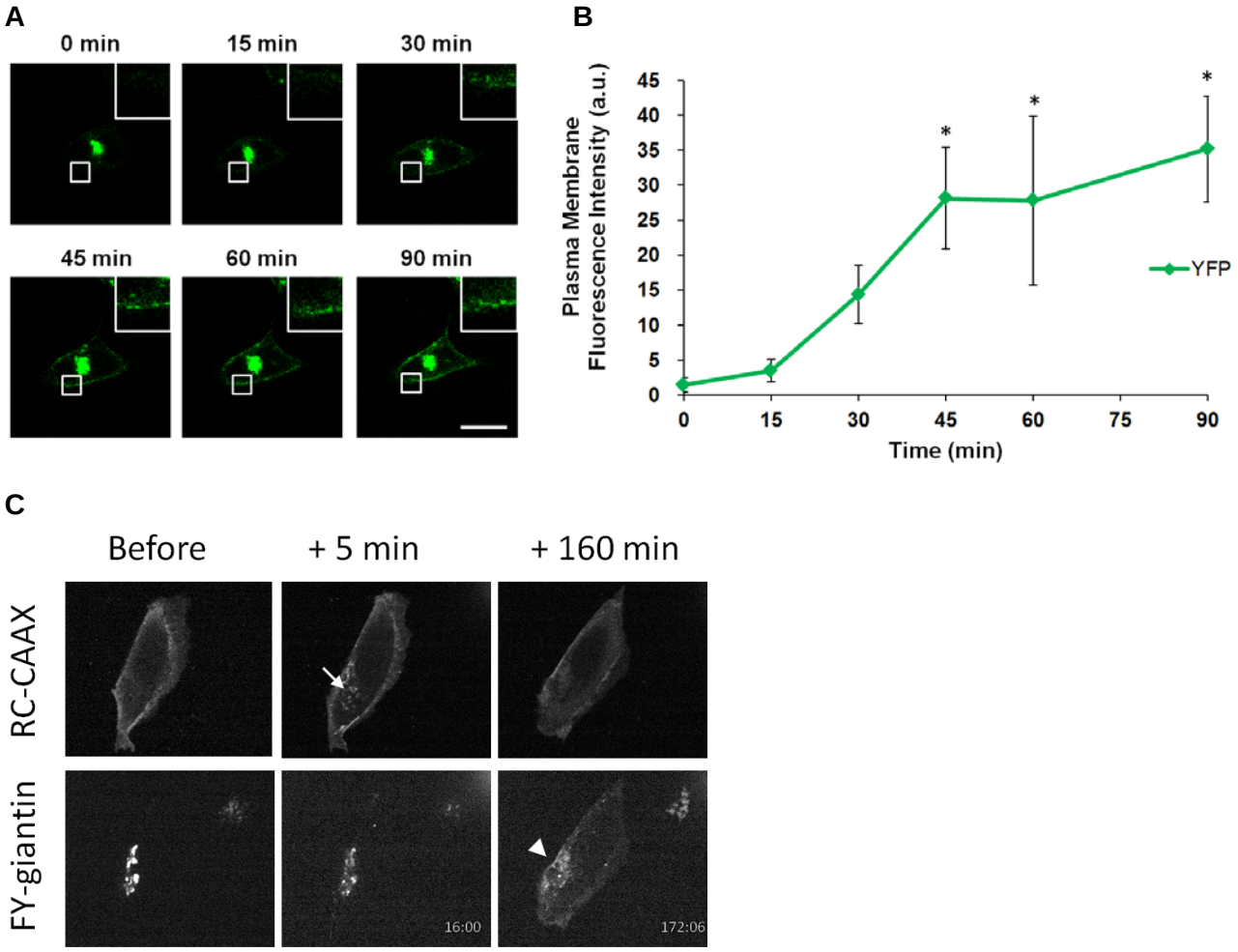

Fig. S3. Analysis of the dynamics and kinetics of the DISplay procedure. (**A**) Fluorescence micrographs of HeLa cells were transfected with plasmids encoding FYG-Myc (green) and RCh-CAAX were analyzed by fluorescence microscopy after 7.5 min after the addition of rapamycin. Scale bar: 20 μm. (**B**) Analysis of the kinetics of the translocation of FYG-Myc to the PM. Data are means ± SD from three independent experiments. **P* < 0.05 compared to the 0-min time point by Student's *t*-test. (**C**) HeLa cells were transfected with plasmids encoding FY-giantin (bottom) and RC-CAAX (top) and then were left untreated or were treated with rapamycin for the indicated times before being analyzed by fluorescence microscopy. The arrow indicates the accumulation of RC-CAAX on the Golgi, whereas the arrowhead indicates the accumulation of FY-giantin at the cell surface. Data are representative of three independent experiments.

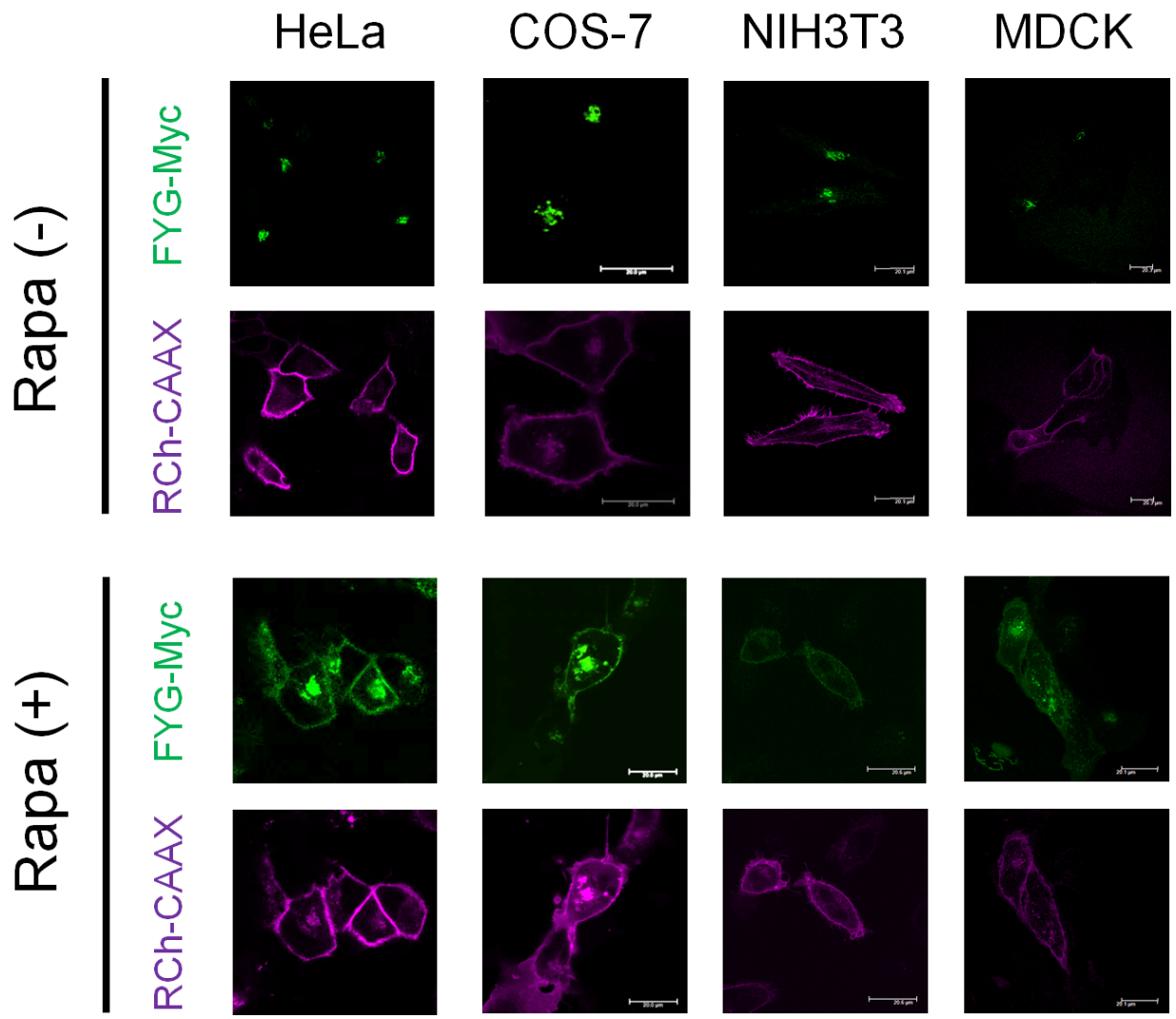

Fig. S4. The DISplay can be used with different cell types. Fluorescence micrographs of HeLa, COS-7, NIH3T3, and MDCK cells transfected with plasmids encoding FYG-Myc (green) and RCh-CAAX (magenta) were treated with rapamycin or DMSO as a control, as indicated, before being analyzed by confocal microscopy. Scale bar: 20 µm. Data are representative of 5 independent experiments for HeLa, COS7, and MDCK cells, and three independent experiments for NIH3T3 cells.

