Golgicide A reveals essential roles for GBF1 in Golgi assembly and function

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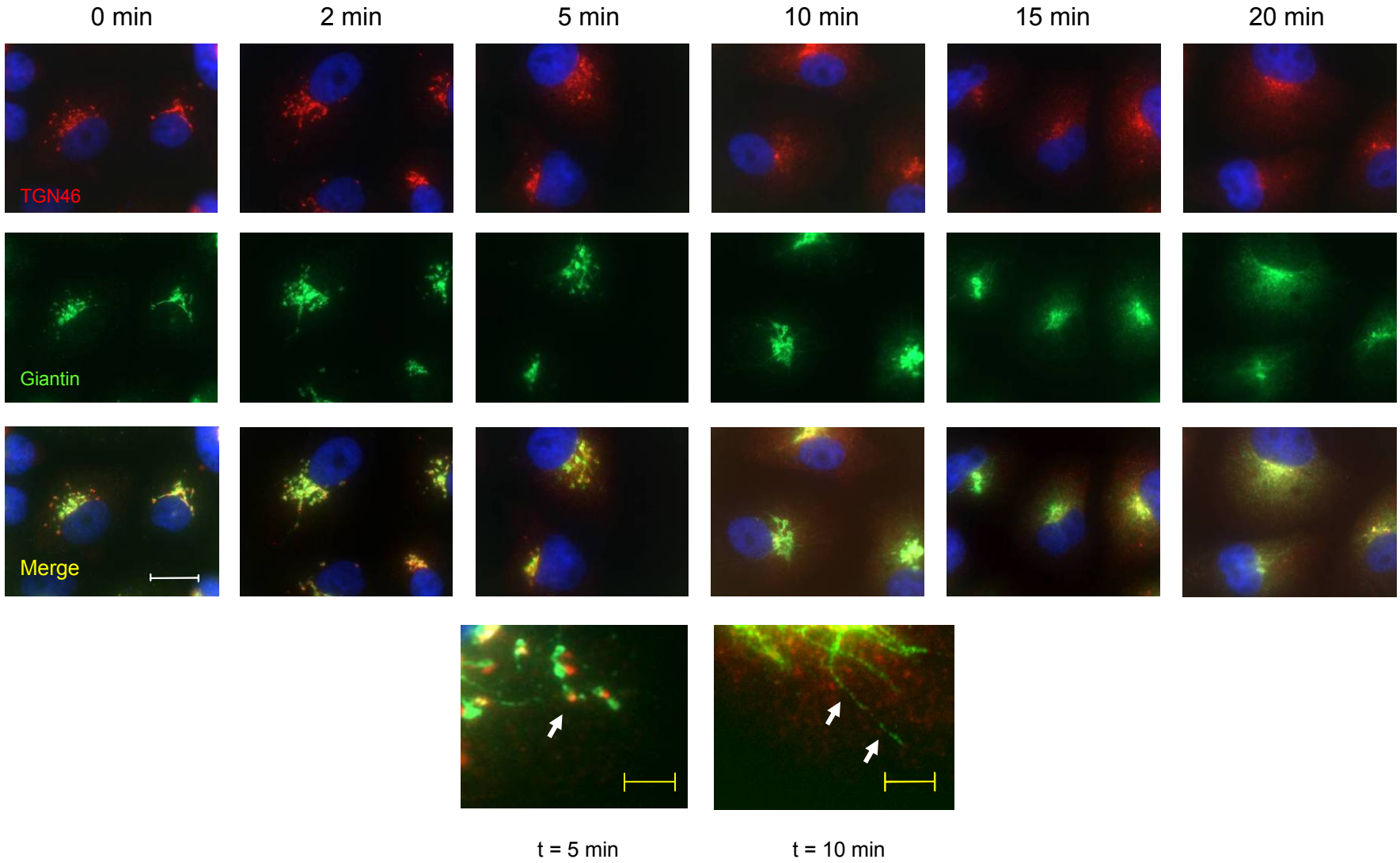
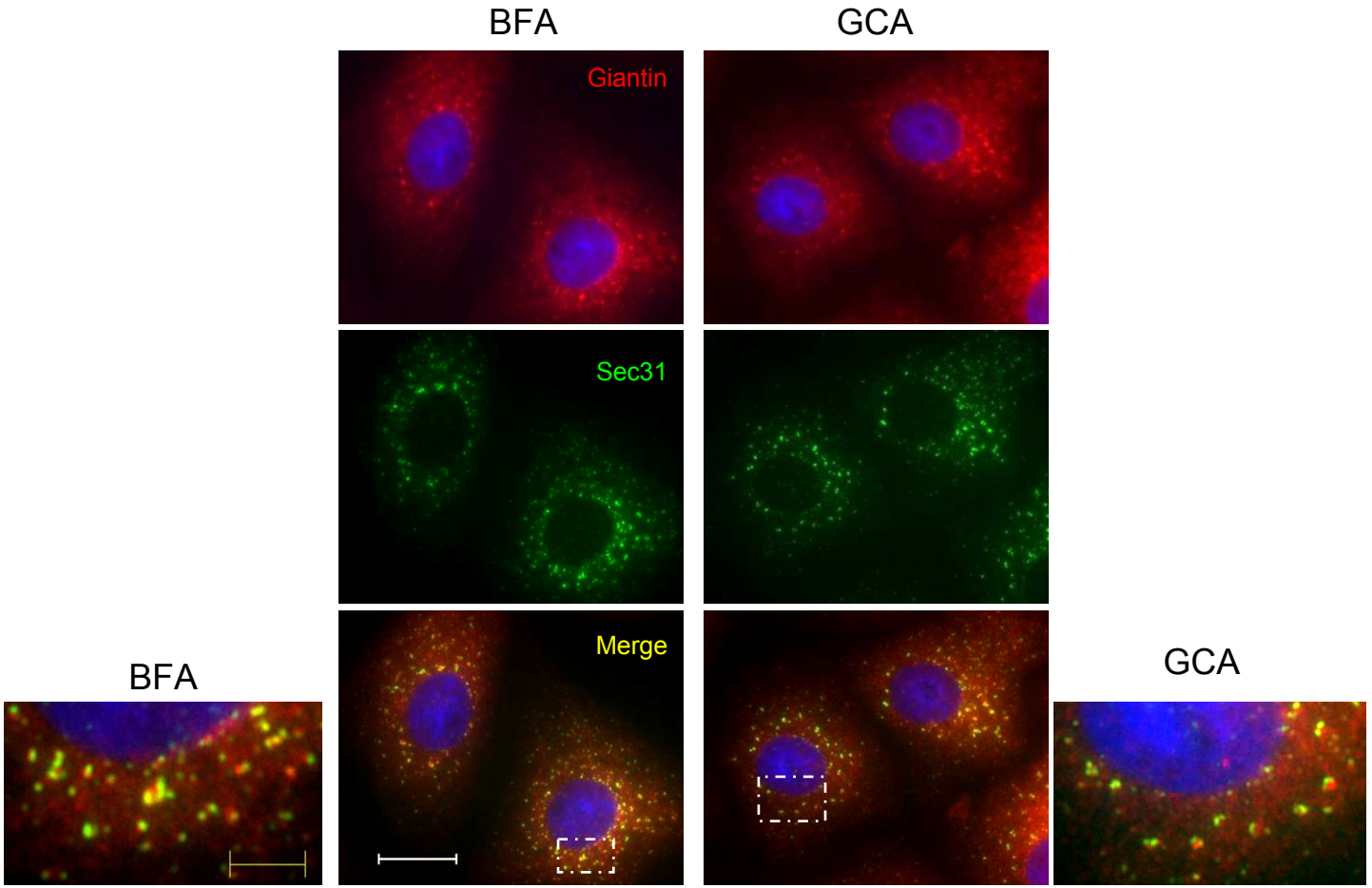
Supplementary Information