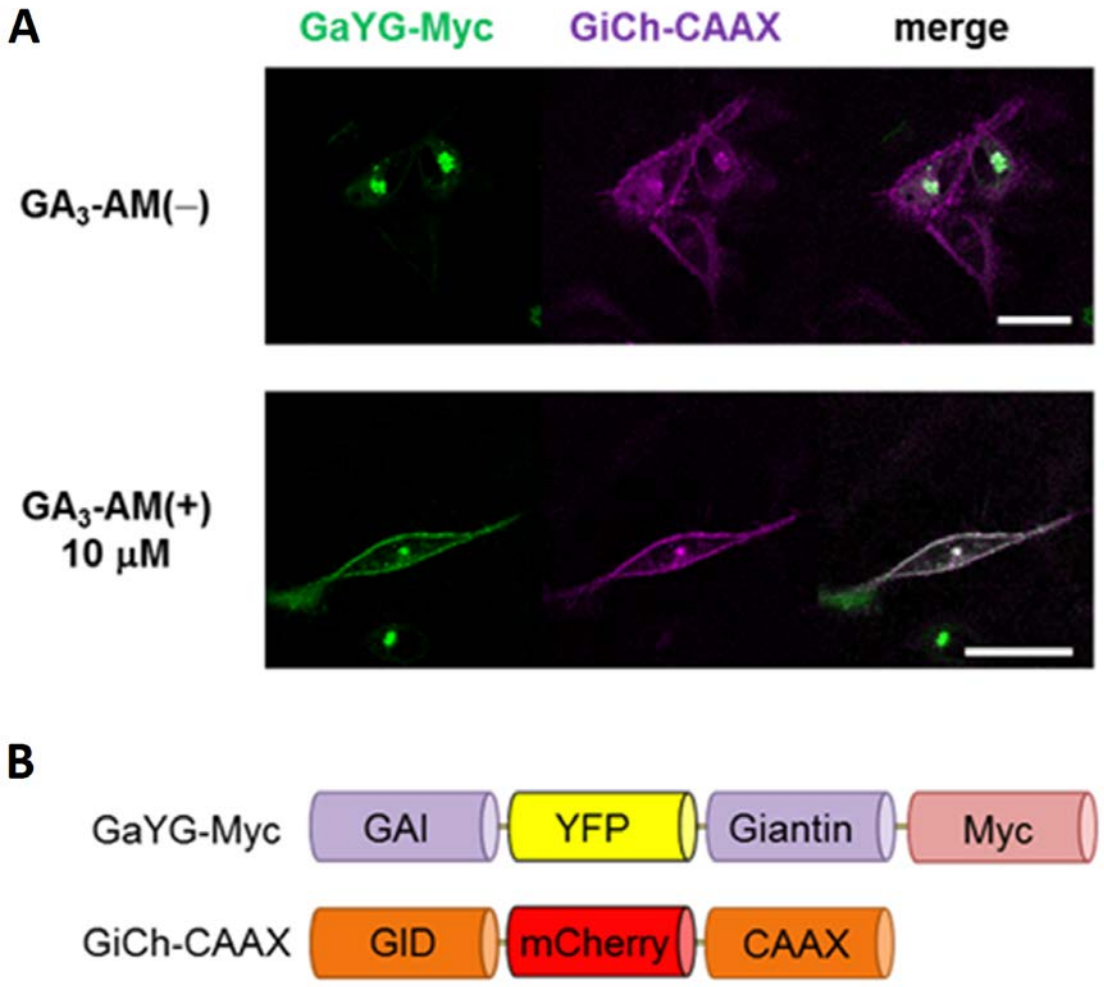

Fig. S5. Gibberellin-based dimerization system. (**A**) HeLa cells transfected with plasmids encoding GAIs-YFP-Giantin-Myc (GaYG-Myc, green) and GID1-mCherry-CAAX (GiCh-CAAX, magenta), a construct pair designed for the gibberellin (GA_3-AM)-based dimerization system, were left untreated or were treated with 10 μM GA_3-AM before being analyzed by confocal microscopy. Scale bar: 20 μm. (**B**) Schematic representation of the GaYG-Myc and GiCh-CAAX constructs. Data are representative of three independent experiments.

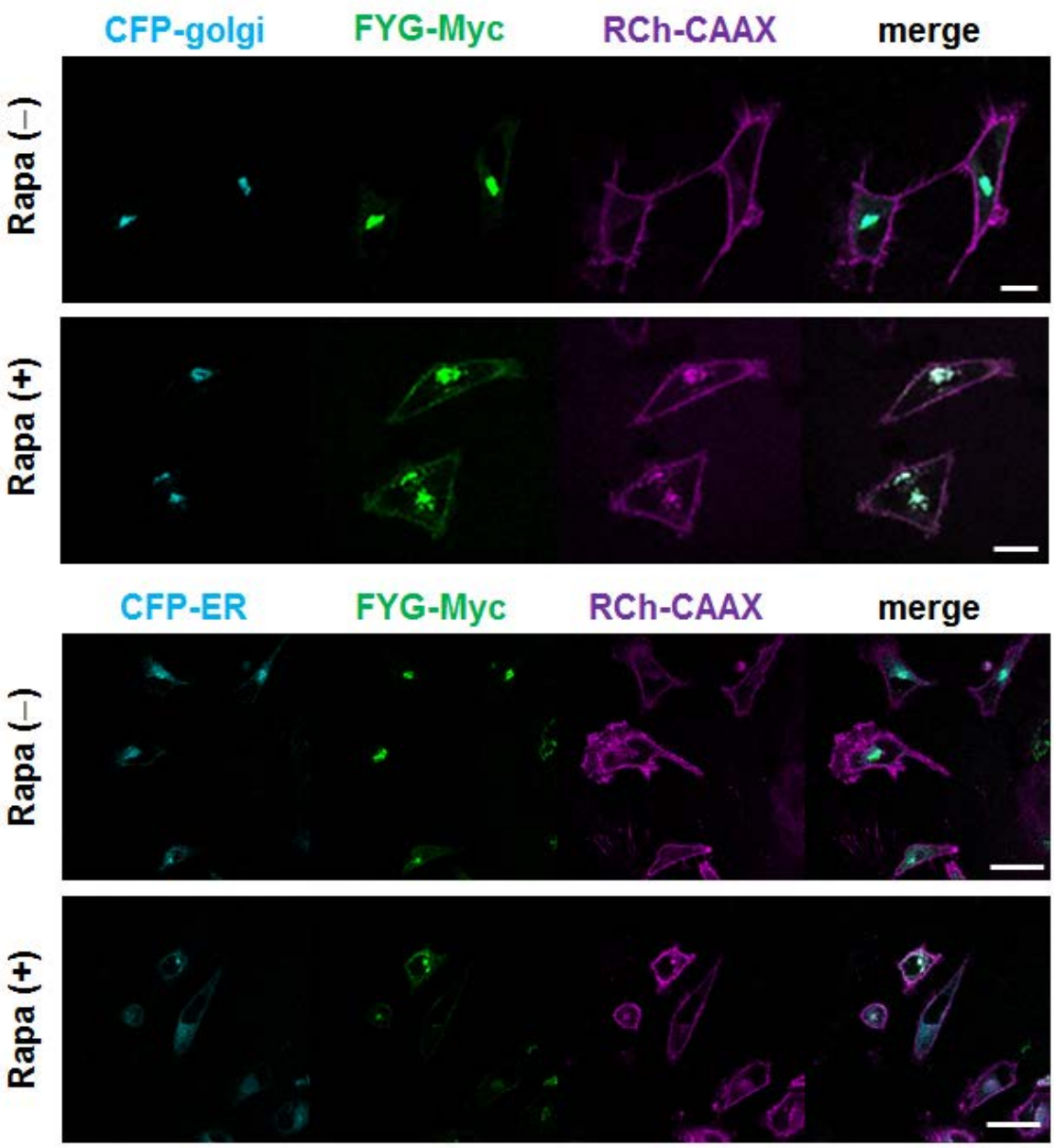

Fig. S6. Analysis of the localization of Golgi-anchored proteins and the effect of rapamycin on organelles. HeLa cells transfected with plasmids encoding FYG-Myc (green), RCh-CAAX (red), and either CFP-golgi or an ER marker fused to CFP (CFP-ER) (cyan) were left untreated or were treated with rapamycin before being analyzed by confocal microscopy. Scale bar: 30 μm. Data are representative of four independent experiments.

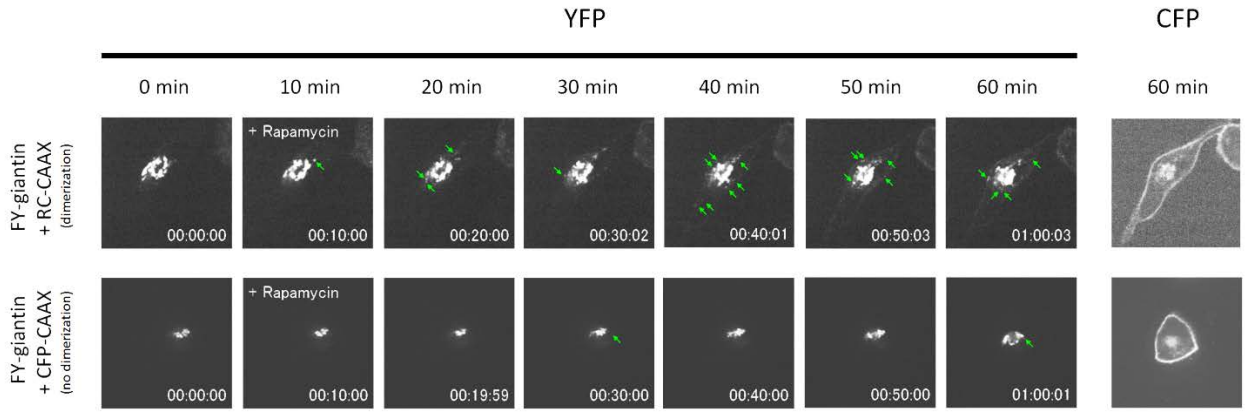

Fig. S7. Analysis of intracellular vesicles formed de novo after dimerization of FYG-Myc and RC-CAAX. MDCK cells transfected with plasmids encoding the indicated constructs were treated with rapamycin and analyzed by fluorescence microscopy at the indicated times. Top: The formation of vesicles containing the giantin TMD was monitored every 10 min in cells expressing FYG-Myc (left panels, time-lapse images every 10 min) and RC-CAAX (rightmost panel, images at 60 min). The newly formed vesicles that were not detected in the previous time points are indicated by green arrows. Rapamycin was added at 10 min. These images correspond to Movie S2. Bottom: The formation of vesicles containing Giantin TMD was monitored in cells in the manner described earlier except for the plasmids used for the transfection: FYG-Myc (left panels) and CFP-CAAX (rightmost panel). These images correspond to Movie S3. Data are representative of three independent experiments.

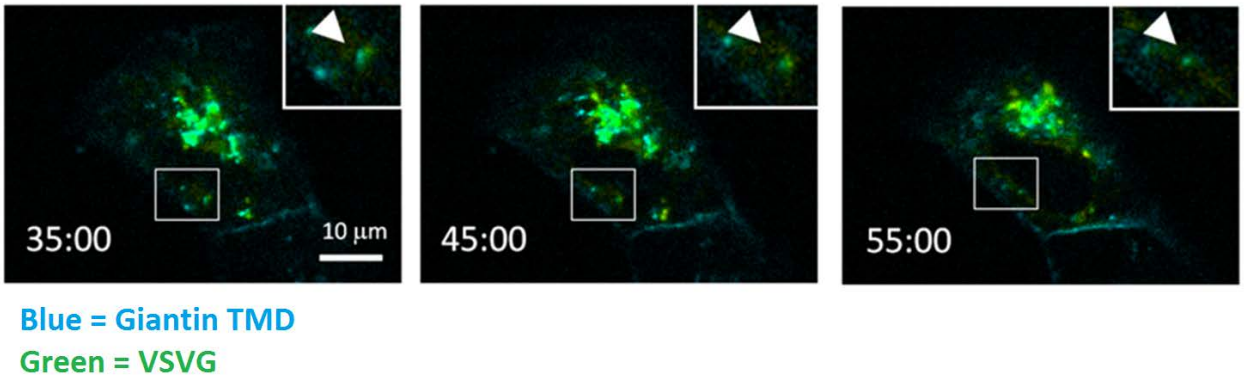

Fig. S8. Colocalization of FY-Giantin and VSVG protein. MDCK cells transfected with plasmids encoding FKBP-CFP-Giantin TMD (green) and VSVG-YFP (red) were treated with rapamycin and analyzed by fluorescence microscopy at the indicated time to detect secretory vesicles (arrowhead) that contained both proteins. The cells were kept at the non-permissive temperature (37°C) to retain VSVG-YFP at the endoplasmic reticulum (ER) and Golgi until the time-lapse imaging was performed at the permissive temperature (25°C). Scale bar: 10 µm. Data are representative of two independent experiments.

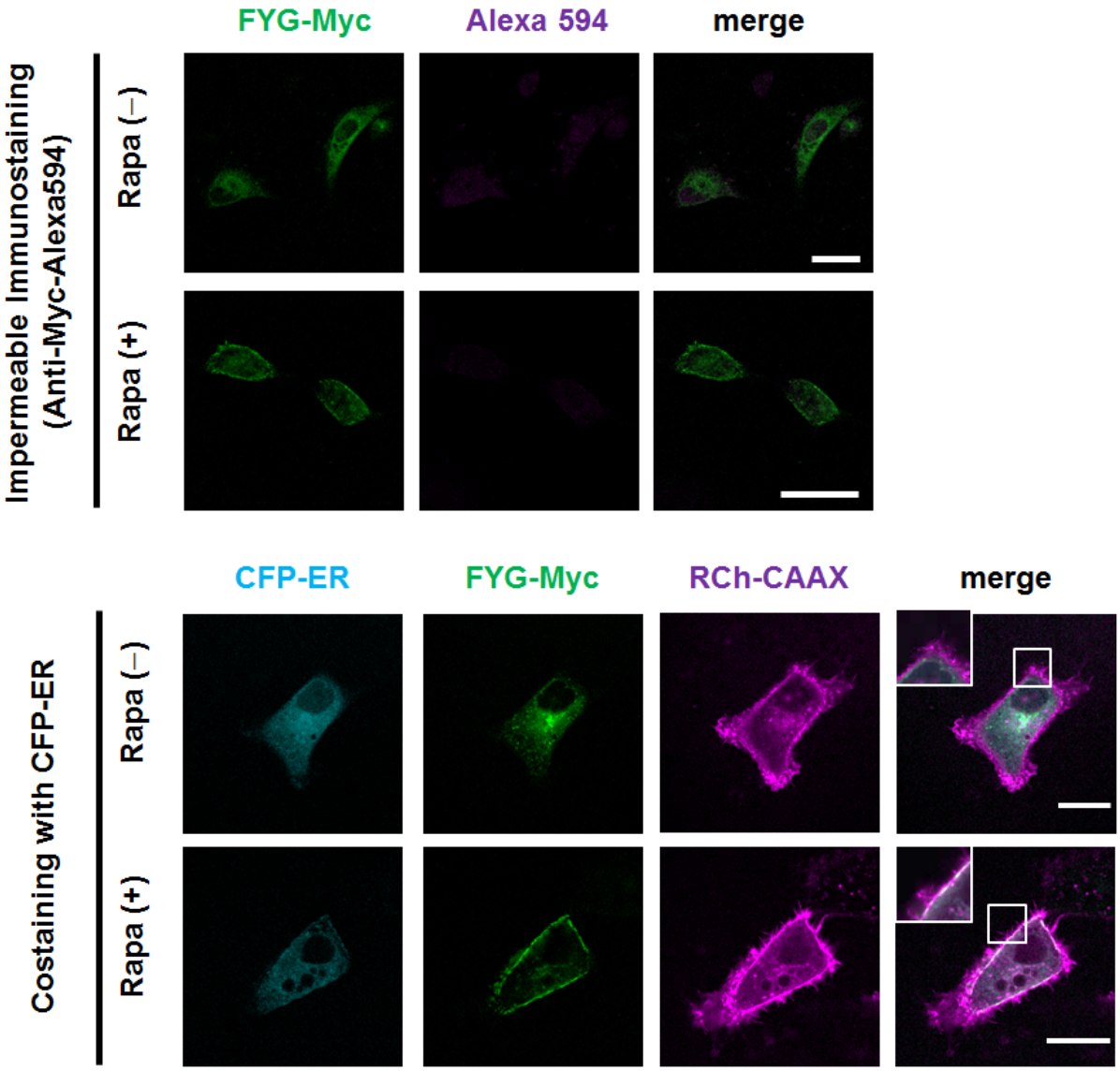

Fig. S9. Localization of Golgi-anchored protein in HeLa cells treated with Brefeldin A. Top: HeLa cells transfected with plasmids encoding FYG-Myc (green) and RCh-CAAX were incubated with Alexa Fluor 594–conjugated anti-Myc antibody (magenta) under non-permeabilized conditions and then were analyzed by fluorescence microscopy. Cells were treated with Brefeldin A (30 μg/m) for 1 hours before the addition of rapamycin. Bottom: HeLa cells transfected with plasmids encoding FYG-Myc (green), RCh-CAAX (magenta), and CFP-ER (cyan) were treated with Brefeldin A (30 μg/ml) for 1 hour before the addition of rapamycin and then were analyzed by fluorescence microscopy. Scale bar: 20 μm. Data are representative of five independent experiments.