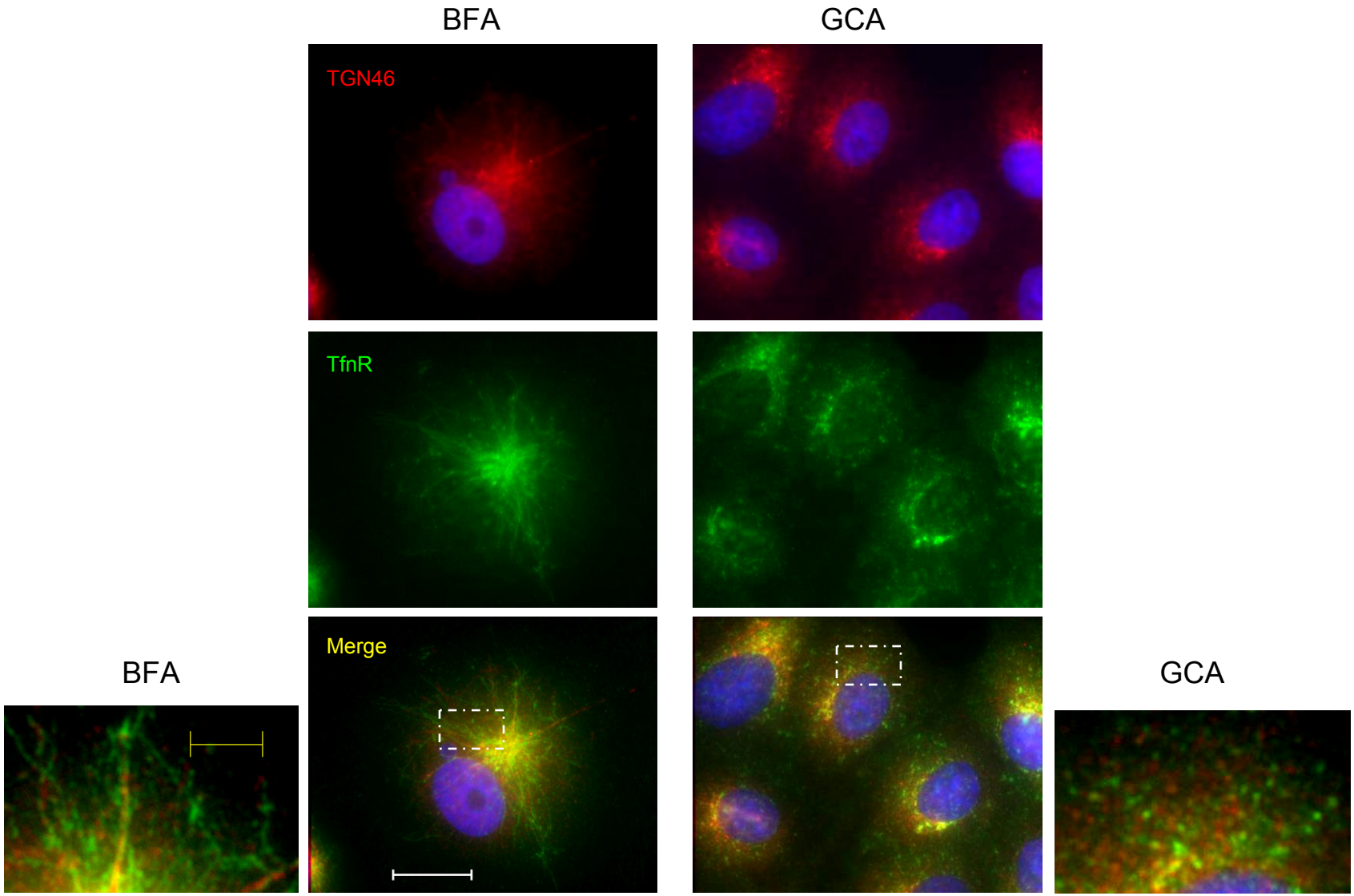
Contents:



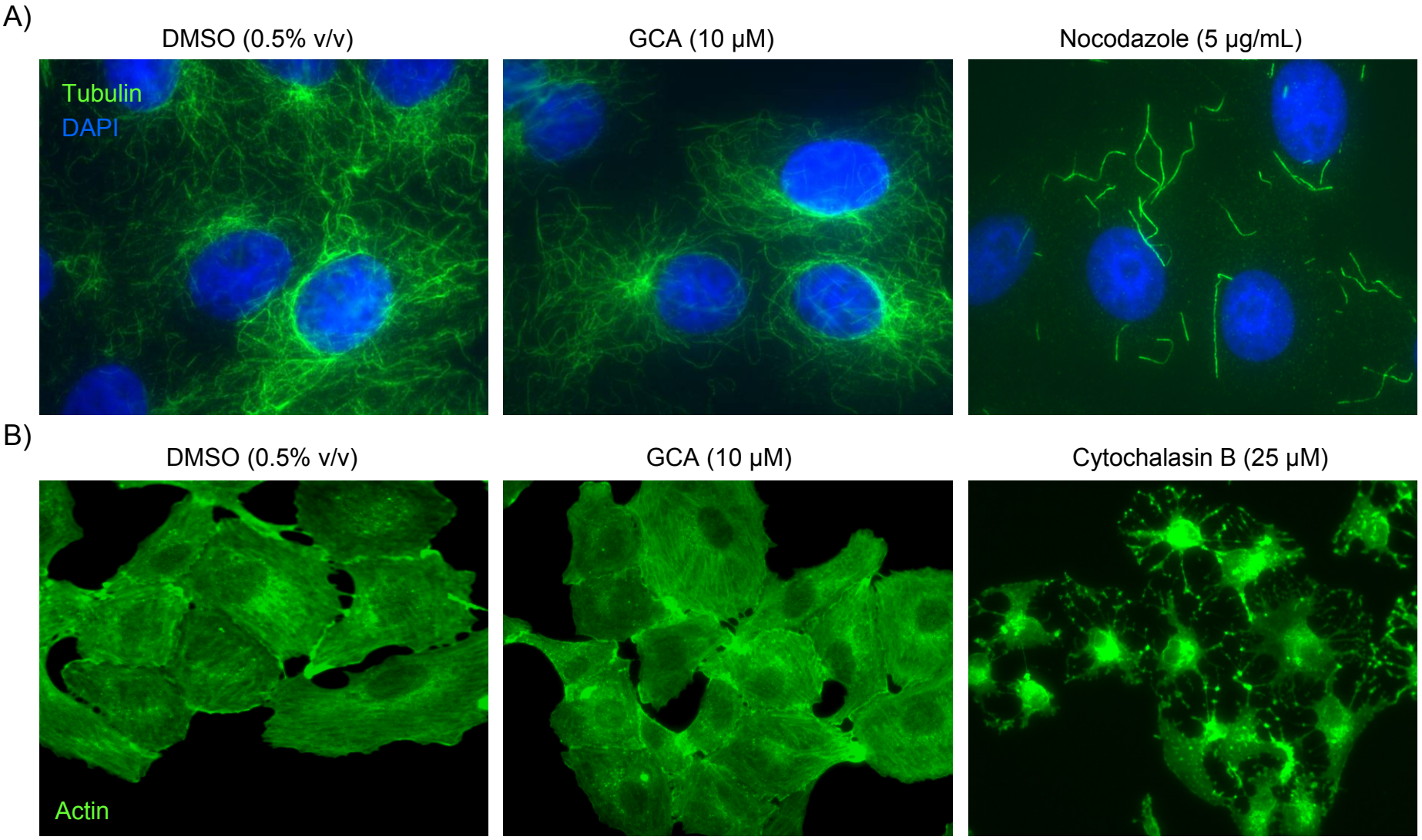
Supplementary Figure 1A. GCA causes tubulation and subsequent dispersal of the Golgi and TGN. (**a**) Vero cells were treated with GCA (10 μM) for the indicated times prior to fixation and labeling with anti-TGN46 (red) and anti-giantin (green) antibodies. Both the TGN and *medial*-Golgi begin to disassemble within 5 min of treatment. Insets demonstrate giantin-positive tubules (arrows) at the indicated times. White scale bars = 20 μm. Yellow scale bars = 5 μm.