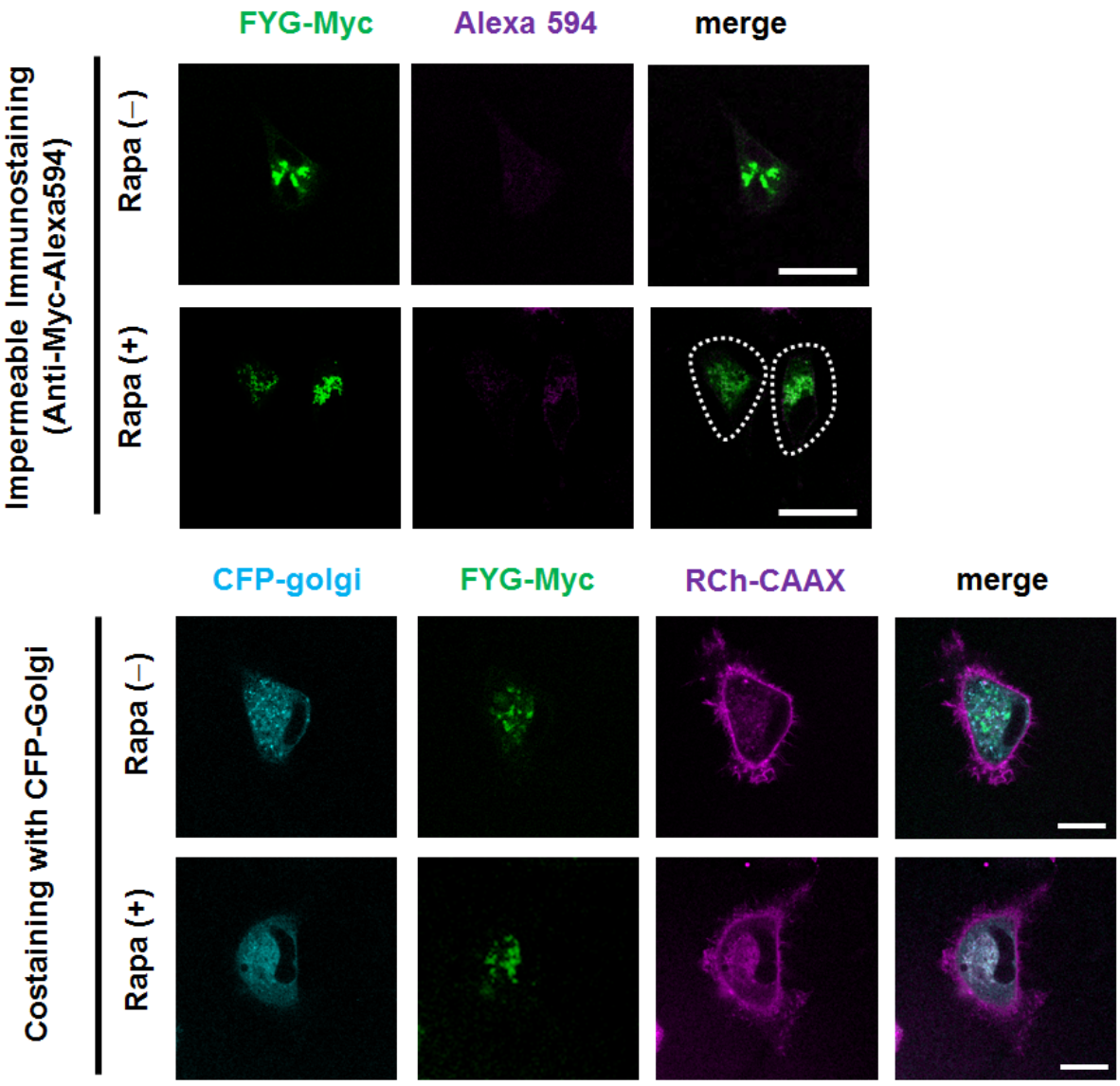

Fig. S10. Localization of Golgi-anchored protein in HeLa cells treated with monensin. Top: HeLa cells transfected with plasmids encoding FYG-Myc (green) and RCh-CAAX were treated with Alexa Fluor 594–conjugated anti-Myc antibody (magenta) under non-permeabilized conditions and then were analyzed by fluorescence microscopy. Cells were treated with 10 μM monensin for 3 hours before the addition of rapamycin. Bottom: HeLa cells transfected with plasmids encoding FYG-Myc (green), RCh-CAAX (magenta), and CFP-Golgi (cyan) were treated with 10 μM monensin for 3 hours before the addition of rapamycin and then were analyzed by fluorescence microscopy. Scale bar: 20 μm. Data are representative of five independent experiments.

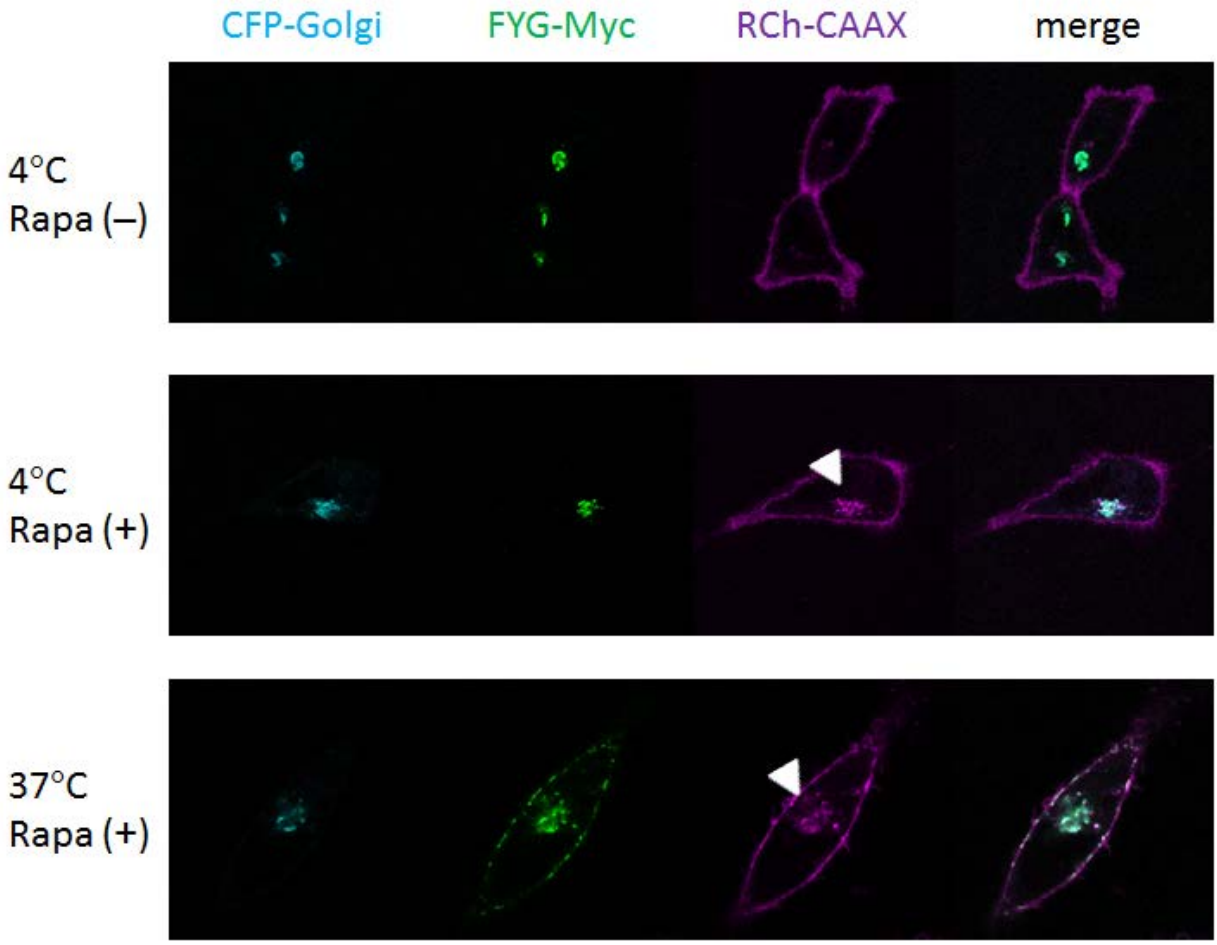

Fig. S11. Localization of Golgi-anchored protein in HeLa cells at 4°C. HeLa cells transfected with plasmids encoding FYG-Myc (green), RCh-CAAX (magenta), and CFP-Golgi (cyan) were maintained at 4 or 37°C from 10 min before the addition of rapamycin and then were analyzed by fluorescence microscopy. Data are representative of three independent experiments.

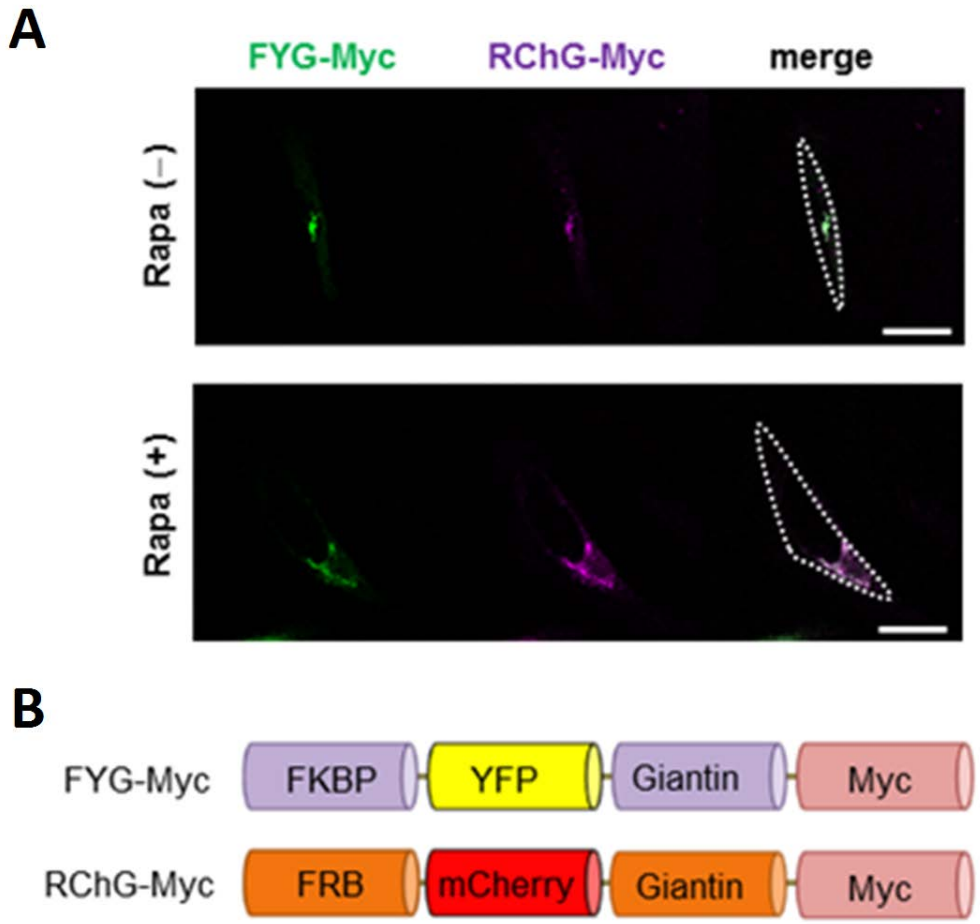

Fig. S12. Dimerization of Golgi-anchored protein constructs. (**A**) HeLa cells transfected with plasmids encoding FYG-Myc (green) and RChG-Myc (magenta), a pair Golgi-anchored protein constructs that can undergo dimerization, were left untreated or were treated with rapamycin before being analyzed by fluorescence microscopy. Scale bar: 20 μm. White dotted lines indicate the cell periphery. Data are representative of three independent experiments. (**B**) Schematic representation of FYG-Myc and RChG-Myc.

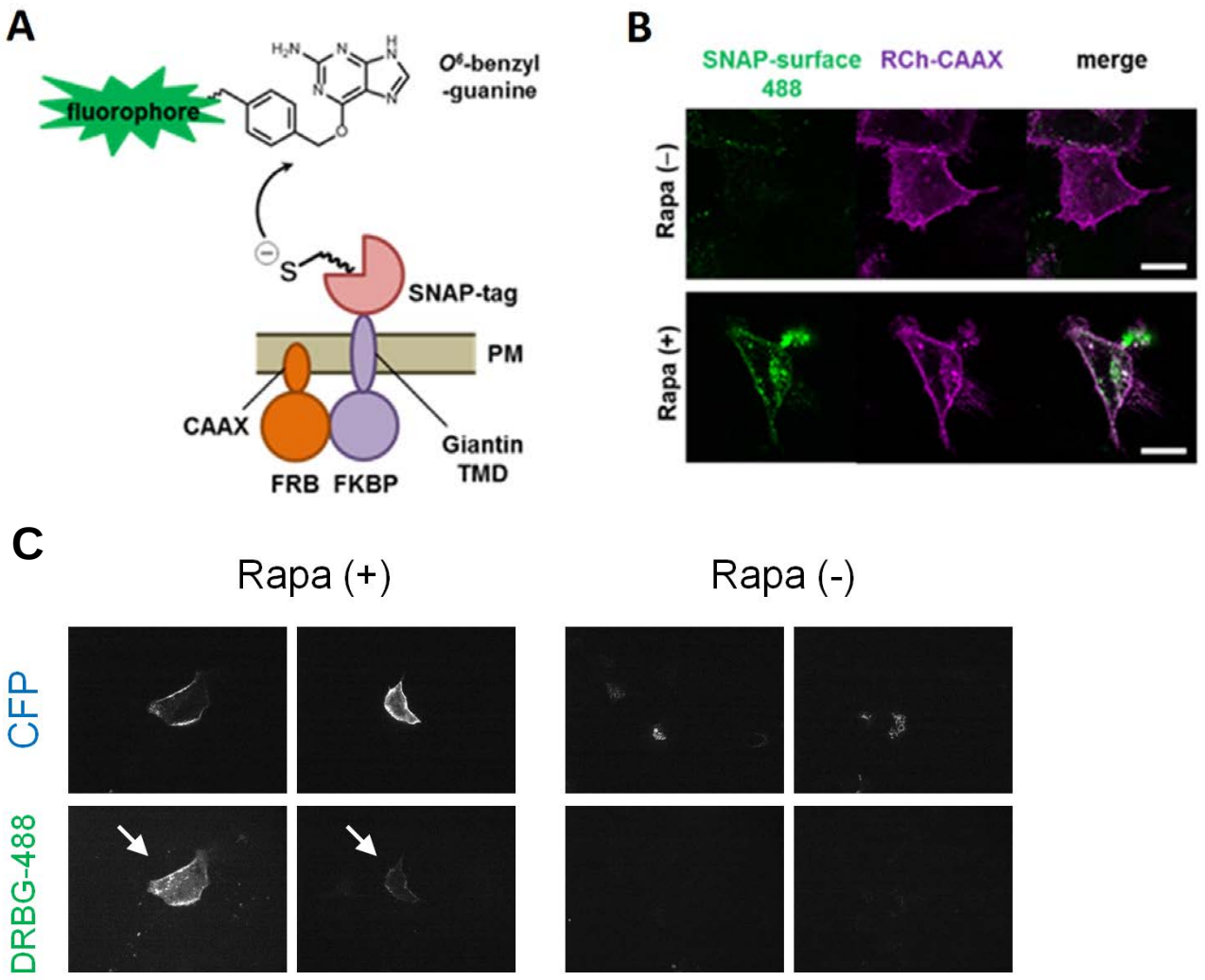

Fig. S13. Labeling at the cell surface with the SNAP-tag. (**A**) Schematic representation of the cell-surface display of a SNAP-tag protein that is covalently modified by a membrane-impermeable SNAP-tag substrate. A membrane-impermeable ligand, such as SNAP-surface 488 or DRBG-488 (*35*), can react with SNAP-tag facing the outer leaflet of the plasma membrane (PM) but not the SNAP-tag contained inside Golgi lumen. (**B**) HeLa cells transfected with plasmids encoding FKBP-Giantin-SNAP (green) and RCh-CAAX (magenta) were left untreated or were treated with rapamycin. Cells were labeled with 5 μM SNAP-surface 488 for 30 min, and were washed three times before imaging by fluorescence microscopy. (**C**) HeLa cells transfected with plasmids encoding FKBP-CFP-Giantin-SNAP and FRB-CAAX were left untreated or were treated with 200 nM rapamycin. Cell-surface proteins were labeled with the fluorescently activatable SNAP-tag labeling probe DRBG-488 (2 μM) for 1.5 hours before being analyzed by fluorescence microscopy. Scale bar: 20 μm. Data are representative of three independent experiments.

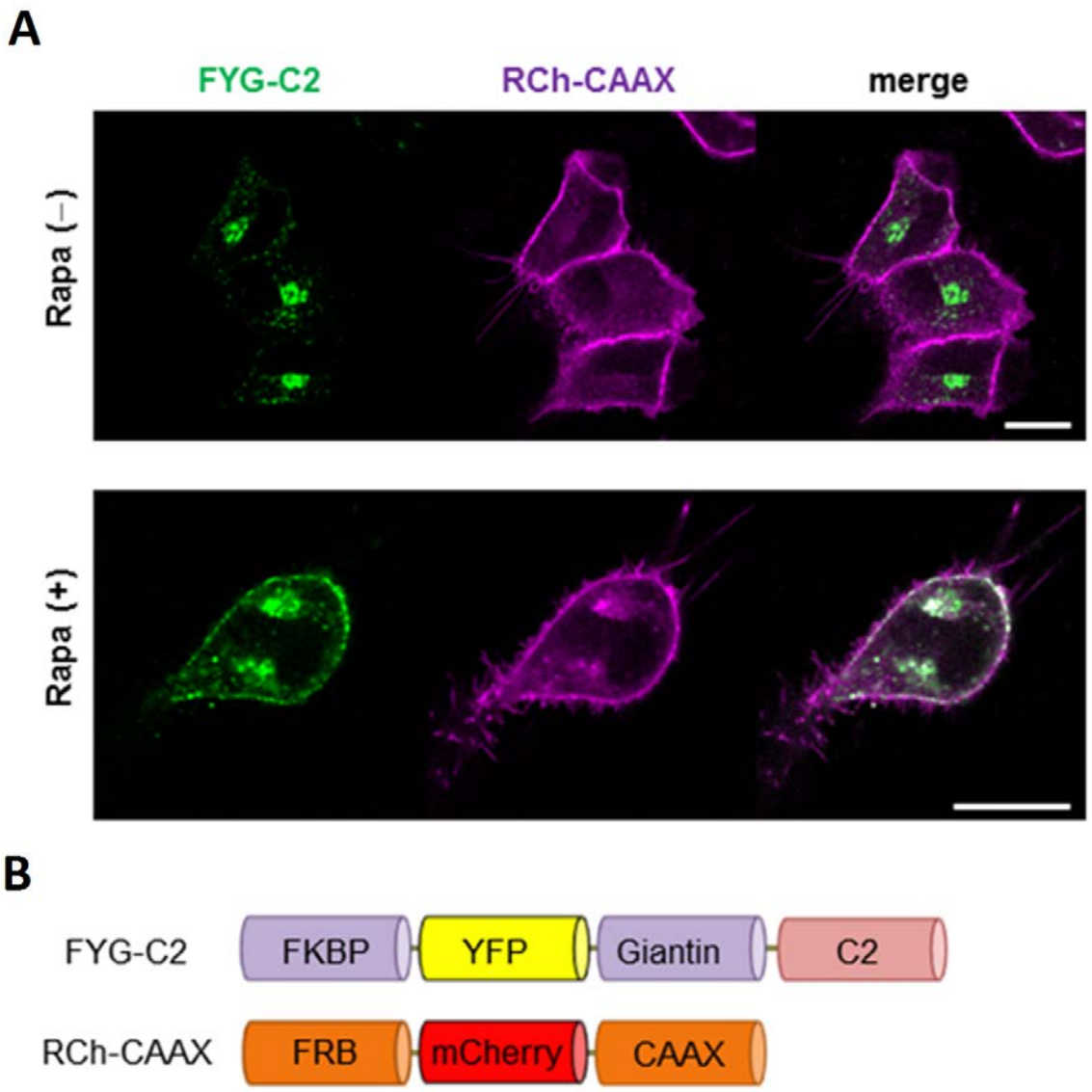

Fig. S14. Localization of C2-fused protein. (**A**) HeLa cells transfected with plasmids encoding FYG-C2 (green) and RCh-CAAX (magenta) were left untreated or were treated with rapamycin before being analyzed by fluorescence microscopy. Scale bar: 20 μm. Data are representative of four independent experiments. (**B**) Schematic representation of the FYG-C2 and RCh-CAAX.