Supplementary Figure 1B. BFA and GCA disperse the medial-Golgi marker giantin to a partially punctate pattern adjacent to ERES. Vero cells were treated with BFA (10 μg/ml) or GCA (10 μM) for 60 mins then fixed and labeled with antibodies against giantin (red) or Sec31 (green). Both compounds cause giantin redistribution into a hazy and punctate pattern. Giantin-positive punctate structures are closely approximated to Sec31-labeled ERES. White scale bars = 10 μm. Yellow scale bars = 5 μm.

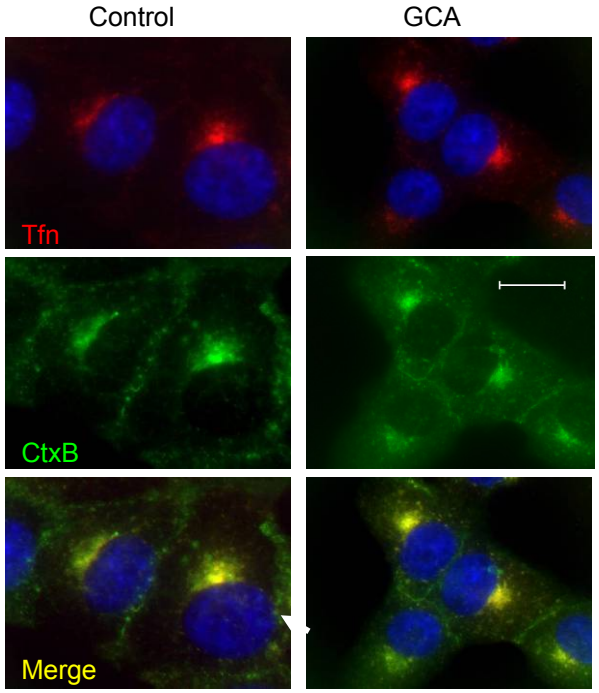


Supplementary Figure 2. BFA and GCA have distinct effects on the TGN and endosomes. Vero cells were treated with BFA (10 μg/ml) or GCA (10 μM) for 15 mins then fixed and labeled with antibodies against TGN46 (red) or transferrin receptor (Tfn; green). Whereas BFA treatment resulted in tubulation and partial overlap of TGN and recycling endosomes, GCA caused both to disperse into punctate structures that did not co-localize.



Supplementary Figure 3. GCA does not affect microtubular or actin cytoskeletons. (**a**) Vero cells were treated for 30 min at 37ºC with DMSO, GCA, or nocodazole at the indicated concentrations prior to fixation and immunostaining, as described in Supplemental Methods. At 10 μM, GCA had no observable effects on microtubules, while nocodazole, an inhibitor of microtubule polymerization, produced drastic morphological effects. (**b**) Vero cells were treated with DMSO, GCA, or cytochalasin B at the indicated concentrations and developed for immunofluorescence, as in (**a**). GCA showed no effects on actin microfilaments compared to DMSO-treated cells, while the actin-depolymerizing agent cytochalasin B induced significant changes to actin morphology.

a)



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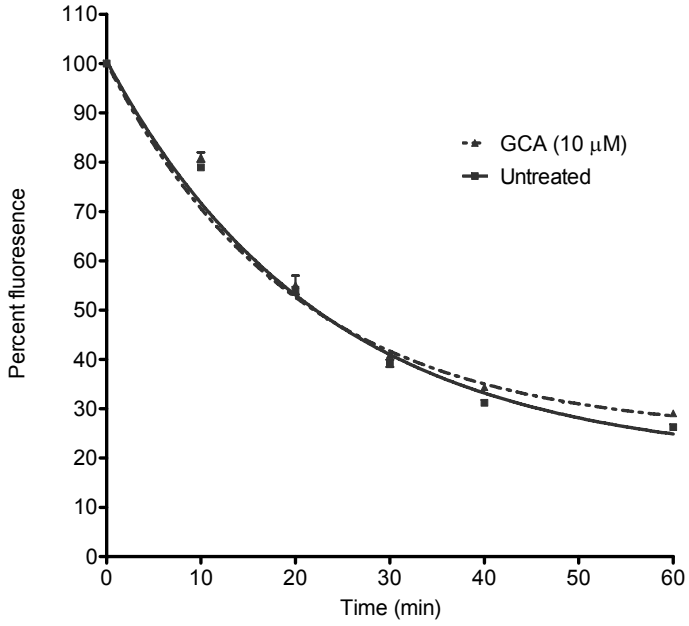
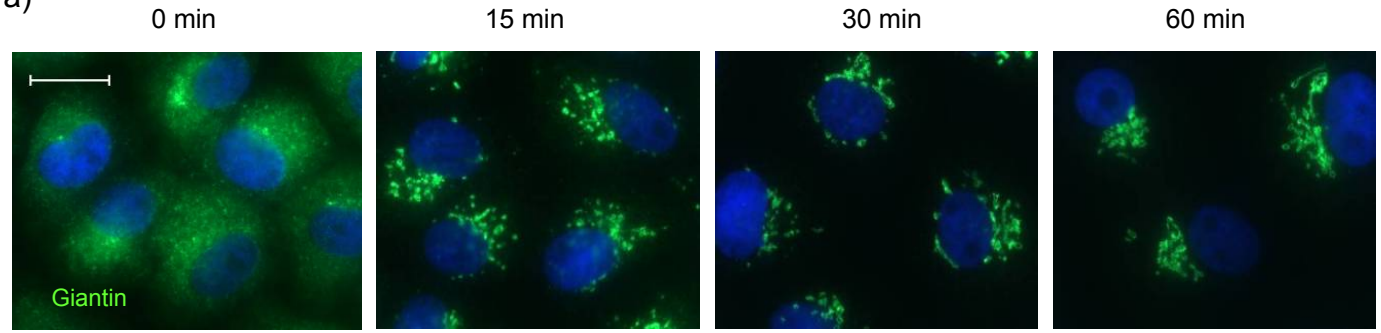


	Untreated	GCA
Half Life	15.90	13.92

Half life (min)

Supplementary Figure 4. GCA does not affect transport through recycling endosomes. (**a**) GCA treatment maintains endocytic transport to recycling endosomes. Vero cells were treated for 15 min with DMSO (control; 0.5% v/v) or GCA (10 μM), then incubated with AlexaFluor 594-labeled CtxB (1 μg/mL) and 488-labeled Tfn (1 μg/mL) for 1 h at 4°C in serum-free medium prior to shifting cells to 19°C for an additional hour. Cells were then fixed and developed for immunofluorescence. GCA, similar to control cells, did not affect CtxB trafficking to a juxtanuclear, Tfn-positive recycling endosome compartment. Blue, nuclei. (**b**) GCA treatment does not affect the kinetics of transferrin recycling. Vero cells were left untreated or were treated with GCA for 1 h. Cells were allowed to internalize AlexaFluor-488 labeled transferrin for 60 min. Fresh media containing quenching anti-AlexaFluor-488 antibodies were added, and at various times the cells were harvested and fixed (see Supplementary Methods). Each time point was performed in triplicate, and the mean and standard deviation of each is presented. All data were fitted by nonlinear regression assuming one phase decay, and half-lives (in min; inset) were calculated using GraphPad Prism. CtxB, cholera toxin B subunit; Tfn, transferrin. White scale bar = 20 μm.