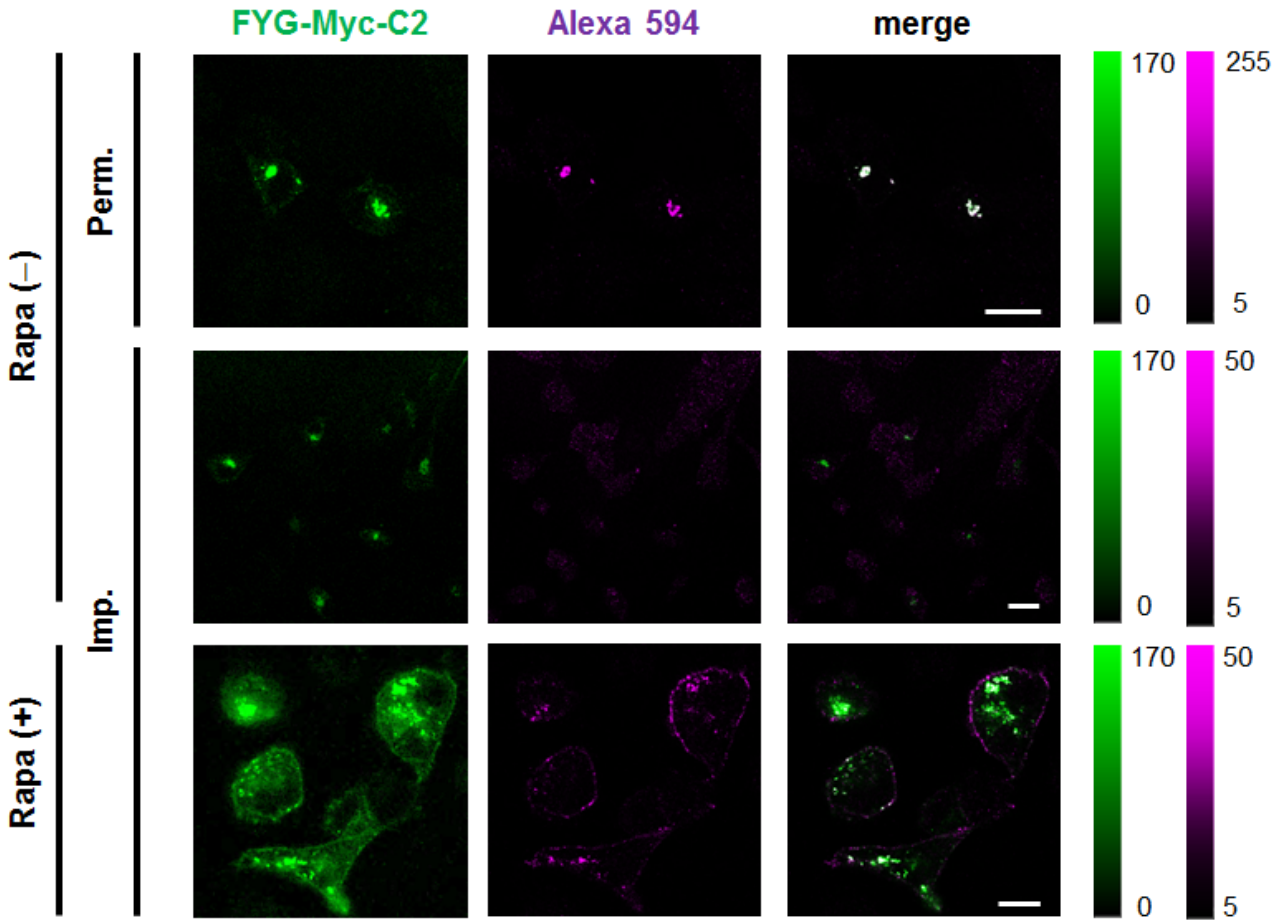

Fig. S15. Immunostaining of the Myc tag in cells expressing FYG-Myc-C2. HeLa cells were transfected with plasmids encoding RC-CAAX and FYG-Myc-C2 (green), were left untreated or were treated with rapamycin, and then were permeabilized (Perm.) or were left unpermeabilized (Imp.) before being stained with an antibody against the Myc-tag . As a secondary antibody, an Alexa Fluor 594–conjugated anti-rabbit antibody (magenta) was used and then cells were analyzed by fluorescence microscopy. Scale bar: 20 μm. Color bars on the right scale to the fluorescence intensity of individual images of FYG-Myc-C2 (for green bars) and Alexa594 (for magenta bars). Data are representative of three independent experiments.

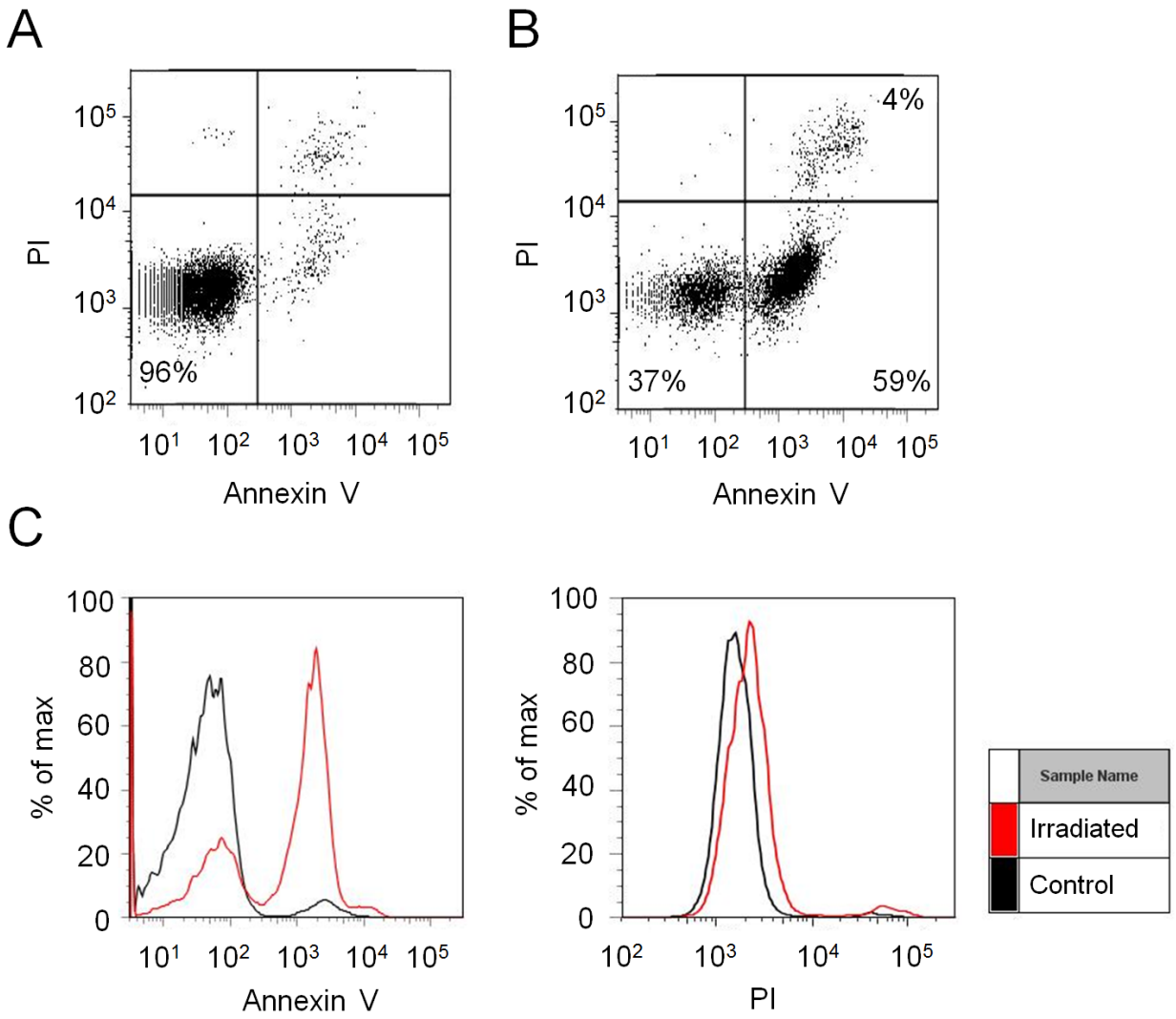

Fig. S16. Flow cytometric analysis of apoptotic Jurkat cells. Flow cytometric analysis of Jurkat cells labeled with Alexa Fluor 647–conjugated Annexin V and propidium iodide (PI) after irradiation with UV light to induce apoptosis. Annexin V: $\lambda_{ex.}$ = 633 nm / $\lambda_{em.}$ = 780 ± 60 nm; PI: $\lambda_{ex.}$ = 488 nm / $\lambda_{em.}$ = 575 ± 26 nm. (**A**) Flow cytometric analysis of non-treated cells. (**B**) Flow cytometric analysis of UV-irradiated cells. (**C**) Histograms showing the mean fluorescence intensities of Annexin V and PI of UV-irradiated and non-treated (control) cells. Data are representative of three independent experiments.