a)



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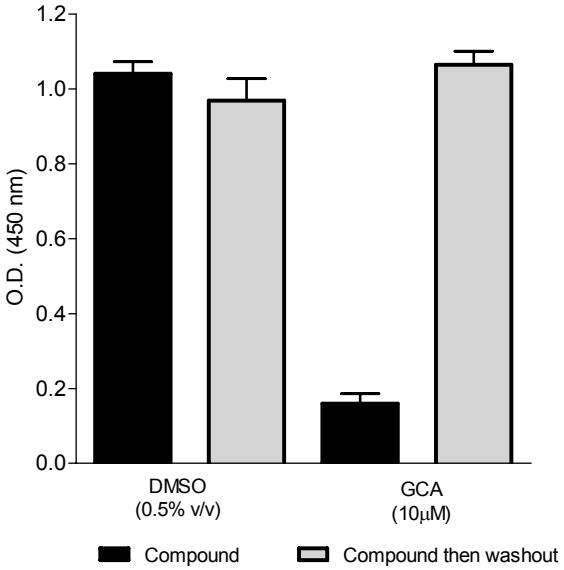
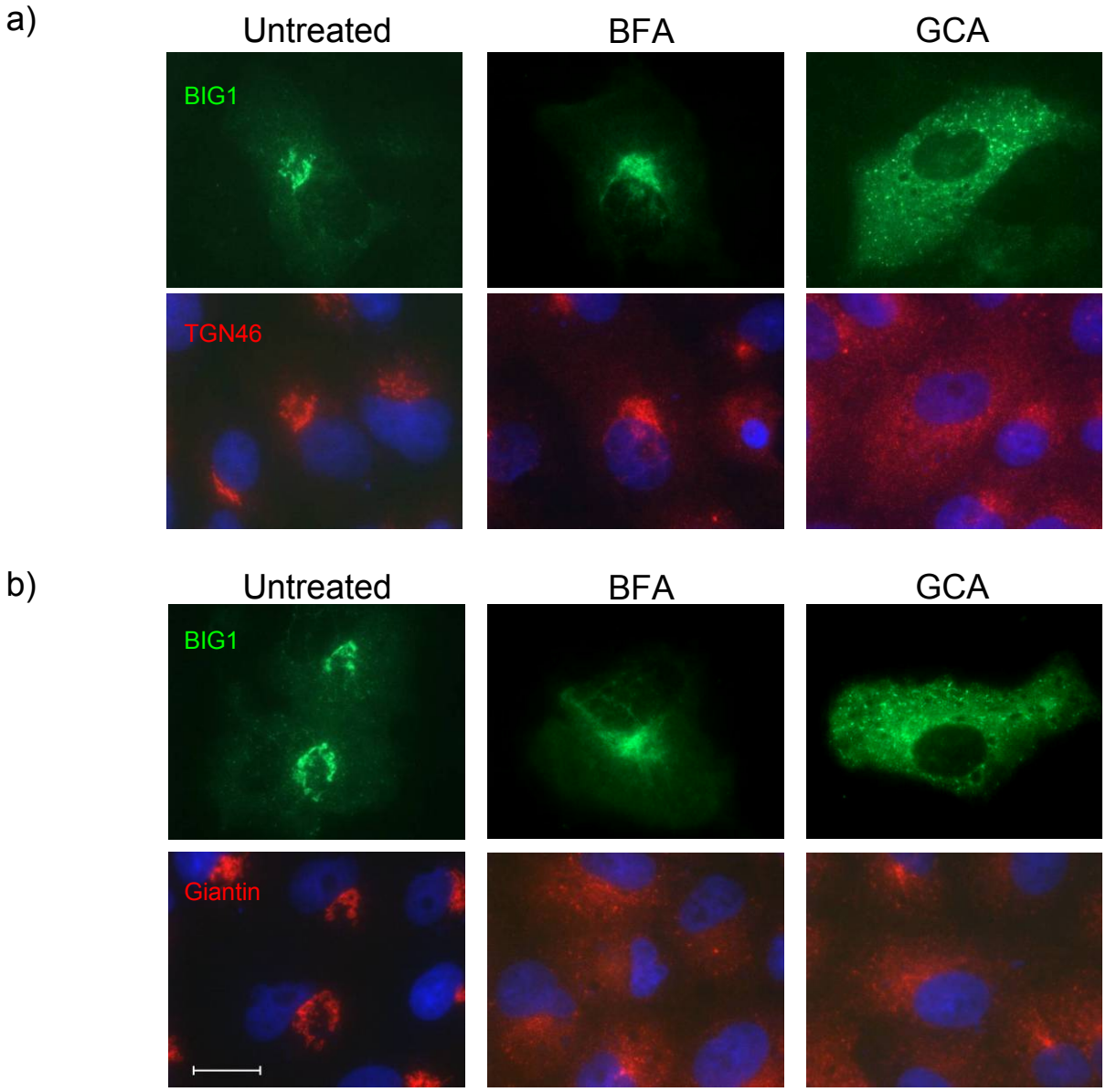
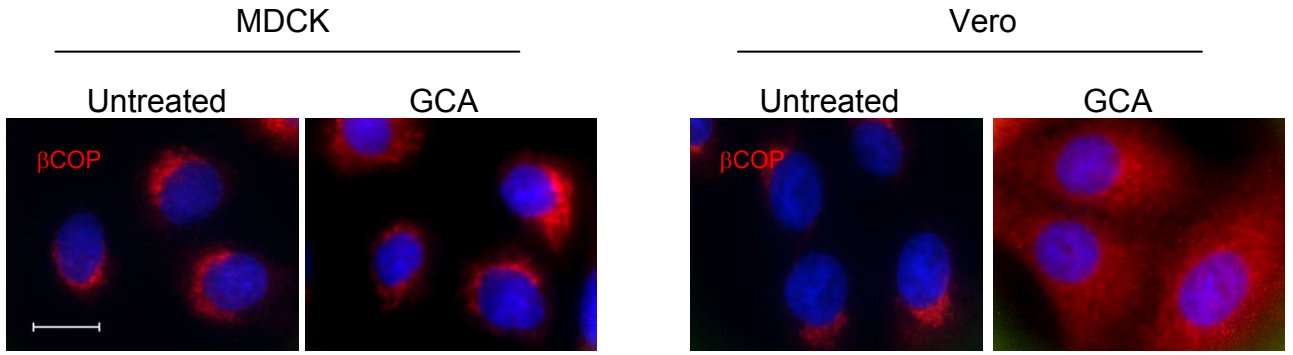


Supplemental Figure 5. The effects of GCA are rapidly reversible. (a) Vero cells were treated with GCA (10 μM) for 1 h. To remove GCA, cells were washed three times with PBS and incubated at 37ºC in media alone for various times (t = 0, 15, 30, and 60 min) prior to fixation and labeling with an anti-giantin antibody. The *medial*-Golgi (green) reassembles by 60 min following GCA removal. White scale bar = 20 μm. (b) The effects of GCA on protein secretion are reversible. Vero cells were transduced with adenovirus expressing NPY-GFP and were treated with DMSO or GCA for 1 h at the indicated concentrations. In cells with compound washout (black bars), DMSO or GCA was removed as described in (a), and cells were incubated in media alone for 1 h. The amount of secreted GFP was assessed 2 h later by ELISA (see Methods). GCA treatment alone (white bars) inhibits NPY-GFP secretion, while NPY-GFP secretion was restored to control levels following GCA removal.



Supplemental Figure 6. Expression of BIG1-HA is partially protective against BFA but has no protective effect against GCA. Vero cells were transiently transfected with BIG1-HA, exposed either to BFA (10 μg/ml) or GCA (10 μM) for 1 hr, then labeled with anti-HA plus either (a) anti-giantin or (b) anti-TGN46. Wheras BIG1-expressing cells are partially protected from BFA at the TGN (a), there is no protective effect of BIG1 expression against GCA at TGN (a) or Golgi (b). White scale bar = 20 μm.

a)



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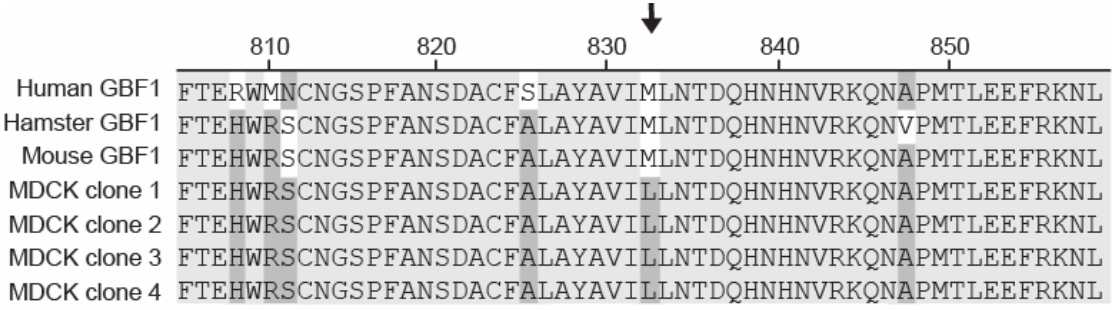
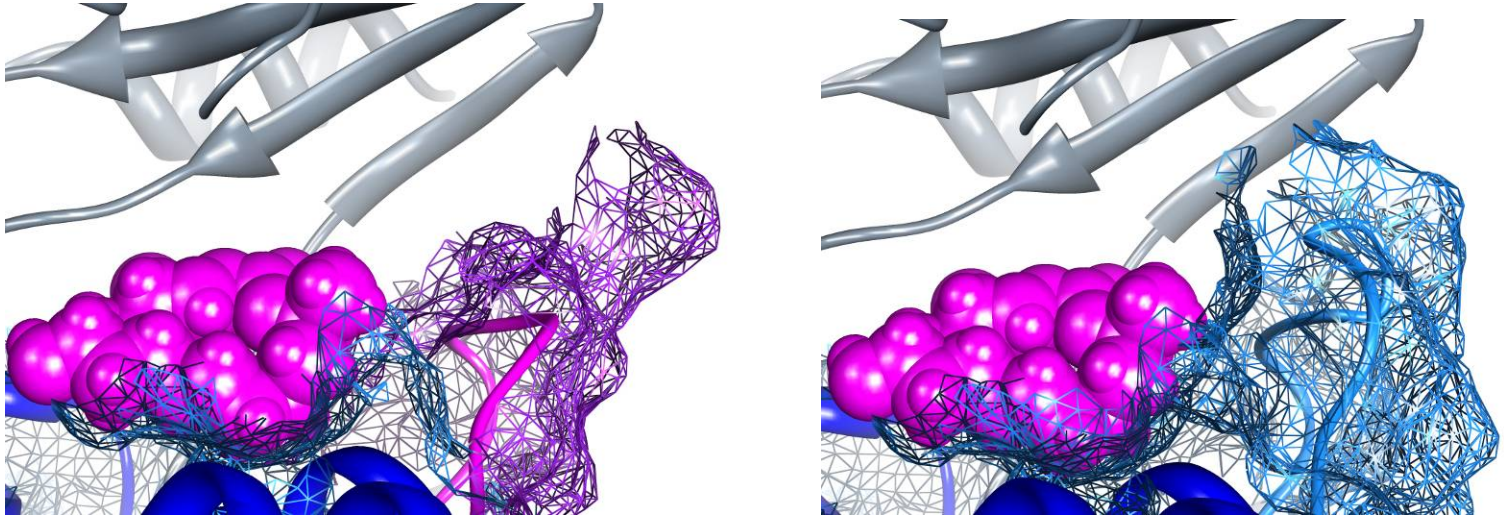
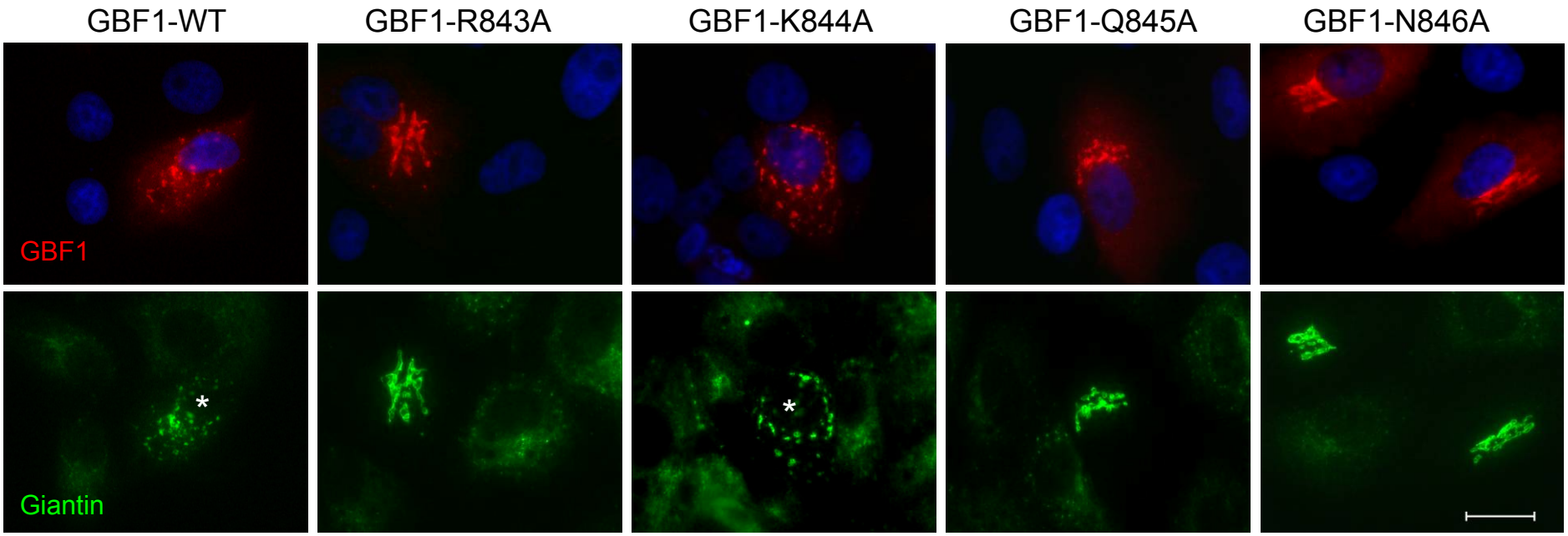