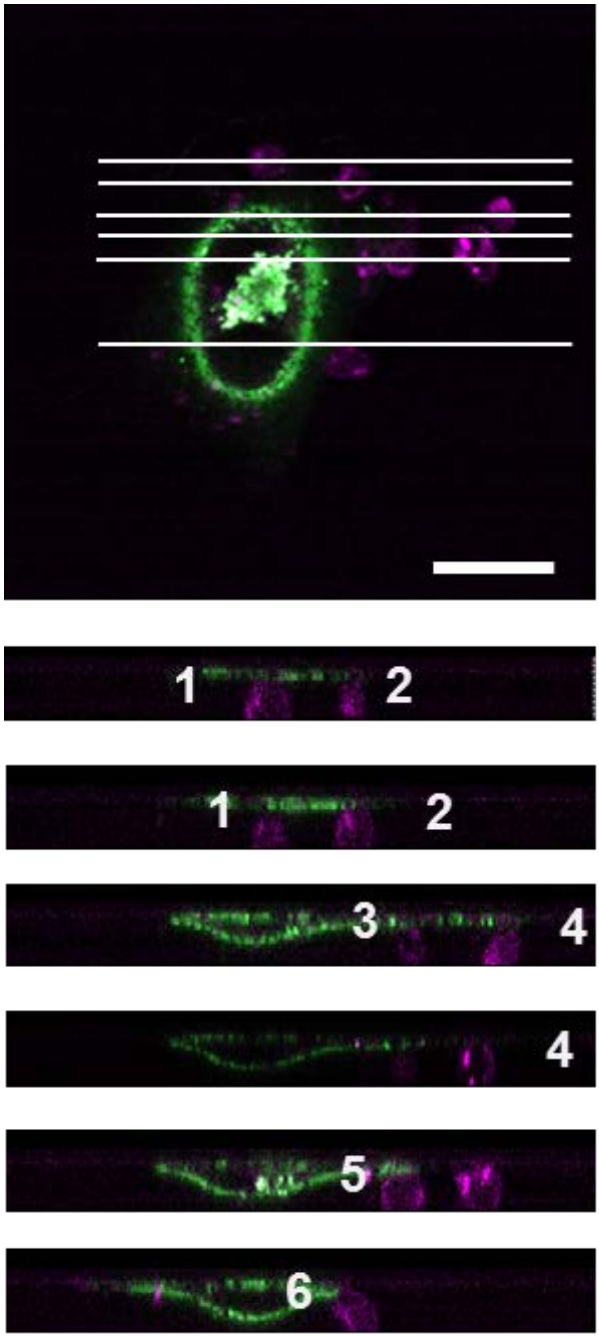

Fig. S17. Fluorescence micrographs of HeLa cells bound to apoptotic Jurkat cells. HeLa cells transfected with plasmids encoding FYG-C2 (green) and RC-CAAX were treated with rapamycin and then incubated with apoptotic Jurkat cells. The HeLa cell shown appears to associate with six apoptotic Jurkat cells (denoted by 1 through 6) that were independently labeled with CellTracker Red (magenta). Bottom panels indicate the x-z plane images whose y coordinates correspond to the white perpendicular lines in the top x-y plane image. Scale bar: 20 μm. The same cell is also shown in Fig. 2B.

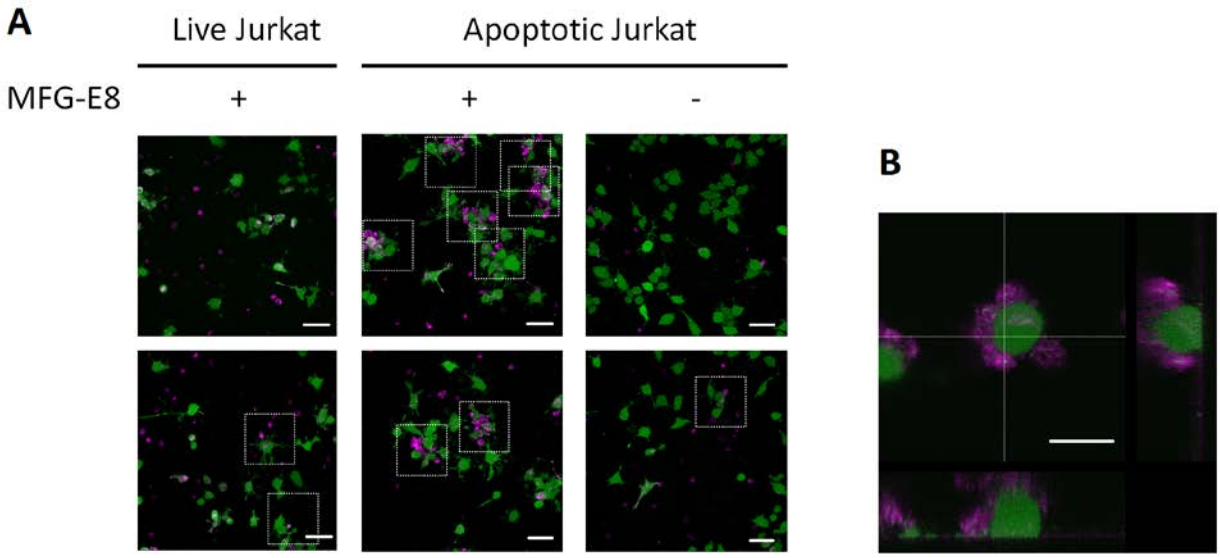

Fig. S18. Attachment of apoptotic Jurkat cells to the surface of RAW264.7 cells. (**A**) RAW264.7 cells that were labeled with calcein-AM (green) were then incubated with either live or apoptotic Jurkat cells. These Jurkat cells were labeled with CellTracker Red (magenta) and incubated in culture medium containing bovine MFG-E8 (15 μg/ml) for 12 hours. Dotted square areas highlight the RAW264.7 cells that are bound to the apoptotic cells. Thirteen of 156 cells (8%) or 48 of 109 cells (44%) bound to apoptotic cells in the absence or the presence of MFG-E8, respectively. Scale bar: 20 μm. (**B**) Fluorescence micrographs of RAW264.7 cells binding to apoptotic Jurkat cells in the presence of MFG-E8. x-z and y-z plane images are also shown. Scale bar: 20 μm. Data are representative of three independent experiments.

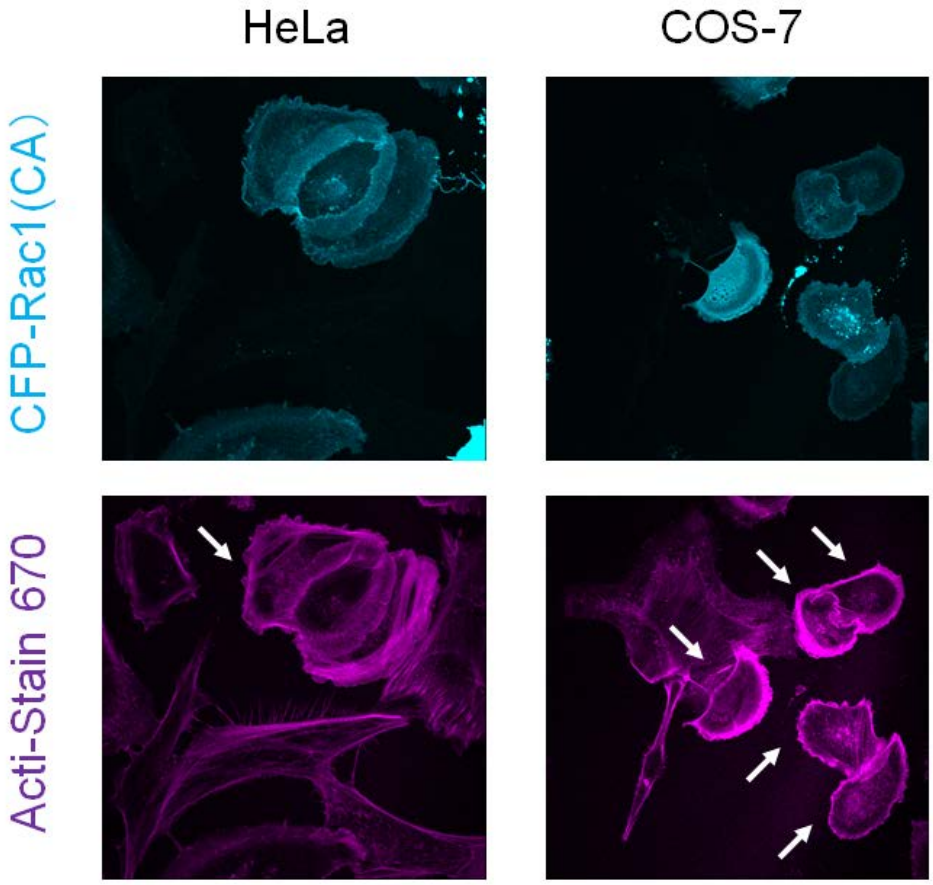

Fig. S19. Actin structures in cells expressing Rac1 (CA). HeLa cells transfected with plasmid encoding CFP-Rac1 (CA) and stained with Acti-stain 670 phalloidin were analyzed by confocal microscopy. White arrows indicate cells expressing CFP-Rac1 (CA). Data are representative of three independent experiments.