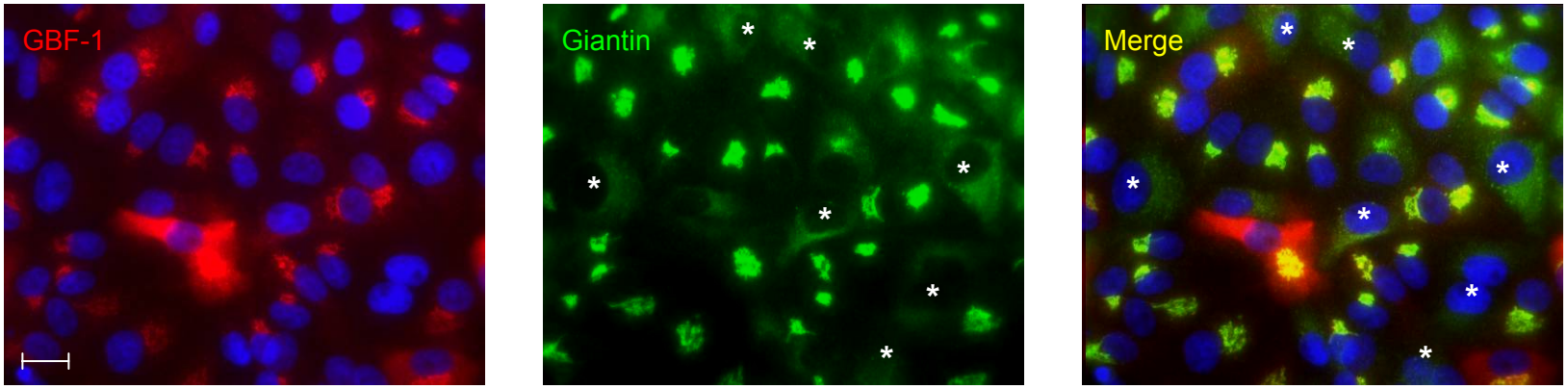
Supplemental Figure 7. MDCK cells are resistant to GCA. (a) MDCK and Vero cells were left untreated or treated for 1 h with GCA (10 μM). GCA did not affect βCOP distribution in MDCK cells, whereas both were dispersed in GCA-treated Vero cells. White scale bar = 20 μm. (b) The Sec7 domain of GBF1 was amplified from MDCK cDNA (see Methods). The sequences of four resulting clones were compared to published mammalian orthologues. A methionine for leucine substitution was identified in the canine peptide (black arrow).



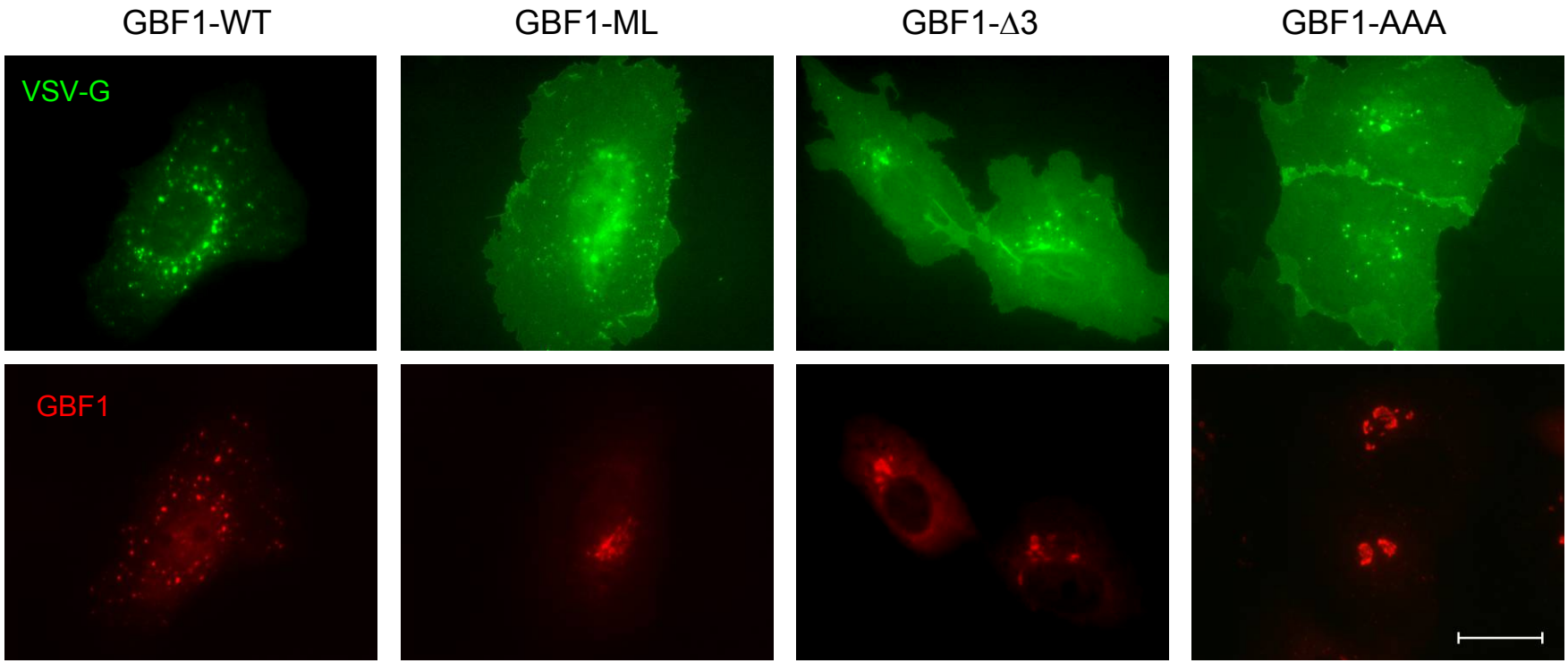
Supplemental Figure 8A. The tripeptide loop of GBF1 does not affect BFA interactions. The left panel presents the previously published structure of ARNO Sec7 domain (blue ribbon) in complex with BFA (purple spheres) and Arf1 (gray ribbon). In the right panel, the GBF1 Sec7 domain has been substituted for ARNO. The loop region accounting for GCA selectivity is shown in purple (left panel) or light blue (right panel). BFA does not contact the loop region in either model.



Supplemental Figure 8B. Expression of GBF1-ML but not GBF1-loop mutants results in resistance to the effects of BFA on Golgi morphology. Vero cells were transiently trasfected with GBF1-WT, or a GBF1-R843A, GBF1-K844A, GBF1-Q845A, or GBF1-N846A. Thirty six hours later the cells were treated for 60 mins with GCA (10μM) then fixed and labeled with anti-HA epitope (GBF1; red) or giantin (green). Cells expressing GBF1-R843A, GBF1-Q845A, and GBF1-N846A are resistant to the effects of GCA on Golgi morphology, whereas cells expressing GBF1-WT and GBF1-K844A are only partially protected. White asterix mark the partially protected cells. White scale bar = 20 μm.



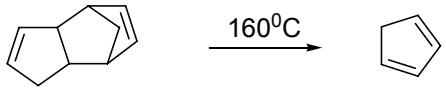
Supplementary Figure 9. Transduction with GBF1-ML is less than 100% efficient. Vero cells were transduced with GBF1-ML to be used in Arf1 activation assay (Figure 5). An aliquot of cells was seeded into a slide chamber. Simultaneous with cells used for the the Arf1-GTP pulldown assay, these cells were treated with GCA for 1 hr then fixed and labeled with antibodies against HA (GBF1; red) or giantin (green). Nuclei were labeled with DAPI. GBF1 expression varies from cell to cell, with approximately 85% of cells expressing detectable GBF1. These cells are protected from the effects of GCA, while approximately 15% express low or undetectable GBF1 and are not protected from GCA (white asterix). White scale bar = 20 μm.

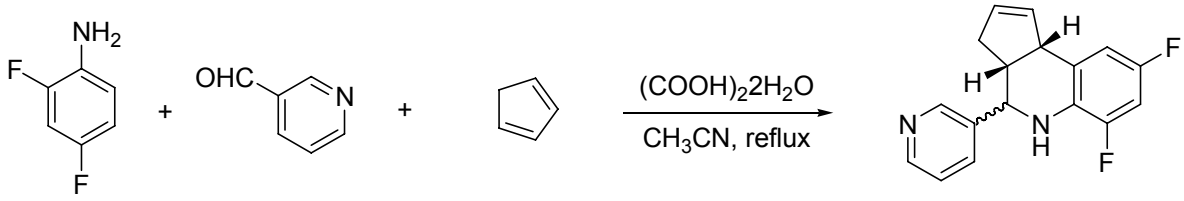


Supplemental Figure 10. Expression of GBF1-ML or GBF1-loop mutants results in resistance of the effects of GCA on *ts* VSVG-GFP transport. Vero cells were co-transfected with *ts*VSVG-GFP and either GBF1-WT or a GBF1-mutant. One day after transfection the cells were incubated for 12 hrs at 42°C to arrest VSVG-GFP in the endoplasmic reticulum. The cells were treated with GCA (10 μM) for 30 mins then shifted to 32°C and incubated 4 hrs. The cells were then fixed and labeled with anti-HA (red) antibodies. Whereas expression of GBF1-WT fails to rescue VSVG-GFP transport in the presence of GCA, GBF1-ML and the GBF1-loop mutants are resistant to the effects of GCA. White scale bars = 20 μm.

Supplementary Methods

GCA synthesis. Dicyclopentadiene (100 mL) was placed in a round-bottomed flask equipped with a magnetic stirbar and a column fitted with a distillation head through which cold water was circulated. The contents of the flask were slowly heated with stirring to 160^0C in oil bath, and 60 mL of cyclopentadiene was collected in a receiver [1].





To a solution of 3-pyridinecarboxaldehyde (25 mg, 0.2 mmol), 2,4-difluoroaniline (30 mg, 0.2 mmol), and cyclopentadiene (30 mg, 0.4 mmol) in CH_3CN (1 mL) was added oxalic acid dihydrate (29 mg, 0.2 mmol) at room temperature. After stirred at 85^oC for 2 hours, the reaction mixture was then cooled to room temperature and then treated with 2 mL of ice water. An equal volume of dichloromethane was added and the organic layer was separated and the aqueous layer was re-extracted with an equal volume of dichloromethane. The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated. The crude product was purified by flash column chromatography using a 98:2 v/v dichloromethane : methanol as solvent to afford title compound (47 mg, 72 % yield as mixture (10 : 1)) as a white solid.

Antibodies and Reagents. Compound GCA was purchased from ChemDiv, reconstituted to 10 mM in DMSO and stored at -20^{o}C. The purity of GCA was confirmed by mass spectroscopy. Shiga-like toxin 1 was from List Biological Laboratories. Recombinant Alexa Fluor 488-labeled cholera toxin B subunit, Alexa Fluor 594-labeled human transferrin, SlowFade Gold mounting reagent containing DAPI, and Alexa Fluor-labeled donkey anti-IgG secondary antibodies were obtained from Molecular Probes. Rabbit anti-giantin polyclonal antibody was obtained from Covance, rabbit anti-hemagglutinin (HA) polyclonal antibody from Sigma-Aldrich, mouse anti-GM130 from BD Transduction, rabbit anti-human TGN38 from Santa Cruz, rabbit anti-βCOP from ABR, and mouse anti-ERGIC-53 from Axxora. DMEM, EMEM, and nonessential amino acids were obtained from Mediatech. DMSO, brefeldin A (BFA), cytochalasin B, and nocodazole were from Sigma. Trans [^{35}S] was purchased from MP Biomedicals, and [^{35}S]O_4 was obtained from American Radiolabeled Chemicals.

Cell Lines and Cell Culture. Vero (African green monkey kidney, CRL-1587), MDCK (Madin-Darby canine kidney, CCL-34), and 293A-HEK (Human embryonic kidney, CRL-1573) cells were obtained from the American Type Tissue Culture Collection (Manassas, VA). 293A cells were obtained from Invitrogen. Vero cells were maintained in DMEM supplemented with 10% fetal calf serum and 1% nonessential amino acids at 37^{o}C under 5% CO_2. MDCK cells were maintained in EMEM supplemented with 10% fetal calf serum and 1% nonessential amino acids at 37^{o}C under 5% CO_2. 293A cells were maintained in EMEM with 10% heat-inactivated horse serum.

Transient transfections were performed using Lipofectamine 2000 (Invitrogen) in OptiMem media, following the manufacturer's recommendations. Following overnight incubation at 37^{o}C under 5% CO_2, cells were collected into chamber slides (Lab-Tek, Campbell, CA) or appropriate dishes and incubated another 24 h before experimentation.

Cloning and sequence analysis of canine GBF1 Sec7 domain. Total RNA was isolated from approximately 10^7 MDCK cells by silica membrane binding (RNeasy, Qiagen) and contaminating chromosomal DNA was removed by DNase treatment (Qiagen). The cDNA was prepared from isolated RNA with random primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The Sec7 domain of the canine GBF1 gene was amplified from MDCK cDNA using primers 5'-CGATTTTCCTGTCTCCTGCCAGATCCACGGG-3' and 5'CCACACATAGTTCT CCCGAACCAAGCC-3' and the resulting product cloned into pcDNA3.1/V5/His. Four resulting clones were sequenced and compared to their human, hamster, and murine counterpart.

Site-Directed Mutagenesis. GBF1-HA was generated by PCR using primers GBF1-HA (5'-GCCGCGCTAGCCTGAGGCATAGTCAGGCACGTCATAAGGATAGCCGTTG ACTTCAGAGGTGGGAATAGGGTCTGTAG-3') and the upstream GBF1 primer (5'-GACAGGTTTGCCAAGATGGTGGATAAGAATATTTACATC-3'). The resulting PCR product was cloned into pcDNA3.1D/V5-His-TOPO (Invitrogen) under control of the cytomegalovirus (CMV) promoter. The insert was sequenced to ensure its fidelity.

The GBF1 mutants were generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). Mutagenic primers were E794K sense (5'-GCCTTCCGTTTGCCCGGGAAGGCACCAGTTATTCACAGGTTGC-3'), E794K antisense (5'-GCAACCTGTGAATAACTGGTGCCTTCCCGGGCAAACGGAAGGC-3'), M832L sense (5'-GGCCTATGCTGTCATCTTGCTTAATACTGACCAGC-3'), and M832L antisense (5'-GCTGGTCAGTATTAAGCAAGATGACAGCATAGGCC-3'). Mutant clones were sequenced to ensure their fidelity.

Radioactive protein synthesis assay. Vero cells cultured overnight at 37°C and 5% CO_2 in 96-well plates (2.5 x 10^4 cells/well) were treated with 0.5% DMSO (v/v) or media containing GCA or BFA at the indicated concentrations. Following a 0.5 h incubation at 37°C, toxin was added to wells in triplicate, and cells were shifted to 37°C for an additional 4 h. Medium containing trans [^{35}S] label at 10 μCi/ml was added, and cells were incubated at 37°C for 45 min, washed with PBS (pH 7.4), and lysed (1 mg/ml BSA, 0.2% deoxycholic acid, 0.1% SDS, 20mM Tris pH 7.4) at 4°C for 12 h. Proteins from the lysed cells were TCA-precipitated (final concentration 15%), transferred to multi-screen HA plates (Millipore), and the filters were washed with ice-cold 20% TCA. Filters were then removed from the plate, placed in 2 mL Bio-Safe II scintillation fluid (RPI), and [^{35}S] incorporation quantitated using a beta counter (Beckman). Independent experiments were performed at least three times for GCA and BFA, and data were analyzed using Prism v4.0 software (2003).

Transferrin Recycling Assay. Transferrin (Tf) recycling was analyzed by modification of a previously published assay for recycling of low density lipoprotein related protein [2]. 2 x 10^5 Vero cells were seeded per well in seven 12-well dishes. The following day, media was removed and replaced with 500 μl serum-free media containing Tf-488 (Invitrogen) at 5 μg/ml and either no compound or B06 (10 μM). The samples were incubated at room temperature for 60 min to allow Tf transport to recycling endosomes. Cells were washed twice with 1 ml PBS, then overlaid with 400 μl media containing 10% FCS, anti-AlexaFluor-488 antibody (Invitrogen) at 15 μg/ml, and either no compound, BFA (10 μg/ml), or B06 (10 μM). T=0 min samples were maintained at room temperature to prevent recycling. The remaining samples were incubated at 37ºC and at various times ranging from 10 to 60 min. Media was removed, and 500 μl prewarmed cell release buffer (Sigma) was added. Cell suspensions were mixed with 250 μl 4% paraformaldehyde, and mean fluorescence was determined by FACS on a Becton Dickenson FACSCaliber System. Mean fluorescence of the T=0 samples was normalized to 100% fluorescence.