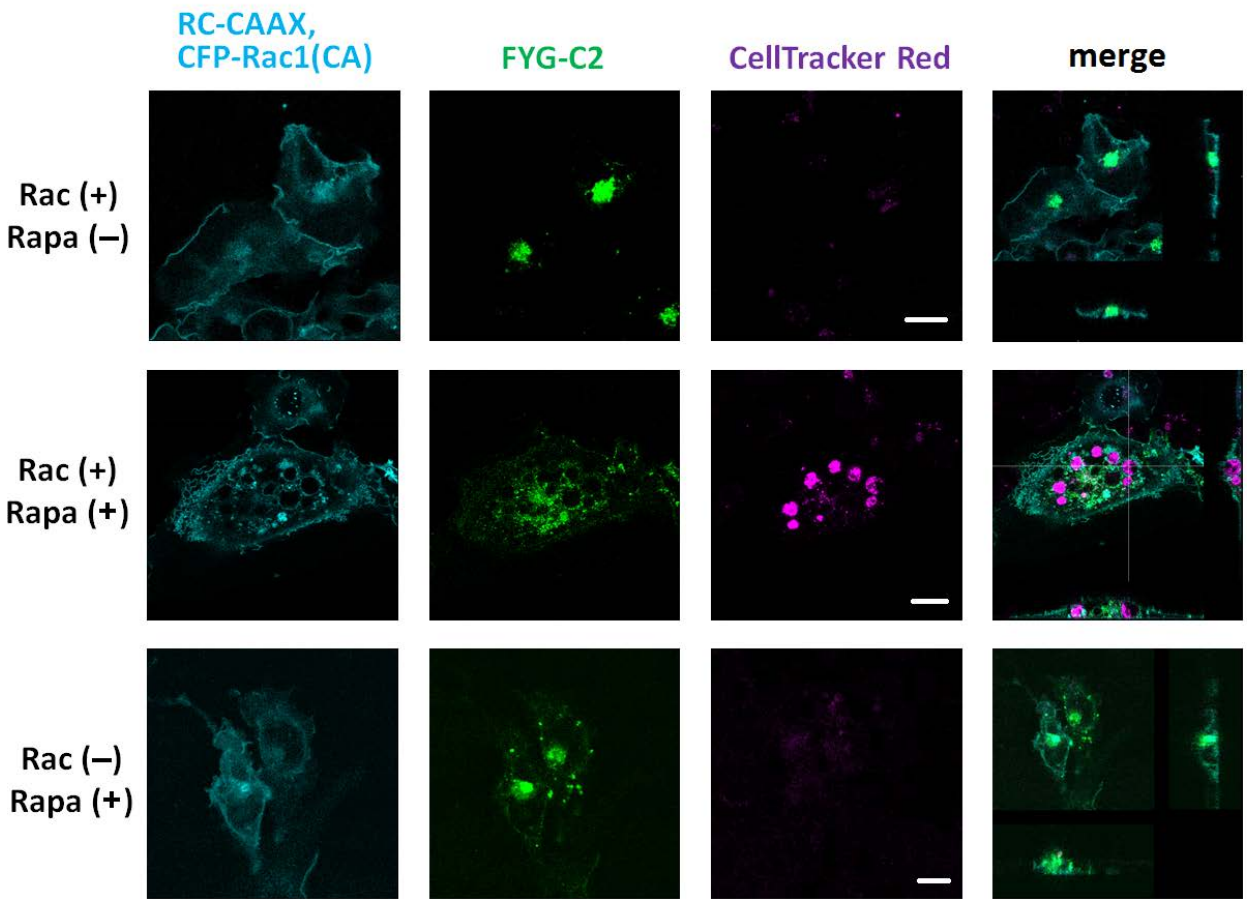

Fig. S20. Fluorescence micrographs of COS-7 cells engulfing Jurkat cells. COS-7 cells transfected with plasmids encoding CFP-Rac1 (CA), FRB-CFP-CAAX (RC-CAAX), and FYG-C2 were left untreated or were treated with 200 nM rapamycin for 60 min, and then were incubated for 12 hours in the presence of apoptotic Jurkat T cells that had been labeled with CellTracker Red before being analyzed by confocal microscopy. Rightmost micrographs were x-z and y-z plane images of the merged fluorescence. Signals from CFP, YFP, and CellTracker Red are presented in cyan, green and magenta, respectively. Scale bars: 20 μm. Data are representative of two independent experiments. Tables S2 and S3 summarize the statistical analysis.

Table S1. Statistical odds ratio analysis of engulfment assays with HeLa cells.

Sample conditions			**Number of cells engulfed**	**Number of cells non-engulfed**	**Odds Ratio[1] C2(+), Rapa(+), apoptosis(+)**	**95% CI[2]**
FYG-X	Rapa	Jurkat				
C2 (MFG-E8)	+	Apoptotic	23	38	–	–
C2 (MFG-E8)	–	Apoptotic	5	49	5.93	2.06 to 17.05
C2 (MFG-E8)	+	Non-apoptotic	3	50	10.09	2.82 to 36.10
Myc	+	Apoptotic	4	47	7.11	2.26 to 22.34

[1]The odds ratio (OR) was calculated based on the equation: OR = ad/bc, where a = the number of cells engulfed (test condition), b = the number of cells non-engulfed (test condition), c = the number of cells engulfed (control), and d = the number of cells not engulfed (control). [2]The 95% CI was calculated based on the equation: 95% CI = exp (ln OR ± SQRT (1/a + 1/b + 1/c + 1/d). In this analysis, statistical significance ($P < 0.05$) was tested by checking whether the 95% CI contains the null number (RR = 1) or not.

Table S2. Statistical risk ratio analysis of engulfment assays with COS-7 cells.

Sample conditions		**Number of cells engulfed**	**Number of cells analyzed**	**Risk [1]**	**Risk Ratio[2] Rac1(CA)(+), Rapa(+)**	**95% (CI)[3]**
Rac1 (CA)	Rapa					
+	+	18	54	0.50	–	–
+	–	5	57	0.096	3.80	1.52-9.52
-	+	0	30	0	–	–

[1]Risk was determined by dividing the number of cells engulfed by the number of cells analyzed. [2]Risk Ratio (RR) was determined by dividing a risk value of the test condition [C2 (+), rapamycin (+), and apoptotic Jurkat (+)] by that of each control condition. [3]The 95% CI was determined for each control condition versus test condition. Calculations were performed based on the equation: 95% CI = exp (ln RR ± SQRT (1/a + 1/b + 1/c + 1/d), where a = the number of cells engulfed (test condition), b = the number of cells analyzed (test condition), c = the number of cells engulfed (control), d = the number of cells analyzed (control). Because the 95% CI does not include the null value (RR = 1), we concluded that the engulfment occurred with statistical significance under the exposure of the C2 domain ($P < 0.05$).

Table S3. Statistical odds ratio analysis of engulfment assays with COS-7 cells.

Sample conditions		n of cells engulfed	n of cells non-engulfed	Odds Ratio[1] Rac1(CA)(+), Rapa(+)	95% Confidence Interval (CI)[2]
Rac1 (CA)	Rapa				
+	+	18	36	–	–
+	–	5	52	5.20	1.77-15.28
-	+	0	30	–	–

[1]The odds ratio (OR) was calculated based on the equation: OR = ad/bc, where a = the number of cells engulfed (test condition), b = the number of cells non-engulfed (test condition), c = the number of cells engulfed (control), and d = the number of cells not engulfed (control). [2]The 95% CI was calculated based on the equation: 95% CI = exp (ln OR ± SQRT (1/a + 1/b + 1/c + 1/d). In this analysis, statistical significance ($P < 0.05$) was tested by checking whether the 95% CI contains the null number (RR = 1) or not.

SUPPLEMENTARY MOVIES

Movie S1
Time-lapse, fluorescence images of FYG-Myc expressed in HeLa cells that are also transfected with RCh-CAAX (not shown here). A chemical dimerizer rapamycin was added at time 0. Note the increase in green fluorescence at the plasma membrane. This movie corresponds to fig. S3A.

Movie S2
Time-lapse, fluorescence images of FYG-Myc expressed in MDCK cells that are also transfected with RC-CAAX. A chemical dimerizer rapamycin was added at an indicated time point. Note the increased appearance of vesicles from Golgi. This movie corresponds to top panels in fig. S7.

Movie S3
Time-lapse, fluorescence images of FYG-Myc expressed in MDCK cells that are also transfected with RC-CAAX. A chemical dimerizer rapamycin was added at an indicated time point. This movie serves as a negative control for Movie S2, and corresponds to bottom panels in fig. S7.