Preparation of Adenovirus Expressing GBF1-HA, GBF1-HA Mutants. Adenovirus expressing GBF1-HA constructs, canine Arf1 switch mutants, and NPY-GFP were prepared by cloning the appropriate cDNA into plasmid pENTR-11 then transferring the cDNA insert to plasmid pAD/CMV/DEST using the Clonase II reaction (all from Invitrogen). Crude adenoviral stocks were isolated from transfected 293A cells, which were then used to generate high titer stocks. Each was aliquoted and frozen at -80^{o}C until

used for transduction. Control experiments were performed with each stock to determine conditions resulting in 100% transduction efficiency.

Arf1-GTP pulldown assay. Human ARF1 bearing a carboxy-terminal HA epitope tag was amplified from a human liver cDNA (Invitrogen) using flanking primers 5'-GTCCTTCCACCTGTCCACAAGCATGGGG-3' and 5'-CCGCGCTAGCCT GAGGCATAGTCAGGCACGTCATAAGGATAGCCGTTCTTCTGGTTCCGGAGCG ATTGGACAGCC-3'. The resulting product was ligated into plasmid pCDNA3.1/V5/His. Nucleotide sequencing was performed to verify fidelity of the HA-tagged wild-type cDNA. Following overexpression, the localization of ARF1 was tracked by indirect immunofluorescence using a mouse anti-V5 (Invitrogen) primary antibody, followed by staining with the corresponding secondary antibody.

The VHS and GAT domains from human GGA3 were amplified from a human cDNA library using primers 5- GGCCGAATTCATGGCGGAGGCGGAAGGGGA-AAGC-3' and 5'- CCGGCTCGAGTCAGTCAGGCAGGGTTAAGGTAGCCACCTCG-3' The resulting product was initially cloned into plasmid pcDNA3.1/V5/His and sequenced to ensure its fidelity. The product was then released by digestion with EcoRI and XhoI and ligated into similarly digested plasmid pGEX-6p1. Expression of the recombinant GGA3-GST protein was induced by the addition of IPTG and the protein purified from crude bacterial lysates on a GSTrap column (Pharmacia). The protein was dialyzed into 50 mM Tris pH 7.5 plus 100 mM NaCl and used in Arf1 pulldown assays using the protocol of Santy and Casanova[3], with the following modifications.

Vero cells were seeded in 10 cm^3 dishes and transduced with adenovirus expressing Arf1-V5 alone, or co-transduced with virus expressing Arf1-V5 and virus expressing GBF1-ML-HA. After overnight incubation, the cells were washed, trypsinized, and seeded into three 25 cm3 flasks each. The following day, monolayers were treated at 37°C for 45 min with media alone, or media containing BFA (10 μg/ml) or GCA (10 μM). The cells were then washed with cold PBS and scraped into 1 ml lysis buffer (50 mM Tris pH 7.6, 100 mM NaCl, 2 mM MgCl2, 1 % SDS, 1 % Triton X-100, and 10% glycerol,). To each sample was added 48 μg of GGA-GST bound to 30 μl of glutathione agarose (Pierce Chemical Company). The samples were incubated rocking at 4°C for 30 mins, then the beads pelleted and washed three times with cold GGA wash buffer (50 mM Tris pH 7.6, 100 mM NaCl, 2 mM MgCl2, 1% NP-40, and 10% glycerol). SDS-PAGE loading buffer was added, the samples were boiled, and equal aliquots separated by SDS-PAGE. Arf1 was detected by Western blot using anti-V5 antibody (Invitrogen) followed by enhanced chemiluminescence. Band intensity was determined using the program ImageJ. The results of duplicate experiments were averaged.

Expression and trafficking of tsVSVG-GFP. Vero cells were transiently transfected with VSVG-GFP *ts*045 using Lipofectamine 2000 (Invitrogen) In some experiments, cells were co-transfected with GBF1-HA WT or M832L plasmids. After overnight incubation at 37°C in 5% CO_2, cells were collected and placed into chamber slides (Lab-Tek) for an additional 8-10 h at 37°C before their transfer to 42°C for 12-16 h. Cells were then treated with cycloheximide (100 μg/ml) to prevent *de novo* protein synthesis and either no compound, GCA (10 μM), or brefeldin A (10 μg/ml) then transferred to

32°C. Cells were fixed following various incubation times at 32°C. Fixation, permeabilization, staining, and imaging were performed as described for immunofluorescence experiments.

NPY-GFP secretion assay. The NPY-GFP assay was similar to that previously reported[4], with slight modifications. Briefly, 10^6 Vero cells were transduced overnight at 37ºC in 5% CO_2 with pAD-NPY-GFP. Cells were then washed, trypsinized, and seeded into a 96-well plate (~1 x 10^4 cells/well). The next day, the cells were washed once with PBS then incubated at 37ºC with media containing DMSO, BFA (10 μg/ml), or GCA (10 μM). Supernatants were collected at various times thereafter. GFP quantitation was performed by ELISA using anti-GFP coated plates (Pierce) and rabbit anti-GFP in solution. Mean absorbance for control wells containing DMEM alone were subtracted from sample wells. Calculation of GFP concentration was performed by comparison to a recombinant GFP (rGFP; Boehring Manneheim) standard curve.

StxB-SS-His Sulfation. An StxB construct containing a tandem of carboxy-terminal sulfation sites and a histidine tag for purification (StxB-SS-His) has been described[4]. Vero cells seeded in a 6-well plate (1 x 10^6 cells/well) were washed three times in serum-free DMEM lacking sulfate (Washington University Tissue Culture Support Center), and then incubated in sulfate-free medium for 3.5 h at 37ºC. After treatment with DMSO (0.5% v/v), BFA (10 μg/ml) or GCA (10 μM) 30 min at 37ºC, media was replaced with sulfate-free media containing these compounds plus StxB-SS-His (1 mg/ml) and 1 mCi/ml $[^{35}S]O_4$ for 3 h at 37ºC. Wells were washed with cold PBS (pH 7.4) and lysed

with PBS containing 1% Triton X-100. Lysates were added to 40 µl Ni-NTA Superflow beads (Qiagen) and rotated at 4ºC overnight. Beads were washed once with PBS containing 1% Triton X-100 and twice with PBS, then resuspended in imidazole (1.5 M in PBS). Eluates were resolved on a 10-20% Tris-HCl denaturing gel, treated with EnHance reagent (DuPont), dried and exposed to film. Band intensity was determined using ImageJ software.

Statistical analysis. All statistical analyses were performed by GraphPad Prism 5. Toxin concentrations were log transformed prior to curve fitting and statistical analyses. Toxin-response curves were generated by nonlinear regression (least-squares fit) to correspond to the observed data, and toxin IC_{50} (the concentration of toxin required to reduce protein synthesis by half) and compound IC_{50} (the concentration of compound required to reduce Stx activity by half) values were calculated using the fitted curves. For the compound-response curves, the GCA concentration varied while keeping the concentration of Stx (1 ng/ml) constant. For the toxin-response curves, the toxin concentration varied, while the concentration of DMSO or GCA was kept constant. Toxin IC_{50} values were compared using the extra sum-of-squares F test applied to the best-fit curves for the data. Differences between toxin IC_{50} values were considered highly statistically significant for $p \leq 0.01$. For assessment of Arf1-GTP levels, values were considered statistically significantly different for $p \leq 0.05$ (*), whereas *ns* denotes a non-significant difference ($p > 0.05$).

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